How glyphosate and its associated acidity affect early development in zebrafish (*Danio rerio*)

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Background. Glyphosate is among the most extensively used pesticides worldwide. Following the ongoing highly controversial debate on this compound, its potential impact on non-target organisms is a fundamental scientific issue. In its pure compound form, glyphosate is known for its acidic properties.

Methods. We exposed zebrafish (*Danio rerio*) embryos to concentrations between 10 μM and 10mM glyphosate in an unbuffered aqueous medium, as well as at pH 7, for 96 hours post fertilisation (hpf). Furthermore, we investigated the effects of aqueous media in the range of pH 3 to pH 8, in comparison with 1 mM glyphosate treatment at the respective pH levels. Additionally, we exposed zebrafish to 7-deoxy-sedoheptulose (7dSh), another substance that interferes with the shikimate pathway by a mechanism analogous to that of glyphosate, at a concentration of 1 mM. The observed endpoints included mortality, the hatching rate, developmental delays at 24 hpf, the heart rate at 48 hpf and the malformation rate at 96 hpf. $LC_{10/50}$, EC_{10} and, if reasonable, EC_{50} values were determined for unbuffered glyphosate.

Results. The results revealed high mortalities in all treatments associated with low pH, including high concentrations of unbuffered glyphosate (>500μM), low pH controls and glyphosate treatments with pH < 3.4. Sublethal endpoints like developmental delays and malformations occurred mainly at higher concentrations of unbuffered glyphosate. In contrast, effects on the hatching rate became particularly prominent in treatments at pH7, showing that glyphosate significantly accelerates hatching compared with the control and 7dSh, even at the lowest tested concentration. Glyphosate also affected the heart rate, resulting in alterations both at pH 7 and, even more pronounced, in the unbuffered system. In higher concentrations, glyphosate tended to accelerate the heart rate in zebrafish embryos, again, when not masked by the decelerating influence of its low pH. At pH > 4, no mortality occurred, neither in the control nor in glyphosate treatments. At 1 mM, 7dSh did not induce any mortality, developmental delays or malformations; only slightly accelerated hatching and a decelerated heart rate were observed. Our results demonstrate that lethal impacts in zebrafish embryos can be attributed mainly to low pH, but we could also show a pH-independent effect of glyphosate on the development of zebrafish embryos on a sublethal level.

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28   **Abstract**

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33   **Methods**. In its pure compound form, glyphosate is known for its acidic properties. We exposed

34   zebrafish (*Danio rerio*) embryos to concentrations between 10 µM and 10 mM glyphosate in an

35   unbuffered aqueous medium, as well as at pH 7, for 96 hours post fertilisation (hpf). Furthermore, we

36   investigated the effects of aqueous media in the range of pH 3 to pH 8, in comparison with 1 mM

37   glyphosate treatment at the respective pH levels. Additionally, we exposed zebrafish to 7-deoxy-

38   sedoheptulose (7dSh), another substance that interferes with the shikimate pathway by a mechanism

39   analogous to that of glyphosate, at a concentration of 1 mM. The observed endpoints included

40   mortality, the hatching rate, developmental delays at 24 hpf, the heart rate at 48 hpf and the

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42   glyphosate.

43   **Results**. The results revealed high mortalities in all treatments associated with low pH, including high

44   concentrations of unbuffered glyphosate (> 500 µM), low pH controls and glyphosate treatments with

45   pH < 3.4. Sublethal endpoints like developmental delays and malformations occurred mainly at higher

46   concentrations of unbuffered glyphosate. In contrast, effects on the hatching rate became particularly

47   prominent in treatments at pH 7, showing that glyphosate significantly accelerates hatching compared

48   with the control and 7dSh, even at the lowest tested concentration. Glyphosate also affected the heart

49   rate, resulting in alterations both at pH 7 and, even more pronounced, in the unbuffered system. In

50   higher concentrations, glyphosate tended to accelerate the heart rate in zebrafish embryos, again, when

51   not masked by the decelerating influence of its low pH. At pH > 4, no mortality occurred, neither in the

52   control nor in glyphosate treatments. At 1 mM, 7dSh did not induce any mortality, developmental

53   delays or malformations; only slightly accelerated hatching and a decelerated heart rate were observed.

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56   embryos on a sublethal level.

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58

59   **Introduction**

60

61   Currently, glyphosate is the most extensively used herbicide worldwide (EC 2007, U.S. EPA 2011), and its

62   approval for application within European Union borders was extended by the European Commission (EC

63   2017) for another five years in December 2017. Glyphosate was first used successfully in the 1970s as

64   the active compound in the formulation *Roundup®* (*Monsanto*, St. Louis, USA) and is nowadays a

65   component in numerous herbicides from various producers. In the U.S., glyphosate tops the list of

66   applied agricultural herbicides and ranks second in home and garden applications (Battaglin et al. 2014).

67   However, in the last couple of years, there have been very controversial debates concerning the effects

68   of glyphosate-based herbicides on non-target organisms (e.g. Folmar, Sanders & Julin 1979, Relyea

69   2005, Benamú, Schneider & Sánchez 2010, Balbuena et al. 2015, Helmer et al. 2015, Motta, Raymann &

70   Moran 2018), whole ecosystems (e.g. Vera et al. 2010) and even on the health of human beings as the

71   IARC rated glyphosate as probably carcinogenic in 2015 (IARC 2017).

72   Glyphosate is a non-selective, broad-spectrum herbicide (Monsanto 2005) that inhibits aromatic acid

73   synthesis in plants by interfering with the shikimate pathway (Steinrücken & Amrhein 1980, Bentley &

74   Haslam 1990, Schönbrunn et al. 2001). Since the shikimate pathway is specific to plants, fungi, bacteria

75   and some protists but is absent in animals, including humans (Maeda & Dudareva 2012), glyphosate was

76   regarded an ideal and safe application option. However, recent studies suggest the metabolisation of

77   glyphosate by humans leading to adverse effects on human health, already caused by chronic low-dose

78   glyphosate ingestion via residues in food and water (Swanson, Hoy & Seneff 2016). Those potential

79   effects include sub-clinical metabolic acidosis induced by shifts in the human gut microbiome,

80   mitochondrial dysfunction, endocrine disruption, DNA damage and the inhibition of the cytochrome

81   P450 enzyme system (Samsel & Seneff 2013, Swanson et al. 2014, Swanson, Hoy & Seneff 2016).

82   Concerning application in the field, glyphosate is advertised as a herbicide with poor rainfastness (Baylis

83   2000), is known to bind to soil particles and is degraded by microbes (Rueppel et al. 1977, von Wirén-

84   Lehr et al. 1997, Forlani et al. 1999). Although Giesy, Dobson & Solomon (2000) stated that less than 1%

85   of glyphosate is washed from the leaves of plants by rainfall directly after application, spray drift and

86   runoff may occur that contribute to the entry of glyphosate into non-target environments, particularly

87   aquatic systems (Giesy, Dobson & Solomon 2000). Furthermore, incidences of e.g. incorrect disposal of

88   packages and expired herbicide remnants or careless cleansing of application instruments might

89   enhance the amount of glyphosate entering the environment.

90   Since glyphosate is applied as formulation, additives like polyoxyethylene tallow amine (POEA), a

91   surfactant that facilitates glyphosate uptake through the plant cuticula, are released into ecological

92   systems inducing adverse effects on non-target organisms at least as severe as glyphosate itself (Perkins,

93   Boermans & Stephenson 2000, Tsui & Chu 2003, Brausch, Beall & Smith 2007, Bringolf et al. 2007,

94   Moore et al. 2012, Defarge, Spiroux de Vendômois & Séralini 2018).   However, glyphosate is not

95   monitored routinely (Uren Webster et al. 2014), and numerous studies show that it is transported from

96 fields (Struger et al. 2008), national parks (Battaglin et al. 2009) and urban areas (Botta et al. 2009) into

97 surface waters and even groundwater. In the environment, glyphosate is degraded to CO_2 and

98 aminomethylphosphonic acid (AMPA), which, in turn, is degraded to inorganic phosphate, ammonium

99 and CO_2. Glyphosate's half-life depends on the surrounding matrix and is estimated between 4.2 and 14

100 days for fresh water (Giesy, Dobson & Solomon 2000, Vera et al. 2010), mainly below 60 days in

101 agricultural soils (Giesy, Dobson & Solomon 2000, Grunewald et al. 2001) and between 47 and 315 days

102 in seawater (Mercurio et al. 2014). Depending on time and location, Struger et al. (2008) measured a

103 mean maximum glyphosate concentration in surface waters between 10 and 20 µg/L, peaking at up to

104 40 µg/L. Botta et al. (2009) could detect 75 to 90 µg/L in storm sewers after rainfall, and Battaglin et al.

105 (2014) even found top concentrations up to 301 µg/L in lake/wetland samples. Although those findings

106 range below the U.S. EPA's maximum contamination level of 700 µg/L (U.S. EPA 2009) or the Canadian

107 long- and short-term freshwater aquatic life standards of 800 and 27 000 µg/L, respectively (Canadian

108 Council of Ministers of the Environment 2012), adverse effects in non-target organisms in the

109 environment have been demonstrated, as already indicated. Therefore, it is of major importance to

110 further elucidate the mechanisms by which glyphosate affects non-target organisms and to assess its

111 risk more realistically. In the long run, it could be conceivable to further establish ecologically sound

112 alternatives, including bioherbicides and synthetic herbicides on the basis of natural phytotoxins (Dayan

113 & Duke 2014), that can potentially substitute for glyphosate.

