Non-target effects of a glyphosate-based herbicide on Common Toad larvae (*Bufo bufo*, Amphibia) and associated algae are altered by temperature

Fabian Baier [1] , **Edith Gruber** [1] , **Thomas Hein** [2, 3] , **Elisabeth Bondar-Kunze** [2, 3] , **Marina Ivanković** [2] , **Axel Mentler** [4] , **Carsten A Brühl** [5] , **Bernhard Spangl** [6] , **Johann G Zaller** [Corresp. 1]

1 Institute of Zoology, University of Natural Resources and Life Sciences Vienna (BOKU), Vienna, Austria

2 Institute of Hydrobiology and Aquatic Ecosystem Management, University of Natural Resources and Life Sciences Vienna (BOKU), Vienna, Austria

3 WasserCluster Lunz - Biologische Station GmbH, Lunz am See, Austria

4 Institute of Soil Research, University of Natural Resources and Life Sciences Vienna (BOKU), Vienna, Austria

5 Institute for Environmental Sciences, Universität Koblenz-Landau, Landau, Germany

6 Institute of Applied Statistics and Computing, University of Natural Resources and Life Sciences Vienna (BOKU), Vienna, Austria

Corresponding Author: Johann G Zaller
Email address: johann.zaller@boku.ac.at

Background. Glyphosate-based herbicides are the most widely used pesticides in agriculture, horticulture, municipalities and private gardens that can potentially contaminate nearby water bodies inhabited by amphibians and algae. Moreover, the development and diversity of these aquatic organisms could also be affected by human-induced climate change that might lead to more periods with extreme temperatures. However, to what extent non-target effects of these herbicides on amphibians or algae are altered by varying temperature is not well known.

Methods. We studied effects of five concentrations of the glyphosate-based herbicide formulation Roundup PowerFlex (0, 1.5, 3, 4 mg acid equivalent glyphosate L^{-1} as a one time addition and a pulse treatment of totally 4 mg a.e. glyphosate L^{-1}) on larval development of Common toads (*Bufo bufo*, L.; Amphibia: Anura) and associated algae communities under two temperature regimes (15°C vs. 20°C).

Results. Herbicide contamination reduced tail growth (-8%), induced the occurrence of tail deformations (i.e. lacerated or crooked tails) and reduced algae diversity (-6%). Higher water temperature increased tadpole growth (tail and body length +66%, length-to-width ratio +4%) and decreased algae diversity (-21%). No clear relation between herbicide concentrations and tadpole growth or algae density or diversity was observed. Interactive effects of herbicides and temperature affected growth parameters, tail deformation and tadpole mortality indicating that the herbicide effects are temperature-dependent. Remarkably, herbicide-temperature interactions resulted in deformed tails in 76% of all herbicide treated tadpoles at 15°C whereas no tail deformations were observed for the herbicide-free control at 15°C or any tadpole at 20°C; herbicide-induced mortality was higher at 15°C but lower at 20°C.

Discussion. These herbicide- and temperature-induced changes may have decided effects on ecological interactions in freshwater ecosystems. Although no clear dose-response effect was seen, the presence of glyphosate was decisive for an effect, suggesting that the lowest observed effect concentration (LOEC) in our study was 1.5 mg a.e. glyphosate L^{-1} water. Overall, our findings also question the relevance of pesticide risk assessments conducted at standard temperatures.

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8 **Author affiliations**

9 [1]Institute of Zoology, University of Natural Resources and Life Sciences Vienna (BOKU), Austria

11 [2]Institute of Hydrobiology and Aquatic Ecosystem Management, University of Natural Resources and

12 Life Sciences (BOKU), Vienna, Austria

14 [3]WasserCluster Lunz - Biologische Station GmbH, Lunz am See, Austria

16 [4]Institute of Soil Research, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria

18 [5]Institute for Environmental Sciences, University Koblenz-Landau, Landau, Germany

20 [6]Institute of Applied Statistics and Computing, University of Natural Resources and Life Sciences

21 (BOKU), Vienna, Austria

23 *corresponding author: johann.zaller@boku.ac.at

24   **Abstract**

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26   horticulture, municipalities and private gardens that can potentially contaminate nearby water

27   bodies inhabited by amphibians and algae. Moreover, the development and diversity of these

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29   more periods with extreme temperatures. However, to what extent non-target effects of these

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32   formulation Roundup PowerFlex (0, 1.5, 3, 4 mg acid equivalent glyphosate L^{-1} as a one time

33   addition and a pulse treatment of totally 4 mg a.e. glyphosate L^{-1}) on larval development of

34   Common toads (*Bufo bufo*, L.; Amphibia: Anura) and associated algae communities under two

35   temperature regimes (15°C vs. 20°C).