114 Since this study is regarded as fundamental research, the zebrafish *Danio rerio*, as a prominent and

115 easy-to-handle standard organism in the field of ecotoxicology, was used, and the glyphosate

116 concentrations chosen were rather high (1 µM [1.69 mg/L] – 10 mM [1.69 g/L]) and less environmentally

117 relevant, as glyphosate concentrations measured in surface waters lie mainly within a range of µg/L (e.g.

118 Struger et al. 2008, Botta et al. 2009, Battaglin et al. 2014). Glyphosate, in its pure compound form, is

119 known to be a highly polar molecule, a polyprotic acid with pK_a values of <2, 2.3 and 5.6 (Bromilow et al.

120   1993, Wade et al. 1993, Borggaard & Gimsing 2008). Consequently, it lowers the pH of the medium

121   when in solution. As an ionisable substance, glyphosate exerts a higher toxicity in low pH environments,

122   where a higher proportion of the molecules are present in a neutral state, in which an uptake through

123   biomembranes is facilitated, compared to glyphosate ions (Erickson et al. 2006, Saparov, Antonenko &

124   Pohl 2006, Ehrl et al. 2018). Thus, it might be expected that glyphosate toxicity is enhanced in systems

125   dealing with acidification already, whereas in systems characterised by more alkaline surroundings, the

126   toxicity might be mitigated. Although glyphosate has been studied regarding its impact on non-target

127   organisms, including adverse effects on early stages in zebrafish (e.g. Sulukan et al. 2017, Zhang et al.

128   2017, Fiorino et al. 2018), the influence of pH on toxicity results have not been considered so far.

129   The aim of our study was to differentiate the effects of glyphosate-induced acidification of the medium

130   and those exerted by the compound itself (independent of low pH) on embryonic and early larval

131   development of *Danio rerio*. This study should provide lethal and effect concentration data (LC/EC$_{10}$ and

132   LC/EC$_{50}$) for a more profound risk assessment. Besides mortality, additional sublethal endpoints,

133   including hatching, the heart rate, developmental delays and malformations, were observed to account

134   for subtler impacts on embryonic health. For that purpose, glyphosate was tested at concentrations

135   between 10 μM and 10 mM in an unbuffered medium and in an aqueous medium adjusted to pH 7. In

136   addition, the critical range of pH values was determined and tested with and without 1 mM glyphosate.

137   To investigate whether possible effects are specific to glyphosate or to general inhibition of the

138   shikimate pathway, we tested the newly described shikimate pathway inhibitor, 7-deoxy-sedoheptulose

139   (7dSh). This substance is produced by cyanobacteria and was used at a concentration of 1 mM, which is

140   more than one order of magnitude above the effective concentration required to inhibit the shikimate

141   pathway (Brilisauer et al. 2019).

142

143

144 **Material and methods**

145

146 *Glyphosate*

147 Glyphosate [N-(phosphonomethyl)glycine, 96% pure substance, molecular weight: 169.07 g/mol, CAS:

148 1071-83-6, *Sigma-Aldrich, Merck KGaA,* Darmstadt, Germany] was used to prepare the test solutions. A

149 stock solution with a concentration of 25 mM was prepared as follows: glyphosate was diluted in

150 reconstituted water (0.23 g KCl, 2.59 g NaHCO$_3$, 4.93 g MgS$_4$O · 7 H$_2$O and 11.76 g CaCl$_2$ · 2 H$_2$O were

151 dissolved separately in 1 L double-distilled water, then 25 mL of each stock solution was added to 900

152 mL double-distilled water). The stock solution was then diluted to the following test concentrations: 10

153 μM, 50 μM, 100 μM, 250 μM, 500 μM, 750 μM, 1 mM and 10 mM glyphosate (respective specifications

154 for mg/L and pH are given in *Table 1*). All those concentrations were tested unbuffered and at pH 7. For

155 pH adjustments, 1 M HCl and NaOH solutions were used as recommended in the OECD 236 (2013)

156 guideline. For investigations of the influence of pH, 1 mM glyphosate was tested at different pH values

157 ranging between pH 3 and pH 8 *vs.* the respective pH controls. Due to preliminary results from the

158 broad-scale pH testing, particular attention was paid to the range between pH 3 and pH 4.

159 Measurements of pH were conducted with a pH meter (*SevenCompactDuo*, *Mettler Toledo*, Gießen,

160 Germany) directly prior to the exposure.

161

162 *7-deoxy-sedoheptulose (7-deoxy-D-altro-2-heptulose, 7dSh)*

163 The compound *7*-deoxy-sedoheptulose (7dSh) was obtained by chemoenzymatic synthesis and purified

164 as described in Brilisauer et al. (2019). This 7dSh was tested at a 1 mM concentration alongside the

165 unbuffered glyphosate treatments in an unbuffered system. Thus, 7dSh was tested as a neutral solution,

166 since it has no influence on pH.

167

168   *Maintenance of zebrafish and test procedure*

169   The embryos used in this study stem from our own breeding stock of the *Danio rerio* [Hamilton 1822]

170   Westaquarium strain established in the Animal Physiological Ecology group, Tübingen University. Adult

171   zebrafish were kept in 90-L aquaria filled with a 1:1 mixture of purified water and filtered tap water (AE-

172   2L water filter with an ABL-0240-29 activated carbon filter, 0.3 μm; *Reiser*, Seligenstadt, Germany) at 26

173   ± 1 °C and an oxygen saturation of 100 ± 5%. Conductivity ranged from 260 to 350 μS/cm, nitrite and

174   nitrate concentrations from 0.025 to 0.1 mg/L, 1 and 5 mg/L, respectively, and total water hardness

175   from 8 to 12 °dH. Fish were subjected to an artificial 12:12 hour day/night cycle and fed three times

176   daily with flake food (*TetraMin®*, *Tetra GmbH*, Melle, Germany) supplemented with frozen black

177   mosquito larvae and glass worms (*Poseidon Aquakultur Freeze,* Ruppichteroth, Germany) prior to

178   spawning to ensure sufficient dietary protein.

179   The day before the test, pre-exposure and test Petri dishes (90 mm and 30 mm in diameter) were filled

180   with the respective solutions and stored at 26 ± 1 °C overnight to saturate the glass (the same was done

181   with the Schott flask used for the stock solution, beforehand). On the morning of the test, Petri dishes

182   were emptied and refilled with 70 mL (pre-exposure) and 3 mL (test Petri dishes) solution. For spawning,

183   Plexiglas® boxes 20 x 20 x 6 cm in size and covered with a mesh grid to keep zebrafish from feeding on

184   their own eggs were used as breeding boxes. They were topped with artificial sea grass acting as an

185   optical spawning stimulus and were placed into the fish tanks the evening before the start of the test.

186   Zebrafish spawn at sunrise; therefore, spawning in the laboratory starts with the onset of light the next

187   morning. Eggs were collected with a sieve, rinsed with tepid tap water, transferred into pre-exposure

188   Petri dishes and incubated for 2 hours at 26 ± 1 °C. Following the pre-exposure, eggs for the test were

189   chosen with regard to their age and developmental stage (0 hours post fertilisation [hpf] ≜ 8 a.m.),

190   placed into the small 30 mm Petri dishes and stored in a heated cabinet at 26 ± 1 °C. A total of 32

191   individuals were used per treatment, i.e. four per Petri dish and eight replicates each. Embryos were

192   checked every 12 to 24 hours. Endpoints investigated under a stereo microscope (*Stemi 2000-C, Zeiss*)

193   included mortality, developmental delays at 24 hpf, heart rate at 48 hpf, hatching success from 60 to 96

194   hpf and malformations at 96 hpf (see *Table 2*). Except for mortality, analysis of all endpoints, including

195   hatching success, was based on living embryos/larvae at the respective time point of evaluation. Heart

196   rates were determined from two out of four individuals per Petri dish for 20 seconds, and values were

197   extrapolated to 1 minute. Coagulated eggs, dead larvae and empty egg shells were removed from the

198   Petri dishes to avoid depletion of oxygen due to biological degradation processes. The embryo test was

199   run three times and conducted according to OECD 236 (2013). The compound 3,4-dichloraniline (98%,

200   CAS: 95-76-1, *Sigma-Aldrich, Merck KGaA*, Darmstadt, Germany) at a concentration of 4 mg/L served as

201   a positive control and reconstituted water, as a negative control.

202   According to the Directive 2010/63/EU of the European Parliament and the Council on the protection of

203   animals for scientific purposes, *Danio rerio* embryos and larvae that do not feed independently are not

204   regarded as animals, thus regulations and permissions for animal testing do not apply. Nevertheless, all

205   embryos in our tests were handled in the least stressful way possible and with the utmost care. After

206   test termination embryos/larvae were euthanised with MS222.

207

208   *Statistics*

209   All statistical analyses were conducted in *JMP® 11.2.0 (SAS Institute Inc. 2013)*. Mortality, hatching

210   success and the malformation rate at 96 hpf, as well as developmental delays at 24 hpf, were analysed

211   with a likelihood-ratio χ^2 test, followed by Fisher's exact test. Finally, the sequential Bonferroni-Holm

212   method was applied accounting for multiple testing. A Cox regression was used to assess mortality and

213   hatching success over time. For the analysis of heart rate, the data were averaged per Petri dish and

214   checked for a normal distribution and homogeneity of variances. Subsequently an ANOVA with Tukey's

215   HSD or Dunnett's test was conducted. If data did not meet the criteria for an ANOVA and transformation

216 of the data did not lead to the desired result, a non-parametric Steel-Dwass test was conducted instead.

217 Additionally, for assessing the pH range in which pH control and glyphosate treatments differed in heart

218 rate across the whole span of tested pH, non-linear regression analysis, including calculation of 95%

219 confidence intervals (*TableCurve 2D v5.01, SYSTAT Software Inc. 1989-2001*), was applied. Non-linear

220 regression analysis by *TableCurve* was also used for determining LC_{10}/EC_{10} and LC_{50}/EC_{50} values of

221 endpoints in unbuffered glyphosate treatments.

222

223

224 **Results**

225

226 After 96 hpf, mortality and hatching success were 0 % and above 80 %, respectively, in control embryos.

227 The 3,4-dichloraniline positive control induced high mortalities, with rates consistently above 80 % after

228 96 hpf. Thus, the validity criteria according to OECD 236 (2013), including sensitivity of zebrafish, were

229 met.

230

231 *Unbuffered glyphosate*

232 At the two highest concentrations tested (1 and 10 mM), it was already difficult to select well-developed

233 eggs after the two hours pre-exposure period. The yolk sac, which usually has a regular spherical shape,

234 was found to be asymmetric and partly oval, and the chorion fluid, which is naturally clear, was murky in

235 some cases and contained indefinable streaks (*Fig. 1*). As early as 12 hpf, all individuals, without

236 exception, in the 10 mM treatment died (*Fig. 2A*). Mortality in the 1 mM exposure experiment was

237 beyond 85 % at 12 hpf and reached 100 % within the first 24 hours. Within the 750 μM glyphosate

238 treatment, only 6 out of a total of 96 individuals survived until the end of the test at 96 hpf, whereas

239 concentrations of 250 μM and below resulted in negligible or no mortality (≤ 3.125 %). Regarding

240   mortality at 96 hpf, all treatments ≥ 500 µM were highly significantly different from the control

241   (likelihood ratio χ^2, p < 0.001). Lethal concentrations were calculated to be 385 µM (LC_{10}) and 582 µM

242   (LC_{50}) at 96 hpf.

243   Heart rates showed a concentration-dependent relationship, decreasing with increasing glyphosate

244   concentration (*Fig. 2B)*. The mean heart rate was 149 beats per minute [bpm] for the control and

245   between 130 and 140 bpm for low (10 µM, 50 µM), 120 and 130 bpm for medium (100 µM, 250 µM)

246   and 110 and 120 bpm for the higher (500 µM, 750 µM) concentrations. Thus, differences between the

247   control and the 750 µM concentration ranged between 30 and 40 beats per minute. The treatments

248   with the highest concentrations of glyphosate (1 mM, 10 mM) could not be evaluated due to 100 %

249   mortality at that time point. Only two individuals out of those exposed to 1 mM glyphosate survived

250   until 60 hpf and seemed to continue the observed relationship between glyphosate and heart rate by

251   showing even lower rates (93 and 96 bpm). As single individuals, they were not included in the statistical

252   analysis. All remaining treatments were significantly different from the control (ANOVA with Tukey's

253   HSD, p < 0.001) and the relationship between glyphosate concentration and heart rate could be

254   described by linear regression analysis (R^2 = 0.546074, p < 0.001). The EC_{10} was 43 µM.

255   Concerning the hatching rate, we observed a clear division between a cluster of treatments that

256   comprised the control treatment and lower concentrations of glyphosate (10 µM, 50 µM, 100 µM) and

257   another treatment cluster comprising higher concentrations (250 µM, 500 µM, 750 µM) (*Fig. 2C)*.

258   Embryos exposed to lower concentrations hatched in 98 to 100 % of cases, whereas hatching success in

259   the experiments with 250 and 500 µM glyphosate was approximately 40 %. All glyphosate treatments

260   showed significant differences compared with the control (Cox regression, p < 0.05). EC_{10} and EC_{50} levels

261   at 96 hpf were 155 and 224 µM, respectively.

262   There were no developmental delays at 24 hpf for glyphosate concentrations between 10 and 100 µM,

263   whereas in treatments with 250 to 750 µM, rates varied from 15 % to 25 % (*data summarised in Table*

264   *3*). The EC_{10} for this endpoint was 126 µM. Results for all concentrations ≥ 250 µM were highly

265   significant (likelihood ratio χ^2, p < 0.001) compared with the control. A direct concentration dependency

266   could not be observed. Rather, it seemed that a distinct concentration threshold had to be exceeded to

267   induce those developmental delays and failures, which later approached the same level. Prevalent

268   defects were a lack of tail detachment, sometimes combined with apically curved tails (*Fig. 3F*); a lack of

269   somite formation and an impairment of eye development was not detected. Occasionally, embryos

270   were fully developed but either the complete tail or just the posterior end of their tails remained

271   attached to the yolk sac (*Fig. 3E*). Under normal conditions, movement begins after tail detachment. Yet,

272   even the embryos in glyphosate treatments that lacked tail detachment, overall development had

273   progressed to a point at which muscular contractions were already visible. But due to the undetached

274   tails, embryos were unable to turn around and their movement was very limited. Additionally, some

275   embryos had the posterior end of their tails detached but displayed severe spine deformations (*Fig. 3G,*

276   *H, I*). Those embryos could not move their tails in the same fluid manner as normally developed embryos

277   could.

278   Malformations could be found in embryos of all glyphosate treatments but with rates below 20 % (*data*

279   *summarised in Table 3*). All glyphosate treatments were significantly different from the control. Among

280   the malformations recorded, lightly pigmented embryos and larvae were particularly frequent (*Fig. 4C*).

281   Furthermore, reduced eye size occurred regularly (*Fig. 4B*), and some individuals suffered from cardiac

282   or yolk sac oedemas (*Fig. 4E*). Two individuals showed a notable shortening of the tail (*Fig. 4D*).

283   Deformations of the spine at 96 hpf were observed surprisingly rarely, despite the high rates of tail and

284   spine malformations at 24 hpf.

285   In contrast to the glyphosate results, unbuffered 7dSh, thus at a neutral pH, did not induce mortality

286   throughout any of the experiments, and no developmental delays or malformations occurred in 7dSh-

287   exposed embryos. Sublethal effects were limited to a slightly decelerated heart rate (140 bpm), which

288   corresponded to the 10 µM glyphosate treatment (138 bpm) but was 8 bpm lower than in embryos of

289   the negative control (ANOVA with Dunnett's test, p < 0.001), and an accelerated hatching over time (Cox

290   regression, p < 0.05) compared with the negative control. At 60 hpf, the hatching rates in the 7dSh-

291   exposed group and the negative control were similar but clearly below those of the groups exposed to

292   low glyphosate concentrations. At 72 hpf, 7dSh outpaced the negative control and converged on the

293   results of 10 to 100 µM glyphosate treatments. In contrast, the hatching success of 7dSh-exposed

294   embryos at 96 hpf did not differ from that of the negative control.

295

296   *Glyphosate at pH 7*

297   When the glyphosate solutions were adjusted to pH 7, almost no mortality or developmental delays

298   occurred, and malformation rates were below 10 % but were still significantly elevated in 1 mM and 10

299   mM treatments (likelihood ratio χ^2, p < 0.001; *Table 3*). In the concentration range of 10 to 500 µM,

300   heart rates showed a similar trend to those in unbuffered treatments but at a lower level: beats per

301   minute decreased with increasing concentration (*Fig. 2B*). Still, treatments between 100 and 500 µM

302   differed significantly from the negative control (Tukey's HSD, p < 0.01). At 750 µM, heart rates increased

303   again, with a higher frequency than at 250 and 500 µM. At the two highest concentrations (1 mM, 10

304   mM), heart rates were, on the one hand, marginally decelerated (1 mM) and on the other hand,

305   marginally accelerated (10 mM) compared with the negative control. Thus, it seems that there is a

306   turning point between 500 and 750 µM, at which the relationship between increasing concentration and

307   heart rate shifts from deceleration to acceleration in comparison with the negative control.

308   As already seen for lower concentrations in unbuffered treatments, glyphosate tends to induce early

309   hatching, even at the lowest concentration and independently of concentration. This effect unfolded to

310   its true extent in the pH-neutral treatments (*Fig. 2D*). At least twice as much larvae had hatched across

311   all glyphosate treatments at 60 hpf compared with the negative control. After 72 hpf, all larvae were

312   hatched in glyphosate treatments, except for single individuals that hatched at 96 hpf or did not hatch at

313   all, whereas in the negative control, only 53 % of the embryos were hatched at 72 hpf and even about

314   15 % remained unhatched at 96 hpf.