36   **Results.** Herbicide contamination reduced tail growth (-8%), induced the occurrence of tail

37   deformations (i.e. lacerated or crooked tails) and reduced algae diversity (-6%). Higher water

38   temperature increased tadpole growth (tail and body length +66%, length-to-width ratio +4%)

39   and decreased algae diversity (-21%). No clear relation between herbicide concentrations and

40   tadpole growth or algae density or diversity was observed. Interactive effects of herbicides and

41   temperature affected growth parameters, tail deformation and tadpole mortality indicating that

42   the herbicide effects are temperature-dependent. Remarkably, herbicide-temperature interactions

43   resulted in deformed tails in 76% of all herbicide treated tadpoles at 15°C whereas no tail

44   deformations were observed for the herbicide-free control at 15°C or any tadpole at 20°C;

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46   **Discussion.** These herbicide- and temperature-induced changes may have decided effects on

47   ecological interactions in freshwater ecosystems. Although no clear dose-response effect was

48   seen, the presence of glyphosate was decisive for an effect, suggesting that the lowest observed

49   effect concentration (LOEC) in our study was 1.5 mg a.e. glyphosate L^{-1} water. Overall, our

50   findings also question the relevance of pesticide risk assessments conducted at standard

51   temperatures.

Introduction

With almost a third of their species threatened, amphibians are the most vulnerable vertebrate group on earth (IUCN 2004). Amphibian declines and extinctions are caused by multiple factors and their interaction: alien species, over-exploitation and land use change, global change, increased use of pesticides and other toxic chemicals and infectious diseases (Aldrich et al. 2016; Blaustein et al. 2011; Brühl et al. 2013; Collins & Storfer 2003). In their natural habitats, especially anuran amphibians are always associated with algae communities that constitute an important source of nutrition during larval development, however, algae are likewise affected by similar environmental factors as amphibians (Whitehead et al. 2009; Wilson et al. 2003). The current study examined the effects of a glyphosate-based herbicide on growth and development of larvae of Common toads (*Bufo bufo* L.) and on the composition of the associated algal communities. Additionally, as climate change scenarios predict more frequent extreme temperature events (IPCC 2013), we also assessed whether different water temperatures alter potential herbicide effects. Moreover, toad spawning ponds can vary considerably in their depths and in their water temperature. Globally, Common toads are mainly distributed in Europe living in habitats from lowland to mountain areas up to 2500 m a.s.l. (Arnold & Ovenden 2002; Recuero et al. 2012). Since Common toads frequently spawn in ponds located near agriculturally used areas or because they need to cross these landscapes during their seasonal migrations from the spawning ponds to woody overwintering sites, they are frequently exposed to pesticides (Berger et al. 2013; Lenhardt et al. 2015). Common toads were selected as a study organism also for ethical reasons because they are among the least threatened of the generally endangered animal class of Amphibia.

74   Glyphosate-based herbicides are the most often and broadly used pesticides in

75   agriculture, horticulture, forestry, vineyards, municipalities and private gardens. Generally,

76   glyphosates' mode of action inhibits an enzyme only known to plants and some microorganisms;

77   therefore, and due to the half-life of 7-14 days in water it is considered only slightly toxic to

78   amphibians (Franz et al. 1997; Giesy et al. 2000). Most glyphosate-based herbicides are not

79   meant to be used in or close to aquatic environments, however, contamination of water bodies

80   through drift, surface runoff and direct overspray has been reported (Battaglin et al. 2009; Berger

81   et al. 2013; Peruzzo et al. 2008). Nevertheless, several studies report detrimental effects of

82   glyphosate-based herbicide formulations to amphibians during their aquatic and terrestrial life