315

316   *pH range*

317   In a first step, 1 mM glyphosate was tested at pH 3, pH 4, pH 5, pH 6, pH 7 and pH 8 in comparison with

318   negative controls at the respective pH but without the pesticide (*Fig. 5*). Mortality was 100 % for both

319   treatments at pH 3, independent of the presence of glyphosate. Only a single individual survived the first

320   12 hpf. In contrast, only one individual died throughout all other exposures within 96 hpf. Morphological

321   aberrations described for high glyphosate concentrations under unbuffered conditions (*Fig. 1 and 3*) also

322   applied to low pH treatments, independent of glyphosate addition. Concerning sublethal endpoints,

323   results between different acidities in the range of pH 4 to pH 8, as well as between control and

324   glyphosate within the same pH range, were inconspicuous for the most part. Thus, the pH 3 to pH 8

325   series was tested just once, and subsequent testing concentrated on the range from pH 3 to pH 4. Thus,

326   in the next step, pH 3, pH 3.25, pH 3.5, pH 3.75 and pH 4 were investigated in detail. As embryos

327   exposed to pH 3.75 and pH 4 did not show any prominent effects, only a single run was conducted, and

328   the final testing scheme was determined from pH 3 to pH 3.5 in 0.1 increments. Additionally, a test with

329   unbuffered glyphosate at a 1 mM concentration (which resulted in a pH of 3.2 in the test solution) was

330   included for direct comparison.

331   Mortality decreased with increasing pH. Treatments with a pH of 3.2 and lower induced 100 % mortality

332   after 96 hpf. Whereas embryos exposed to pH 3 and pH 3.1 died within 48 hpf at the latest, embryos in

333   pH 3.2 treatments survived considerably longer (*Fig. 5*). Apart from pH 3.5 without glyphosate, all

334   treatments showed elevated mortality rates compared with the negative control (Cox regression,

335    $p < 0.05$). There were no differences between control and glyphosate treatments with corresponding pH

336   values, except for the elevated mortality in unbuffered glyphosate compared with the respective pH 3.2

337   control.

338   Compared with the negative control, hatching was significantly delayed and also reduced in both

339   glyphosate and pH control treatments (Cox regression, p < 0.001). Whereas 30 % of the control embryos

340   hatched at 60 hpf, in the pH control and glyphosate exposures, the hatching rate at 60 hpf was

341   consistently below 5 % (see supplementary file, hatching rate). The tendency toward glyphosate-

342   induced premature hatching at 60 hpf that was observed in pH-neutral treatments was not evident at

343   low pH. Although not statistically significant (except for pH 3.5: Cox regression, p < 0.001), embryos

344   exposed to glyphosate tended to hatch earlier and more frequently than embryos in the respective pH

345   controls (see also *Table 3*).

346   Heart rates were significantly lowered by glyphosate at pH 3.3 to 3.5, as well as by the corresponding

347   control pH treatments (Steel-Dwass, p < 0.001). Differences between glyphosate and the respective

348   controls at the same pH value could only be detected when the full pH range dataset (including results

349   for pH 3 to pH 8) was analysed. At a pH between 5.55 and 6.02, glyphosate elevated the embryonic

350   heart rate significantly compared with pH controls (*TableCurve 2D v5.01; Fig. 6*). Developmental delays

351   and malformations occurred in the low pH treatments, but they did not vary in a pH-dependent manner,

352   and there was no detectable difference between glyphosate and the respective pH controls.

353

354   *Comparison*

355   When datasets for the unbuffered glyphosate treatment and the pH range were merged regarding

356   mortality in relation to pH (*Fig. 5*), interestingly, embryos exposed to unbuffered glyphosate showed

357   higher mortalities at 500 and 750 µM compared with their 1 mM counterparts at pH 3.5 and pH 3.4,

358   respectively. The unbuffered 750 µM treatment with a pH of 3.4, in particular, resulted in a mortality

359   rate more than twice as high as that in the glyphosate pH 3.4 treatment (1 mM), mirroring mortality

360   effects seen in treatments ranging rather between pH 3.25 and pH 3.3.

361

362

363   **Discussion**

364

365   The results we obtained for unbuffered glyphosate treatments are in close agreement with those

366   reported in the literature. Fiorino et al. (2018) found mortality rates below 10 % for concentrations

367   between 0.005 and 10 mg/L (< 100 µM, compare to *Table 1*), as well as 17 % mortality in 50 mg/L (≈ 295

368   µM) treatments after 96 hpf. Uren Webster et al. (2014) and Stehr et al. (2009) found no effect on

369   mortality in embryos exposed to concentrations up to 10 mg/L after 96 hpf. Those findings are in line

370   with our results for unbuffered glyphosate treatments. By contrast, Bortagary et al. (2010) also tested

371   higher glyphosate concentrations and detected mortalities of 35.4 % (25 mg/L), 74 % (75 mg/L) and 82 %

372   (150 mg/L) as early as 24 hpf. Their rates were remarkably higher than ours, in particular concerning

373   concentrations of 25 and 75 mg/L; however, the exposure temperature in Bortagary's set-up was 27.9 °C

374   and thus about 2 K higher than in our experiments. Although zebrafish easily tolerate temperatures

375   around 28 °C (López-Olmeda & Sánchez-Vázquez 2011), a 2 K temperature difference may result in a

376   higher metabolic rate. Thus, the effects of substances set in faster and might be more severe.

377   Nevertheless, the abundance and characteristics of developmental delays and malformations from

378   Bortagary's experiment matched our findings. They described delays in separation of the yolk sac that

379   very likely coincide with the lack of tail detachment observed in this study. Additionally, they found

380   deformations of the spine and deformities in the tail area that are in line with the findings presented

381   here. Similar effects occurred in glyphosate tests with Java medaka (*Oryzias javanicus*) embryos. Yusof,

382   Ismail & Alias (2014) detected increased alterations in the curvature pattern at concentrations ranging

383   from 100 to 500 mg/L glyphosate. Morphological alterations described for zebrafish embryos included

384   yolk sac and pericardial oedema, deformations of the skeleton and spine and misshapen yolk sacs

385   (Bortagary et al. 2010, Sulukan et al. 2017, Fiorino et al. 2018) and thus corresponded, at least partly, to

386   our findings of oedema, light pigmentation and small eyes. In Java medaka, embryo malformations like

387   disproportional head and body sizes, bends in tails and a lack of cornea were found (Yusof, Ismail & Alias

388   2014). Similar findings were reported in *Xenopus laevis* embryos incubated with 1/5000 dilutions of

389   glyphosate-based herbicides. Those embryos showed alterations in cephalic and neural crest

390   development and a shortening of the anterior-posterior axis, and when glyphosate was injected, optic

391   vesicles were reduced, resulting in the formation of smaller eyes or in their complete reduction. In chick

392   embryos, such an effect could be attributed to an increase in the activity of endogenous retinoic acid

393   (Paganelli et al. 2010). Retinoic acid is a derivative of vitamin A that is crucial to various processes in

394   vertebrates and thus ensures the proper development of zebrafish embryos on numerous levels (Holder

395   & Hill 1991, Marsh-Armstrong et al. 1994, Stafford & Prince 2002, Kawakami et al. 2005, Keegan et al.

396   2005, Laue et al. 2008). As a consequence, interference in retinoic acid signalling has the potential to

397   cause developmental delays and malformations during embryonic development in vertebrates. In this

398   context, Sulukan et al. (2017) investigated potential correlations of malformation induction with

399   carbonic anhydrases (CA), an enzyme family that is involved in various biological processes, including

400   respiration, CO_2 and ion transport, acid-base balance and the formation of reactive oxygen species

401   (ROS). They found malformations in all treatments (1–100 mg/L), with simultaneous inhibition of CA and

402   an increase in ROS leading to apoptosis, which was interpreted to be causally responsible for the

403   observed malformations. As CA inhibition can directly or indirectly be related to increasing ROS (Sulukan

404   et al. 2017) and, considering our results concerning pH, it might be questionable whether glyphosate

405   itself is the sole inducer of the observed effects or whether a low pH contributes to the elevation of ROS

406   levels and thus, might be at least as relevant regarding the development of malformations.

407   Although our results for mortality, developmental delays and malformations are in line with findings

408   reported in literature, which did not consider glyphosate-induced pH decrease, they were not

409   reproduced by glyphosate at pH 7. In a comprehensive analysis of data from pH control and glyphosate

410   treatments, it can be seen that the described effects occurred independently of glyphosate or its

411   concentration, as long as an acid milieu below pH 3.75 was provided. In the case of those endpoints in

412   zebrafish embryos, sublethal effects may be detected only between pH 3.3 and pH 3.75. A pH lower

413   than 3.25 induced 100 % mortality within 96 hpf and within 12 hpf at pH 3, whereas pH 3.75 to pH 8 did

414   not affect survival. However, it is not unlikely that sublethal effects caused by glyphosate itself are

415   masked by the severe consequences of low pH.

416   Even though our data revealed that the effects detected here and also reported in the literature are

417   likely to be caused mainly by low pH values, our data indicate that even at neutral pH, glyphosate affects

418   embryonic development. Early hatching reported, for example, by Uren Webster et al. (2014) and

419   Bortagary et al. (2010) at concentrations between 10 and 50 mg/L could be observed in pH neutral

420   treatments in particular and was not concentration dependent. As all embryos seemed to be well

421   developed at the point of hatching, early hatching should be interpreted as accelerated chorion

422   degradation rather than as premature hatching. According to Kimmel et al. (1995), the hatching period

423   of *Danio rerio* starts as early as at 48 hpf already, and there is no developmental difference under

424   normal conditions between larvae that hatch spontaneously and those remaining in the chorion. Thus,

425   glyphosate at pH 7 seems to contribute to more spontaneous hatching instances. During embryo

426   handling throughout the test, it was striking that the chorion of individuals within low pH and/or

427   glyphosate treatments was more sensitive to physical contact, e.g. while positioning embryos, compared

428   with negative control embryos. Chorions were never injured during the test procedure but became

429   dented easily. Through movements within eggs, embryos bulged out the dents by themselves rapidly.

430   Hence, accelerated hatching might be correlated with a more damageable chorion that has ruptured

431   sooner as a result of movements of the embryo with or without direct hatching intention. The fact that

432   the effect of accelerated hatching was found to be more pronounced in pH 7 treatments might be due

433   to the developmental status. Developmentally retarded embryos with late detachment of the tail in

434   lower pH treatments were less agile than normally developed individuals in neutral treatments at the

435   same time point and thus were less likely to rupture the chorion accidently. This assumption is basically

436   supported by observations from Yusof, Ismail & Alias (2014), who found a thinning of the embryonic

437   chorion of Java medaka following a 100 to 500 mg/L glyphosate exposure, and Zhang et al. (2017), who

438   detected a decrease in surface tension of the chorion combined with an increase in locomotion that

439   might lead to accelerated hatching in zebrafish embryos.

440   Regarding the heart rate, concentration dependency was still apparent at neutral pH, although it was

441   less pronounced than in the unbuffered glyphosate treatments. At a high glyphosate concentration, the

442   decreasing effect on the heart rate seemed to reverse, as the heart accelerated again. The outcomes of

443   the pH range treatments corroborate the importance of pH to the heart rate but also the potential of

444   glyphosate at 1 mM to accelerate the heart rate considerably, at least between pH 5.55 and pH 6.05.

445   Few studies have evaluated the effect of glyphosate on the heart rate in zebrafish embryos. Bortagary et

446   al. (2010) reported a decrease in the heart rate following exposure to concentrations exceeding 25

447   mg/L, whereas Roy et al. (2016) studied the effect of glyphosate on the cardiovascular system in detail

448   but solely regarding a single concentration. Their finding of a heart rate of 129 bpm in response to 50

449   mg/L glyphosate matches our results of 130 bpm at a slightly lower concentration of 250 µM glyphosate

450   at neutral pH (compare *Table 1*), whereas it differed from the unbuffered treatment with a delta of

451   about 4. Differences between studies might be attributed to temperature impact. Zebrafish embryos in

452   this study were reared and observed at 26 °C, whereas Roy et al. (2016) conducted the entire test at

453   28.5 °C. The specific effect of temperature on the zebrafish heart rate has been addressed previously

454   (Schweizer et al. 2017). The observed progressive decrease in heart rate might be correlated to

455 structural abnormalities found by Roy et al. (2016) for the impact of glyphosate on heart development.

456 In that study, the shape and size of the atrium and ventricle changed and decreased, the endocardium

457 was thickened and the vascular network throughout the body was poorly connected. As a result,

458 erythrocytes could not move properly and were slowed down on their way through the organism,

459 contributing to the formation of cardiac oedema. Although there are no references for the effects of

460 higher glyphosate concentrations on the zebrafish heart rate, our results are in agreement with a study

461 on Java medaka embryos by Yusof, Ismail & Alias (2014). The authors used 100 to 500 mg/L glyphosate

462 and detected only acceleration of the heart rate. A glyphosate concentration of 100 mg/L corresponds

463 to a glyphosate concentration of 591.47 µM and thus relates approximately to the turning point

464 between 500 and 750 µM, at which the decreasing trend in the heart rate was reversed.

465 Our study supports previous findings of negative effects of glyphosate on fish embryonic stages but, for

466 the first time, distinguishes between direct consequences of the substance itself (hatching, heart rate)

467 and indirect effects by lowering the pH of the surrounding medium (mortality, developmental delays,

468 malformations). Since the primary target of glyphosate is absent in metazoans, the toxic side-effects of

469 glyphosate may be caused either by secondary target sites or indirectly by inhibition of the shikimate

470 pathway in the associated microbiome of the tested animals (Samsel & Seneff 2013, Motta, Raymann &

471 Moran 2018) or impairment of symbiotic microorganisms in engineered plants with the glyphosate-

472 insensitive EPSP synthase class II (Zobiole et al. 2010), respectively. Since the control compound 7dSh,

473 which also inhibits the shikimate pathway, did not cause the side-effects observed for glyphosate, the

474 observed effects were more likely caused by secondary target sites of glyphosate.

475

476 Although the tested concentrations were rather high compared with measured field concentrations, the

477 inherent adverse potential of glyphosate in combination with further increasing application rates is

478 worrying. Lethal and severe sublethal effects might be prominently driven by glyphosate's ability to

479   drastically lower pH but, given that its acidic character contributes largely to its efficacy, the herbicide's

480   action and the pH can hardly be considered separately. Due to their acidity, glyphosate anions have a

481   high affinity to bind to cations and build neutral salt complexes, which enhance uptake through

482   biomembranes (Rendal, Kusk & Trapp 2011). Nevertheless, their high binding capacities also apply to

483   strongly charged cations in soil (e.g. Fe^{3+}, Al^{3+}), whose binding inactivates glyphosate (Borggaard &

484   Gimsing 2008, Helander, Saloniemi & Saikkonen 2012), which, in turn, seems to make leaching into

485   surface water or groundwater – per se – a minor problem. However, glyphosate competes with

486   phosphate for binding sites (Borggaard & Gimsing 2008). In phosphate-saturated soils that prevail in

487   areas of intensive agriculture, in which glyphosate is preferably applied, soil particles have a very limited

488   capacity to bind glyphosate anions in addition to phosphate (Helander, Saloniemi & Saikkonen 2012).

489   Consequently, in these cases, leaching of glyphosate into waters must be taken into account. If

490   glyphosate is leached into surface waters with little buffering capacity, side effects of the pesticide

491   should not be underestimated (e.g. Folmar, Sanders & Julin 1979, Howe et al. 2004, Relyea 2005,

492   Paganelli et al. 2010, Vera et al. 2010, Lopes et al. 2014, Uren Webster et al. 2014, Roy et al. 2016,

493   Fiorino et al. 2018). In particular, when glyphosate comes in contact with organisms under slightly acidic

494   conditions: Ehrl et al. (2018) showed that the permeability of glyphosate through biomembranes is

495   increased in a slightly acidic compared to a neutral surrounding. Thus, it may be assumed that

496   glyphosate effects increase with lower pH based on a facilitated uptake of glyphosate into cells.

497   Although recent studies even suggest, that the toxicity exerted by glyphosate in the field is rather

498   caused by certain additives within applied formulations, including POEA, than by glyphosate itself (Tsui

499   & Chu 2003, Moore et al. 2012, Defarge, Spiroux de Vendômois & Séralini 2018), we could clearly show

500   that glyphosate as pure compound is able to induce adverse effects in a non-target organism. And in the

501   light of potential health effects even in human beings induced by a chronic but low dose exposure to

502   glyphosate (Samsel & Seneff 2013, Swanson et al. 2014, Swanson, Hoy & Seneff 2016), the ideal

503   prospective solution for this issue would be a long-term replacement of glyphosate. The compound

504   7dSh, which bases its efficacy on interfering with the shikimate pathway, similar to glyphosate, seems to

505   be a promising candidate. Its effectiveness in plants has been demonstrated (Brilisauer et al. 2019), and

506   in contrast to glyphosate, it neither lowers pH nor induces severe effects in zebrafish embryos, as shown

507   in our study. No mortality, developmental delays or malformations could be detected in embryos

508   exposed to 1 mM 7dSh.

509

510

511   **Conclusions**

512

513   Although the severe effects detected seemed to be mainly caused by a low (glyphosate induced) pH, the

514   compound glyphosate itself affects embryonic development in *Danio rerio* on a sublethal level. Direct

515   comparison between pH controls and 1 mM glyphosate at the respective pH showed only minor

516   differences, but severe pH effects might mask the impacts of glyphosate. From a non-target perspective,

517   it would be advantageous to buffer glyphosate to a neutral level to avoid the severest effects on fish if

518   glyphosate were leached into the aquatic environment at higher concentrations. As glyphosate's acidity

519   elevates its efficacy as a herbicide, such a recommendation is highly unlikely to be implemented by

520   producers and users. In view of the protection of non-target species from side effects, alternatives

521   should be considered that might potentially substitute glyphosate in the long term, and we have shown

522   that 7dSh may be a promising emerging candidate.

523

524

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527

528

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530

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710

Figure 1

Figure 1: *Danio rerio* embryos at 3.3 hpf ("high stage", blastula period, according to Kimmel et al. (1995)).