83   stages (Baylis 2000; Brühl et al. 2011; Duke & Powles 2008; Fryday & Thompson 2012; Relyea

84   2011; Tsui & Chu 2003). Glyphosate-based herbicides tested in concentrations ranging between

85   0.021 mg acid equivalent (a.e.) L^{-1} to 6.0 mg a.e. L^{-1}affected growth and development of North

86   American larval anurans (Edge et al. 2014; Lanctôt et al. 2014; Navarro-Martín et al. 2014;

87   Relyea 2004), showed other sub-lethal morphological and behavioral effects (Cauble & Wagner

88   2005; Mann & Bidwell 1999; Moore et al. 2015; Muñoz et al. 2014) and even lead to acute

89   mortality when exposed to high doses (Fuentes et al. 2011; Moore et al. 2012; Relyea 2005b;

90   Williams & Semlitsch 2010). However, concerning effects of glyphosate-based herbicides on

91   native European amphibian species only very little is known (Wagner & Lötters 2013).

92   Moreover, it has been shown that herbicide formulations, i.e. the products that are actually used

93   in the field, are more detrimental to tadpoles and other aquatic organisms than the active

94   ingredient glyphosate itself (Folmar et al. 1979; Howe et al. 2004; Relyea 2005b). However,

95   unfortunately these adjuvants are usually not declared and considered company secrets (Mullin et

96   al. 2016; Wagner et al. 2013). Besides amphibians, several studies also show that freshwater

97   microalgae are particularly vulnerable to glyphosate-based herbicides because of their

98   physiological and biochemical similarity with terrestrial plants (Annett et al. 2014; Tsui & Chu

99   2003) mainly by initiating oxidative stress in algae (Annett et al. 2014).

100   Temperature has long been known to play an important role in the physiology and

101   ecology of tadpoles (Katzenberger et al. 2014; Ultsch et al. 1999) and algae (Butterwick et al.

102   2005; Schabhüttl et al. 2013). Although climate warming is not considered to be directly lethal to

103   amphibians, increasing water temperatures in amphibian breeding ponds may accelerate larval

104   development and hence reduce the duration of herbicide exposure for these species (Li et al.

105   2013; Rohr & Palmer 2013). Potential interactions between herbicides and temperature are of

106   particular interest as human-induced climate change can increase mean temperatures and will

107   most likely also lead to weather events with more extreme temperatures (IPCC 2013). On the

108   other hand, pesticide use is also expected to rise with rising temperatures because certain pests

109   and diseases are expected to benefit from climate change (Kattwinkel et al. 2011). Interactions of

110   pesticide and global warming effects have been predicted and outlined as a major point of

111   concern for wildlife (Köhler & Triebskorn 2013; Rohr & Palmer 2013). Only very few studies

112   investigated combined effects of herbicides and temperature on amphibians (Baier et al. 2016;

113   Rohr et al. 2011). These studies show that increasing temperatures generally do not enhance the

114   toxicity of the herbicide, but rather temperature ameliorates the adverse effects of the herbicide

115   by accelerating development and reducing the duration of herbicide exposure (Baier et al. 2016;

116   Rohr et al. 2011).

117   Thus, the objectives of the current study were to assess whether (i) herbicide

118   concentrations affect the development of Common toad larvae and associated algae communities

119   and (ii) temperature alters potential herbicide effects. To investigate these objectives we

120 conducted a climate chamber experiment using a full factorial design with five herbicide

121 concentrations at two temperature levels over 24 days. We hypothesized that a higher

122 temperature will accelerate the growth and development of tadpoles, hence reducing the duration

123 of their exposure to the herbicide. When higher temperature reduces exposure to the herbicide

124 more than it enhances detrimental effects of the herbicide, then its net effect could be beneficial.

125 Regarding herbicide effects we also hypothesized that increasing temperatures would increase

126 detrimental effects of the herbicide with increasing concentrations because multiple stressors

127 might decrease resources of amphibians for detoxification and/or temperature regulation (Noyes

128 et al. 2009; Rohr et al. 2003).