A) Control embryo; B), C), D) exemplary embryos with irregularly growing cellular "hood" or misshapen yolk sacs from 1 mM glyphosate concentration; as seen at five times magnification under a stereo microscope (*Stemi 2000-C*, *Zeiss*).
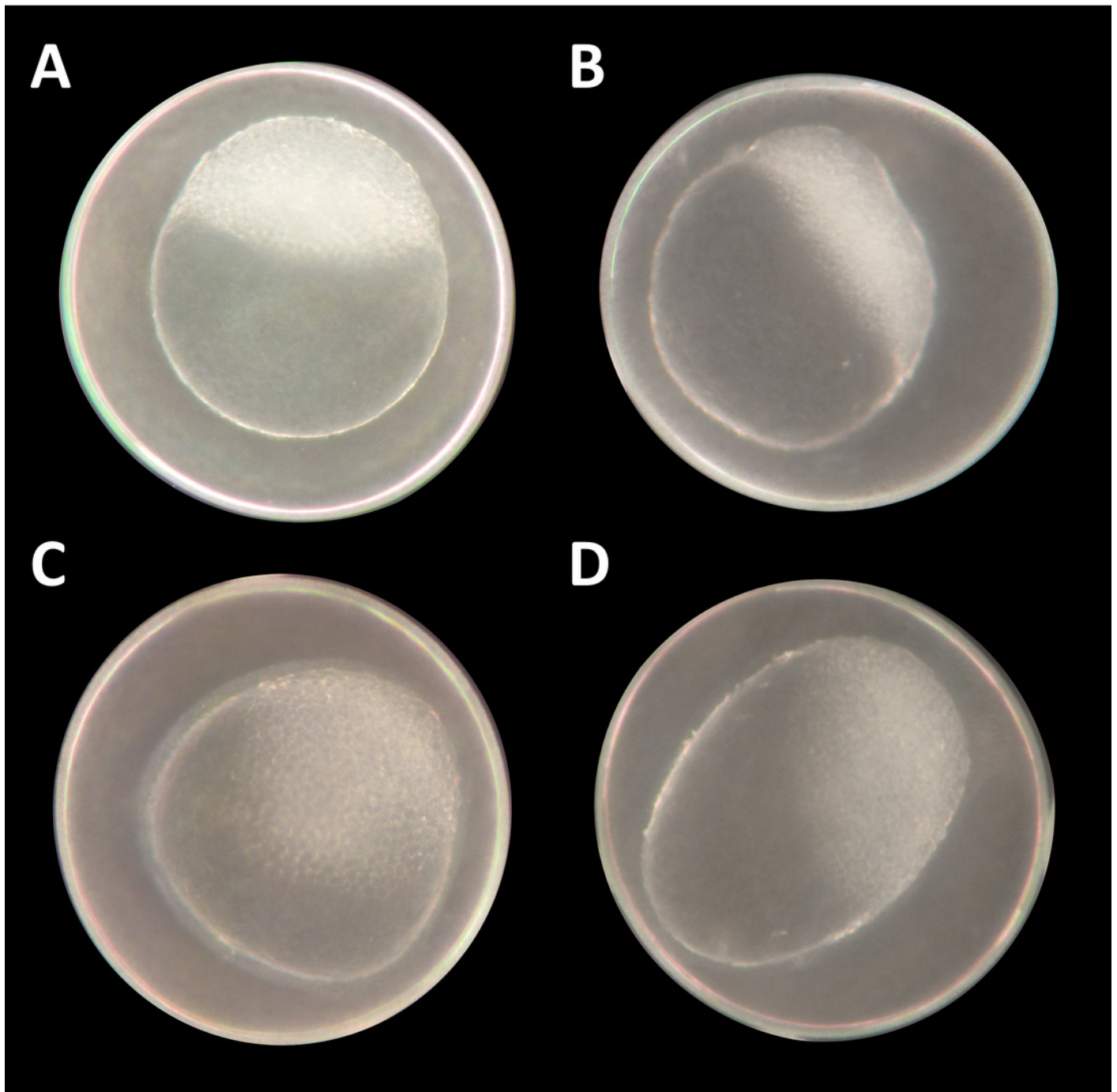
Figure 2

Figure 2: Mortality, heart rate and hatching success in percentage of unbuffered and pH 7 treatment.

A) Mortality after 96 hpf (likelihood ratio χ^2, Fisher's exact test, Bonferroni-Holm, $p< \alpha$), B) heart rate at 48hpf relative to the negative control (Steel-Dwass, $p < 0.01$), C) hatching rate over time in unbuffered treatments (Cox regression, $p < 0.05$), D) hatching rate in pH 7 treatments over time (Cox regression, $p < 0.001$); shaded bars mark treatments with n < 5 that show tendencies but are not included in the statistical analyses.
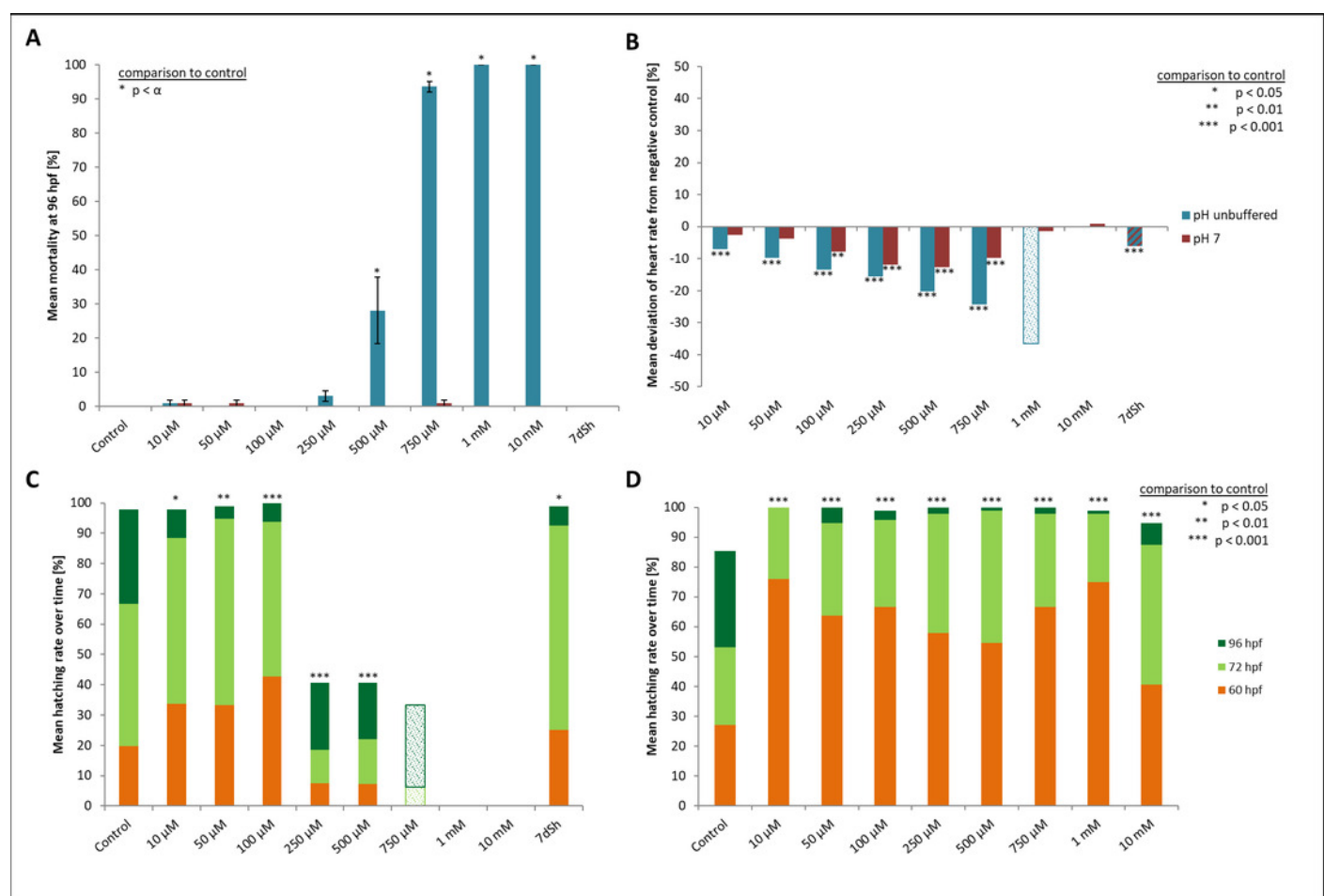
Figure 3

Figure 3: Danio rerio embryos at 24 hpf.

A) Coagulated control embryo; B) coagulated embryo in glyphosate (1 mM); C) streaks within the chorion; D) control embryo; E) no detachment of the tail; F) attached tail growing curved at the distal end; G) dorsal dent; H) irregular curved spine; I) anomalously straight tail; as seen at five times magnification under a stereo microscope (*Stemi 2000-C, Zeiss*).