129

130

131 **Material and Methods**

132 **Experimental Setup**

133 The study was conducted between April 14 and May 9, 2015 in a laboratory at the University of

134 Natural Resources and Life Sciences Vienna (BOKU), Austria. This study was carried out in

135 strict accordance with the recommendations in the Austrian animal experimentation law

136 (Tierversuchsgesetz 2012, BGBl. I Nr. 114/2012) with a permission from the Austrian Federal

137 Ministry of Science, Research and Economy (permit number BMWFW-66.016/0002-

138 WF/V/3b/2015). Anesthetic MS222 (Triacin, Sandoz, Switzerland) was used when necessary to

139 ameliorate animal suffering. The experiment was carried out using a full-factorial design, with

140 herbicide concentration (5 levels) and temperature (2 levels) as factors. Two climate chambers

141 set at different temperatures (15°C and 20°C) with a 12 hours light/dark-cycle were used to set

142 up the two temperature conditions. Light intensity of both climate chambers was measured using

143   a Lux Meter (Voltcraft LX-1108 Lux-Meter; Conrad electronics, Hirschau, Germany) resulting

144   in 5575 ± 106 lux (mean ± SD) in both chambers. We established the following herbicide

145   concentrations: 0, 1.5, 3, 4 mg a.e. L^{-1} (acid equivalent; which equals 0, 3.13, 6.25, 8.33 µl L^{-1} of

146   the Roundup PowerFlex® formulation) from the start and 4 mg a.e. L^{-1} applied as a pulse

147   treatment starting initially with 1.5 mg a.e. L^{-1} and adding another 1.5 mg a.e. L^{-1} on day 5 and 1

148   mg a.e. L^{-1} (2.08 µl L^{-1}) on day 11 after starting the experiment, respectively. In the field,

149   glyphosate concentrations in amphibian breeding ponds are generally highly variable depending

150   on potential pesticide drift or input through leaching by water or soil erosion. Worst-case

151   expected environmental concentration of 7.6 mg a.e. glyphosate L^{-1} have been calculated

152   (Wagner et al. 2013), however during most of the year concentrations in spawning ponds are

153   likely to be lower (Peruzzo et al. 2008; Struger et al. 2008; Trumbo 2005). Five 25-L plastic

154   canisters were used to prepare herbicide solutions. First each canister was filled with 22 L of coal

155   filtered tap water, then the glyphosate-based herbicide Roundup PowerFlex (containing: 588 g L^{-1}

156   ^{1} potassium salt of glyphosate, which equals 480 g L^{-1} glyphosate acid; Monsanto Europe

157   S.A./N.V., Belgium) was added in the desired concentrations using a micro-pipette. This

158   Roundup formulation is available in Europe since 2014 and admitted for usage in agriculture,

159   horticulture, forestry, vineyards, municipalities and private gardens until 2022 (AGES 2015).

160   Afterwards the solutions were mixed thoroughly by shaking and turning the canisters upside

161   down. Four liters of pure coal-filtered water (in case of the control) or the mixtures were then

162   filled into each experimental unit, i.e. polypropylene plastic tubs (volume: 5-L, length: 28 cm,

163   width: 19 cm, height: 14 cm), accounting for 5 replicates per treatment. Each climate chamber

164   contained 25 tubs, making up a total of 50 tubs for the two temperature treatments.

165   Common toad (*Bufo bufo* L.) spawn containing approx. 750 eggs from different clutches

166   was hand-collected on 14 April 2015 from a pond in Neuwaldegg, Vienna (48°14'26.31"N;

167   16°16'34.945"E; altitude 290 m a.s.l.). Toad eggs were sampled with permission of the pond

168   owner (Vienna Municipal Department 49 - Forestry, permit number MA49-808754/2014/3) and

169   the respective authority for nature conservation (Vienna Municipal Department 22 -

170   Environmental Protection, permit number MA22-1629490/2014). Five randomly chosen eggs of

171   the whole spawn were added into each experimental tub, accounting for a total of 250 eggs used

172   for this experiment.

173

174   **Measurements and Analyses**

175   Larval development was determined according to Gosner (Gosner 1960) using a binocular

176   microscope (Nikon SMZ 745; Nikon, Tokyo, Japan). Conforming to this scheme the

177   development of anurans is assigned to 46 stages (Gosner stage, Gs), from undivided eggs (Gs 1)

178   to completely metamorphosed individuals (Gs 46). At the start of our experiment all tadpoles

179   were in Gs 8. As additional morphological parameters we measured body length, body width and

180   tail length from photographs taken of tadpoles from all treatments at May 8, when the maturity

181   of larval amphibians reached Gs 30 and Gs 35 at 15°C and 20°C, respectively. Measurements on

182   photographs were conducted using the free image analysis software ImageJ (version 1.48v,

183   Wayne Rasband, National Institute of Health, USA, http://imagej.nih.gov/ij). Body condition

184   was calculated as body length-to-width ratio. Tail deformations (Bantle et al. 1998; Cooke 1981)

185   were assessed when taking the tadpoles out into Petri dishes for morphological measurements at

186   the end of the experiment. A clear distinction could be made between undeformed (Figure 1A),

187   lacerated (Figure 1B) or curved tails (Figure 1C); only curved tails were considered deformed

188   (Cooke 1981) as lacerated tails could also originate from injuries by intraspecific competition.

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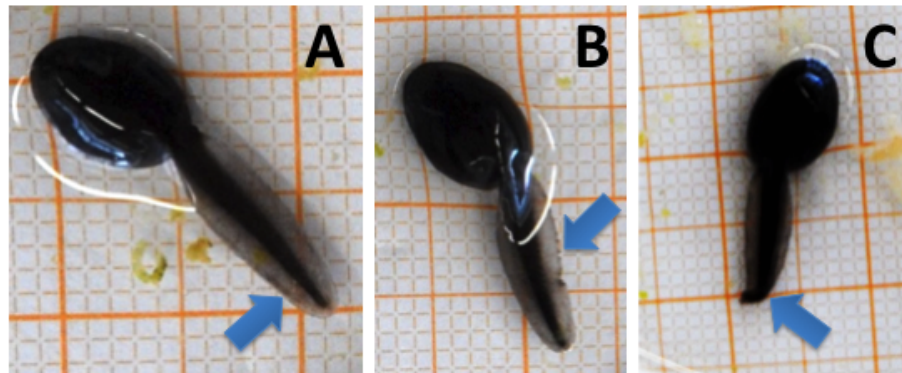
190   Figure 1: Tadpoles observed in the current experiment with undeformed (A), lacerated (B) and
191   curved tail tips (C). Only curved tail tips were considered deformed and included in the analyses.

192

193   Each tub was inspected daily for dead individuals. All larvae were fed ad libitum with

194   same amounts of ground fish food (Tetramin) after reaching Gs 22. When most of the 20°C-

195   treated tadpoles reached Gs 35 (indentations 1-2 of the hind limbs develop at this stage) the

196   experiment was terminated. Water samples of all tubs (also the 15°C ones) were taken after

197   thoroughly mixing with a glass stirring rod using a 100 ml plastic beaker; samples were

198   preserved by adding 5 drops of Lugol iodine solution.

199   In these water samples algae species composition was examined. Algae were introduced

200   into the experimental units via the tadpole eggs; as eggs were randomly assigned to the

201   treatments we considered this as a similar initial situation for all treatments. For algae species

202   determination, the phytoplankton samples were gently mixed by inverting the sample bottle.

203   Initially a preliminary scan was made of a settled 5 ml sample in order to determine the volume

204   to be used for the analyses. For the qualitative analysis at least 5 ml of the sample were

205   analyzed. As a high number of algal cells were present, 1 ml was loaded into a sedimentation

206   chamber of appropriate volume for quantitative analysis. The chamber was topped with a round

207   glass top plate and algae were allowed to settle onto the base of the settling chamber. The

208   method consists of two parts - analysis of non-diatom phytoplankton ("soft algae") and analysis

209   of diatoms. For the non-diatom phytoplankton analysis, algal cells were enumerated in a settling

210   chamber using an inverted microscope at 400x magnification. Only living forms (i.e. chloroplast

211   containing cells) were counted. At this magnification diatoms are enumerated as a group. For

212   diatom analyses, the samples were pretreated with strong oxidants, cleaned and embedded on

213   glass slides in Naphrax. Actual identification and enumeration of diatoms was done under oil

214   immersion (1000x) and for the quantitative analyses a minimum of 500 frustules was counted (2