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.
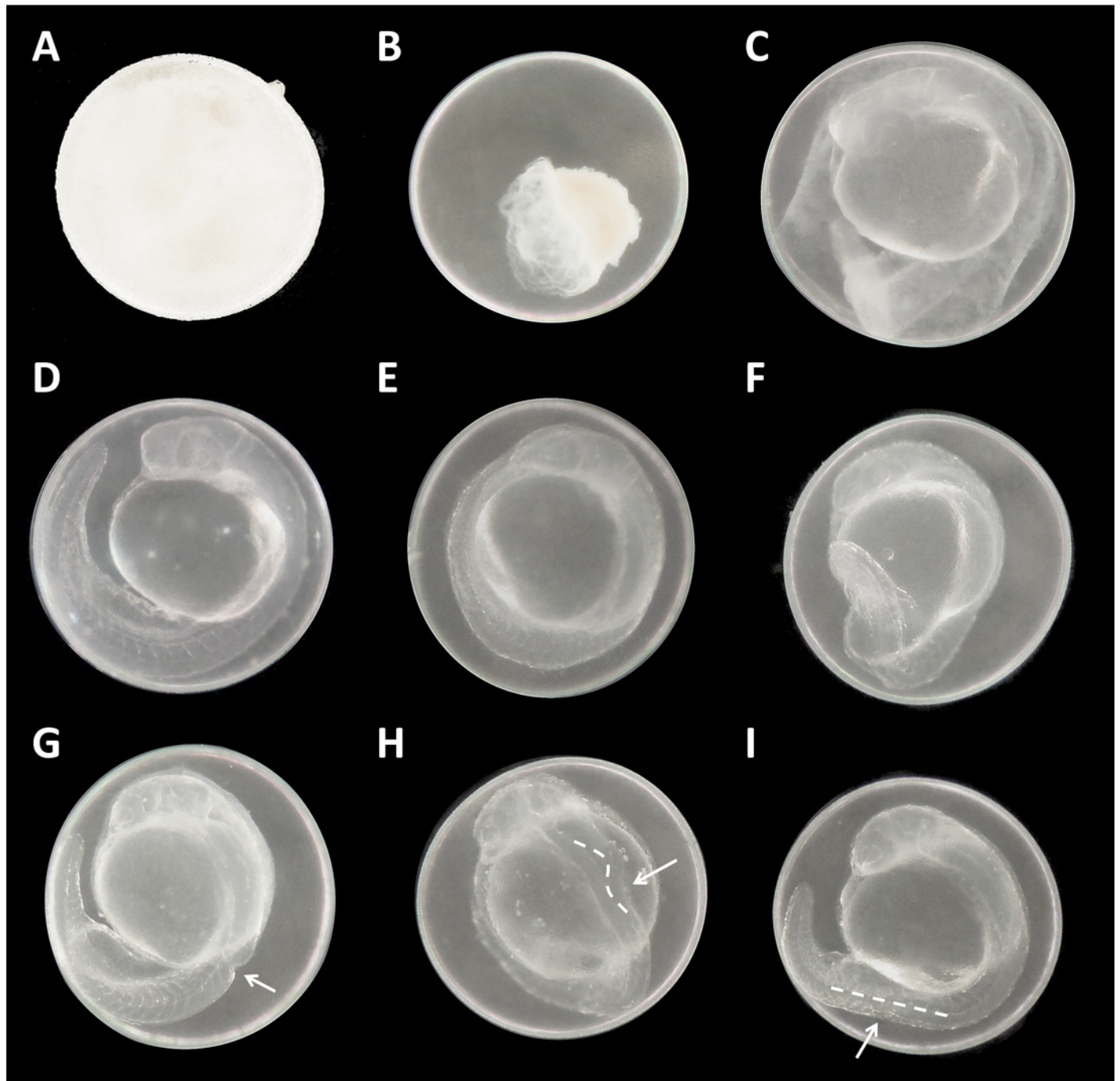
Figure 4

Figure 4: *Danio rerio* larvae at 96 hpf.

A) Control; B) upper larvae with smaller eyes compared with control individual below; C) right larvae with light pigmentation compared with control individual on the left; D) larvae with a shortened tail; E) larvae with cardiac oedema; F) larvae with spine deformation; D)-F) larvae show smaller eyes as regular, additionally; as seen at three to five times magnification under a stereo microscope (*Stemi 2000-C, Zeiss*).

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.
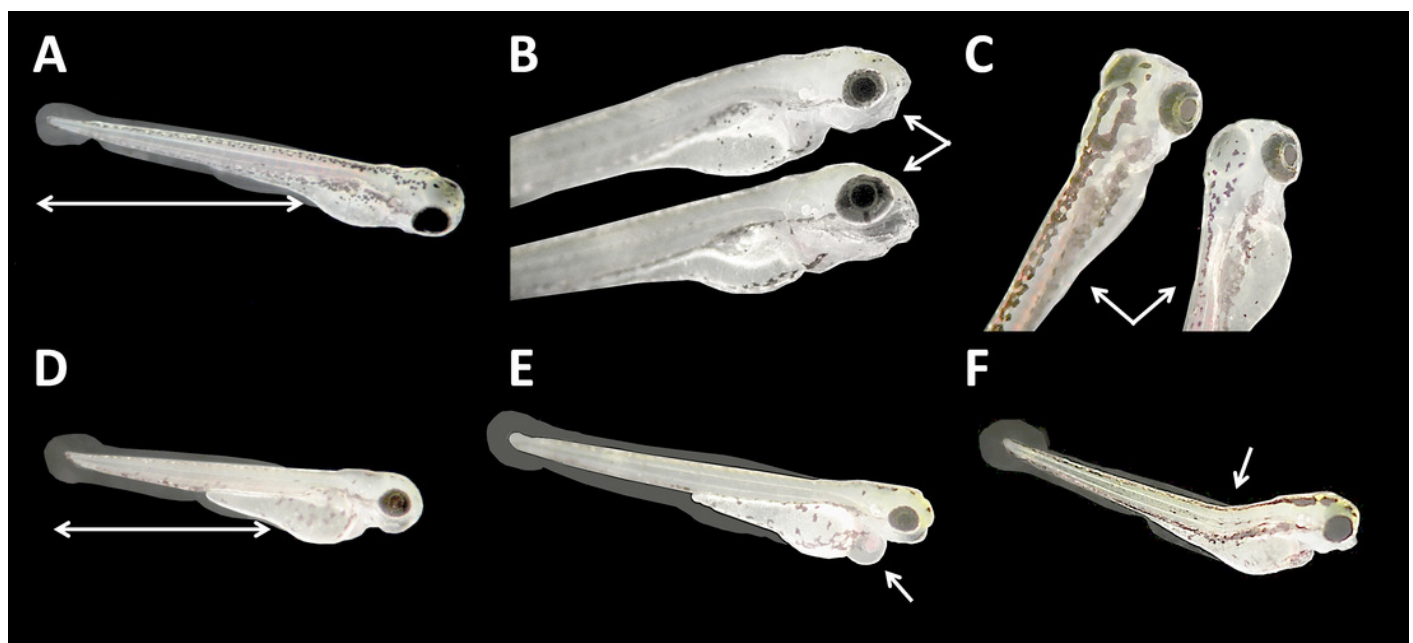
Figure 5

Figure 5: Mortality over time as percentages of embryos exposed to the pH control [A] and glyphosate [B].

Respective concentrations of glyphosate are given in brackets. Results from unbuffered treatments (50 µM–10 mM glyphosate; highlighted in red) are combined with pH range results and positioned according to their measured pH. Treatments not conducted in the pH control or glyphosate scheme are labelled n.a. (not available). Significant differences from the negative control are marked with asterisks (*), except glyphosate pH 3.4 with an additional significant comparison between unbuffered and pH 7 treatment. Significances between pH control and glyphosate treatments within respective pH ranges are denoted with letters (a, b) (Cox regression, p<0.01).
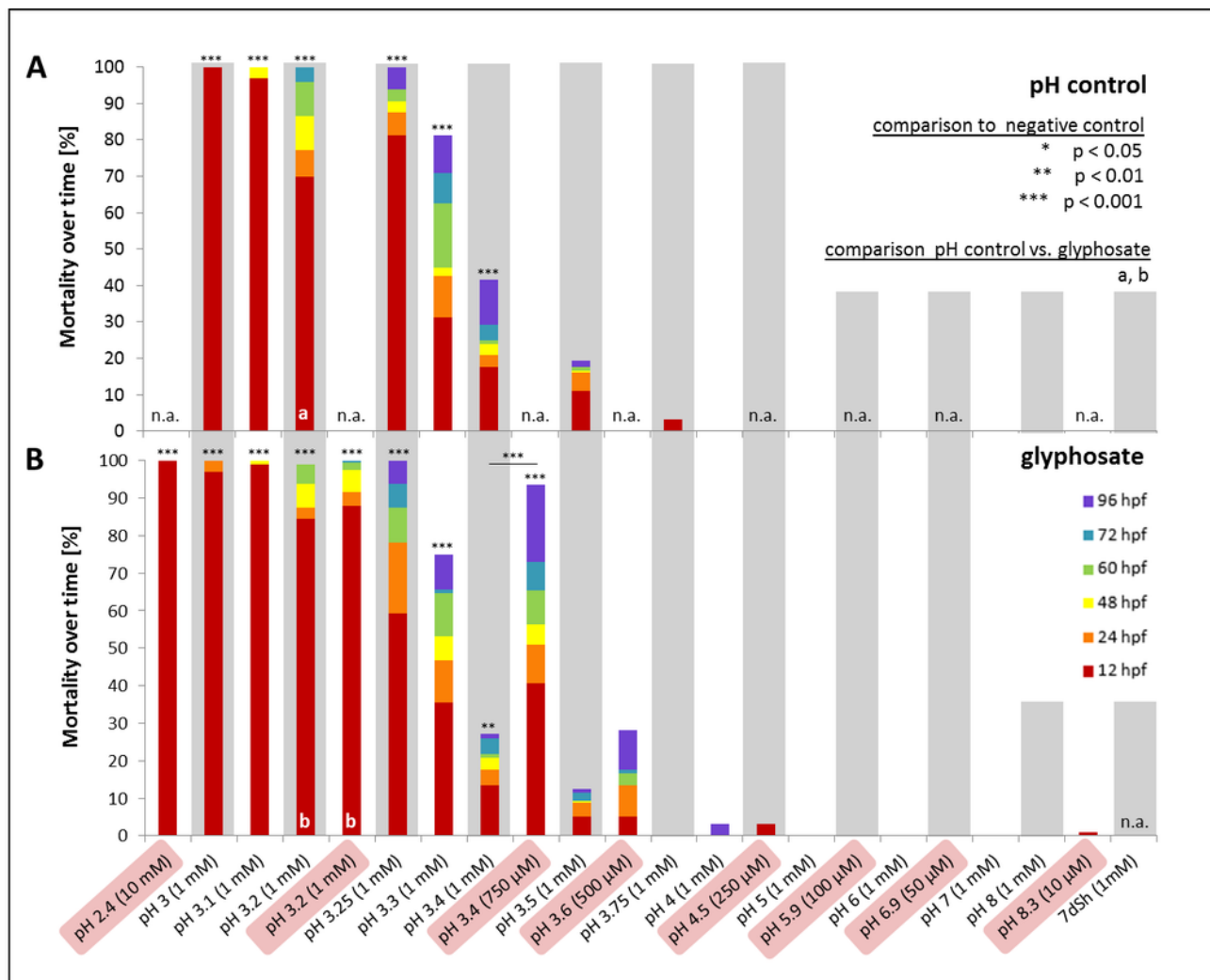
Figure 6