215   frustules = 1 diatom cell) per sample (slide). For the quantitative analyses a minimum of 30

216   microscopic fields is required, including no less than 300 cells. If 300 cells were not observed

217   within the 30 microscopic fields, enumeration was continued until at least 300 were counted. The

218   area counted was recorded to calculate cell number per volume. Algal taxa were identified to the

219   lowest taxonomic rank possible. The taxonomical identification was done using standard

220   literature for species determination (Hindák et al. 1978; Krammer & Lange-Bertalot 1986;

221   Krammer & Lange-Bertalot 1988; Krammer & Lange-Bertalot 1991a; Krammer & Lange-

222   Bertalot 1991b). Species names were checked at the algae base database (algaeBase 2015).

223   Quantitative assessment was conducted according to Utermöhl (Utermöhl 1958). The density is

224   presented as cell number L^{-1}.

225   Water temperature and water oxygen concentration were measured using an Oxymeter

226   (WTW Oxi 90, WTW GmbH, Weilheim, Bavaria, Germany) and pH was measured using a pH-

227   Meter (WTW pH-95, WTW GmbH, Weilheim, Bavaria, Germany). Solved oxygen content, pH-

228   value and temperature of the water in the tubs was first measured on April 14, shortly before

229   introducing the eggs. Henceforth measurements were performed every third day until May 8. A

230   data logger measuring temperature every 15 minutes was placed in the middle section of each

231   climate chamber at the start of the experiment. The mean temperature during the experiment was

232   14.4°C vs. 18.3°C for the 15°C vs. 20°C treatment, respectively.

233   Water samples (500 ml) of each tub were taken at the start and end of the experiment and

234   immediately stored in a freezer at -18°C before they were analysed for glyphosate and its main

235   metabolite aminomethylphosphonic acid (AMPA) in the laboratories of the BOKU Department

236   of Forest and Soil Sciences using a HPLC-MS/MS method (Popp et al. 2008; Todorovic et al.

237   2013).

238

239   **Statistical Analyses**

240   All statistical analyses were carried out using the open-access software R (version 3.2.1, The R

241   Foundation for Statistical Computing, http://www.r-project.org); α was set at 0.05. Shapiro-

242   Wilks tests and QQ-plots were used to check normality of data. Percentage mortality was arcsin-

243   transformed prior to analysis. To examine whether herbicide concentrations and temperature

244   affected toad larvae morphology, analyses of covariance (ANCOVA) with pH, measured water

245   temperature and oxygen levels as co-variables were conducted. Effects of herbicide and

246   temperature on pH and dissolved oxygen were tested using a two-way ANOVA. To analyze

247   effects of herbicide, temperature, pH and dissolved oxygen on algae density and diversity a

248   different ANCOVA model was used as samples were only taken at one point of time. To assess

249   the effects of algae density and diversity on body length, body width, tail length and body length

250   to body width ratio of tadpoles a one-way ANCOVA was conducted. When significant effects

251   were given by the ANCOVA (P < 0.05) a Tukey HSD test was conducted for mean comparisons

252   of herbicide and temperature levels. Water pH and oxygen concentrations were used as

253   covariates in all ANCOVA models. Correlations were tested using Pearson's test. Values in the

254   text are means ± standard deviation.

255

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Results

Herbicide contamination significantly affected tadpole tail length (tl) at 15°C but not at 20°C, occurrence of tail deformations only at 15°C and algae diversity (Shannon- and evenness-index) mainly at 20°C (Figure 2, Figure 3, Table 1). Herbicide concentrations neither affected larval development, body length (bl), body width (bw), body condition (bc), mortality of tadpoles or algae density (Figure 2, Figure 3, Table 1).

Temperature significantly affected larval growth parameters (bl, bw, tl and bc), larval development (Gosner stage) and occurrence of tail deformations of tadpoles as well as algae diversity (Shannon- and evenness-index); however it did not affect tadpole mortality or algae density (Figure 2, Figure 3, Table 1). Algal communities contained between 25 to 43 species, with the highest species number in the 15°C treatment (Figure 3). Diatom algae dominated in species number and abundance in all mesocosms.