Figure 6: Mean deviation of the heart rate from the negative control in percent as a function dependent on pH; pH control: $y = a + bx + cx^{1.5} + dx^{2.5} + ex^{0.5}$; glyphosate: $y = a + bx + cx^2 + de^x + e(\ln x)[su$

Coloured areas mark the 95 % confidence interval. Green lines confine the pH range (between pH 5.55 and pH 6.05) in which the 95 % confidence intervals of pH control and glyphosate do not overlap and thus, where heart rates differ significantly from each other.
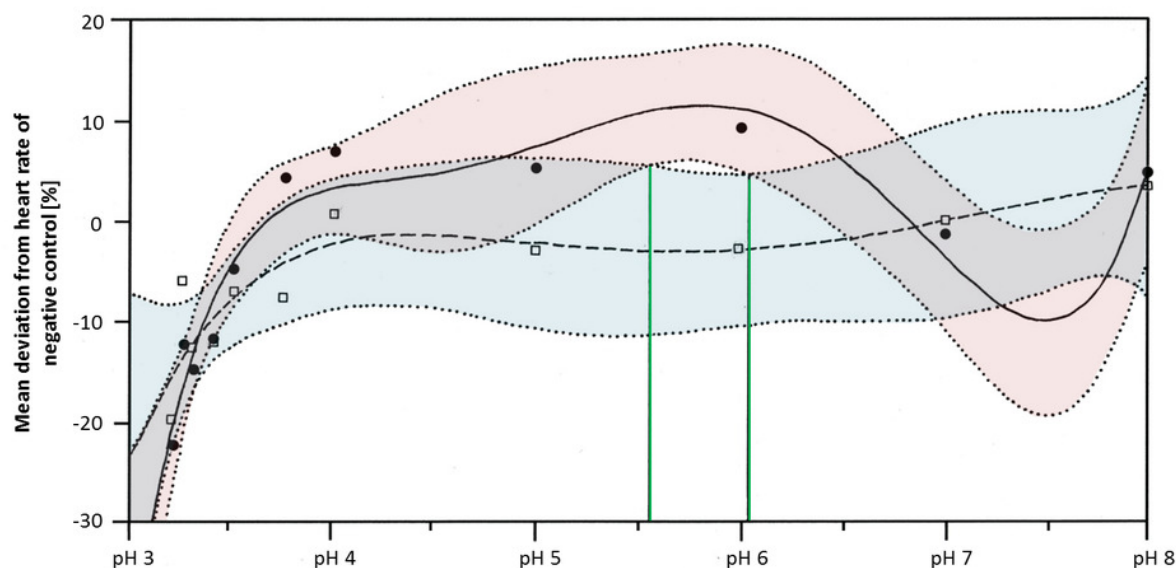
Table 1: Tested glyphosate concentrations including their corresponding values in mg/L and resulting pH in solution.

MOLARITY	mg/L	pH
10 µM	1.69	8.3
50 µM	8.45	6.9
100 µM	16.91	5.9
250 µM	42.27	4.5
500 µM	84.54	3.6
750 µM	126.80	3.4
1 mM	169.07	3.2
10 mM	1 690.7	2.4

Table 2: Overview of observed lethal and sublethal endpoints at respective time points.

ENDPOINT	12 hpf	24 hpf	48 hpf	60 hpf	72 hpf	96 hpf
Mortality	✓	✓	✓	✓	✓	✓
Developmental delays		✓				
- no somites		✓				
- non-detachment of the tail		✓				
- no development of the eyes		✓				
Heart rate			✓			
Hatching success				✓	✓	✓
Malformations						✓
- oedema						✓
- eye/brain defects						✓
- deformation of the spine						✓
- light pigmentation						✓

Table 3: Results for concentration-dependent glyphosate treatments, as well as for pH-dependent control and glyphosate treatments, as percentages.

Asterisks (*) indicate statistically significant differences from the negative control (Cox regression, ANOVA: * p < 0.05, ** p < 0.01, *** p < 0.001; Likelihood ratio χ^2, Fisher's exact test, Bonferroni-Holm: * p< α). Crosses (⬚) denote additional statistical significances between pH control and glyphosate within the same pH range or, in the case of 7dSh, differences from 1mM glyphosate at pH 7. For unbuffered glyphosate concentrations, endpoint-related LC_{10}/EC_{10} and LC_{50}/EC_{50} values are given. HR – heart rate, D – developmental delays, M – malformations, n.s – not significant, n.a. – not available (no sufficient sample sizes for statistical analysis).

	MORTALITY		HATCHING		HR	D	M
	96 hpf [%]	over time	96 hpf [%]	over time	48 hpf [bpm]	24 hpf [%]	96 hpf [%]
UNBUFFERED							
Neg. control	0	-	97.92	-	148.75	0	0.26
10 µM	1.04	n.s.	97.92	*	138.38***	0	2.36*
50 µM	0	n.s.	98.96	*	134.19***	0	5.47*
100 µM	0	n.s.	100	***	128.69***	0	4.69*
250 µM	3.13	n.s.	40.65*	***	125.56***	22.23*	13.57*
500 µM	28.13*	*	40.58*	***	118.63***	20.36*	16.06*
750 µM	93.64*	***	33.33*	n.a.	94.50***	19.04*	18.06*
1 mM	100*	***	n.a.	n.a.	n.a.	n.a.	n.a.
10 mM	100*	***	n.a.	n.a.	n.a.	n.a.	n.a.
LC$_{10}$/EC$_{10}$	385 µM		155 µM		43 µM	126 µM	179 µM
LC$_{50}$/EC$_{50}$	582 µM		224 µM		-	-	-
7dSh							
neutral	0	n.s.	98.93	*	139.75***,⑨	0.35	0.27⑨
pH 7							
Neg. control	0	-	85.42	-	148.04	0	0.52
10 µM	1.04	n.s.	100*	***	144.25	0	1.04
50 µM	1.04	n.s.	100*	***	142.56	1.04	1.87
100 µM	0	n.s.	98.96*	***	136.57**	0	2.60
250 µM	0	n.s.	100*	***	130.31***	0	0.26
500 µM	0	n.s.	100*	***	129.19***	0	1.56
750 µM	1.04	n.s.	100*	***	133.50*	0	2.35
1 mM	0	n.s.	98.96*	***	145.94	0.69	6.56*
10 mM	0	n.s.	94.79*	***	149.38	0	7.29*
pH RANGE - CONTROL							
Neg. control	0	-	94.69	-	160.38	0	0.26
pH 3	100*	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	100*	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	100*	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	84.03*	***	61.11*	***	140.19***	9.36*	4.17*

pH 3.4	**51.04***	*******	**77.78*,⑤**	*******	**141.38*****	**6.93***	**3.51***
pH 3.5	**8.33***	n.s.	**15.77*,⑤**	*****,⑤**	**144.25*****	**8.32***	**2.0***

pH RANGE - GLYPHOSATE						
pH 3	**100***	*******	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	*******	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	*******	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**72.57***	*******	83.33	*******	**136.46*****	**13.33***	0
pH 3.4	**28.47***	*******	**60.61*,⑤**	*******	**141.49*****	**4.54***	**3.80***
pH 3.5	**17.36***	n.s.	**66.57*,⑤**	*****,⑤**	**143.50*****	**10.31***	**2.04***