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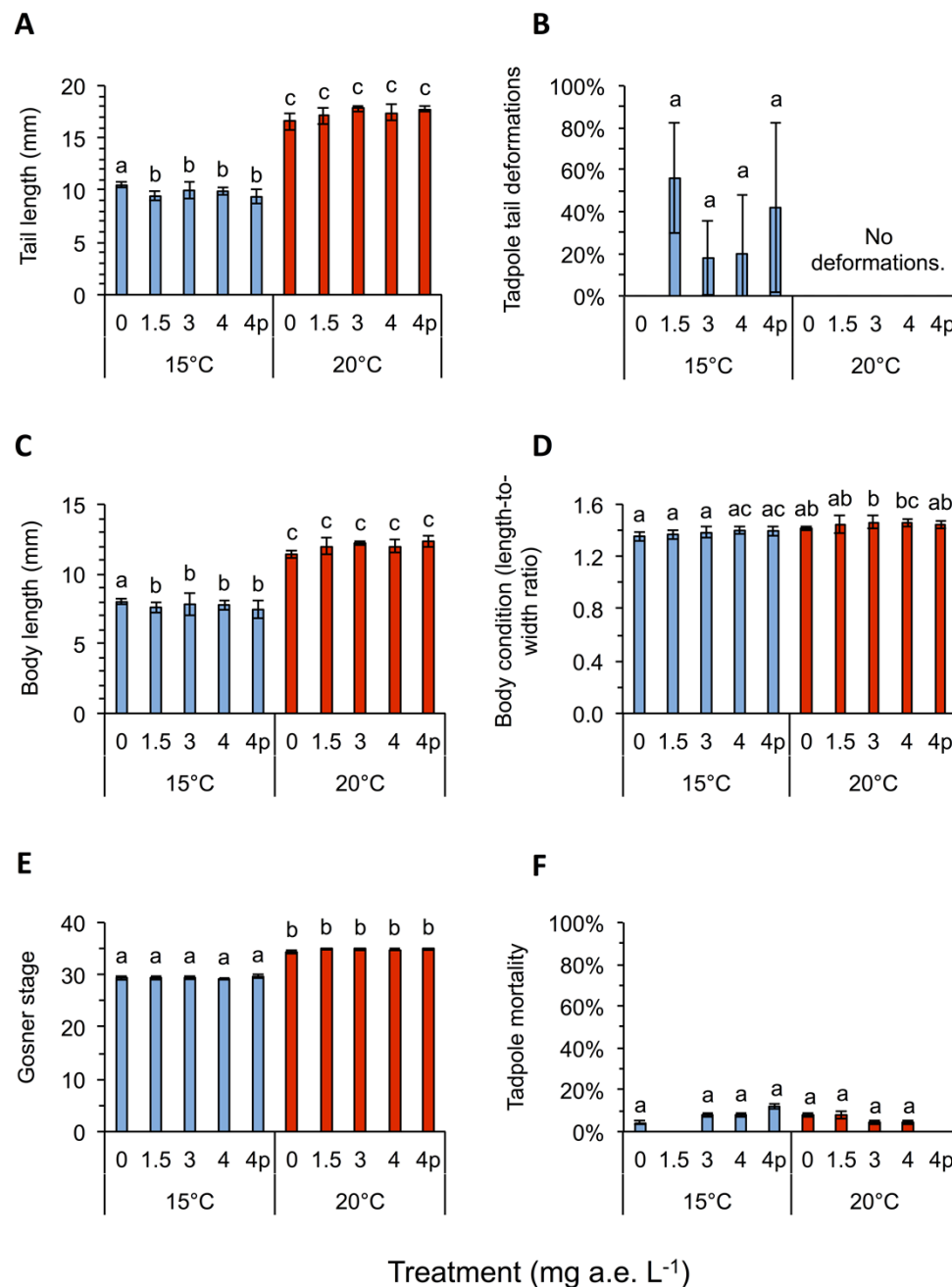
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Figure 2: Tail length (A), tail deformation (B), body length (C), body condition (D), development (E) and mortality (F) of Common toad tadpoles in response to different herbicide treatments (mg a.e. L^{-1}) at 15°C or 20°C, measured 24 days after experiment start. Mean ± SD, n = 5. Means with different letters are significantly different (Tukey HSD, $p < 0.05$).

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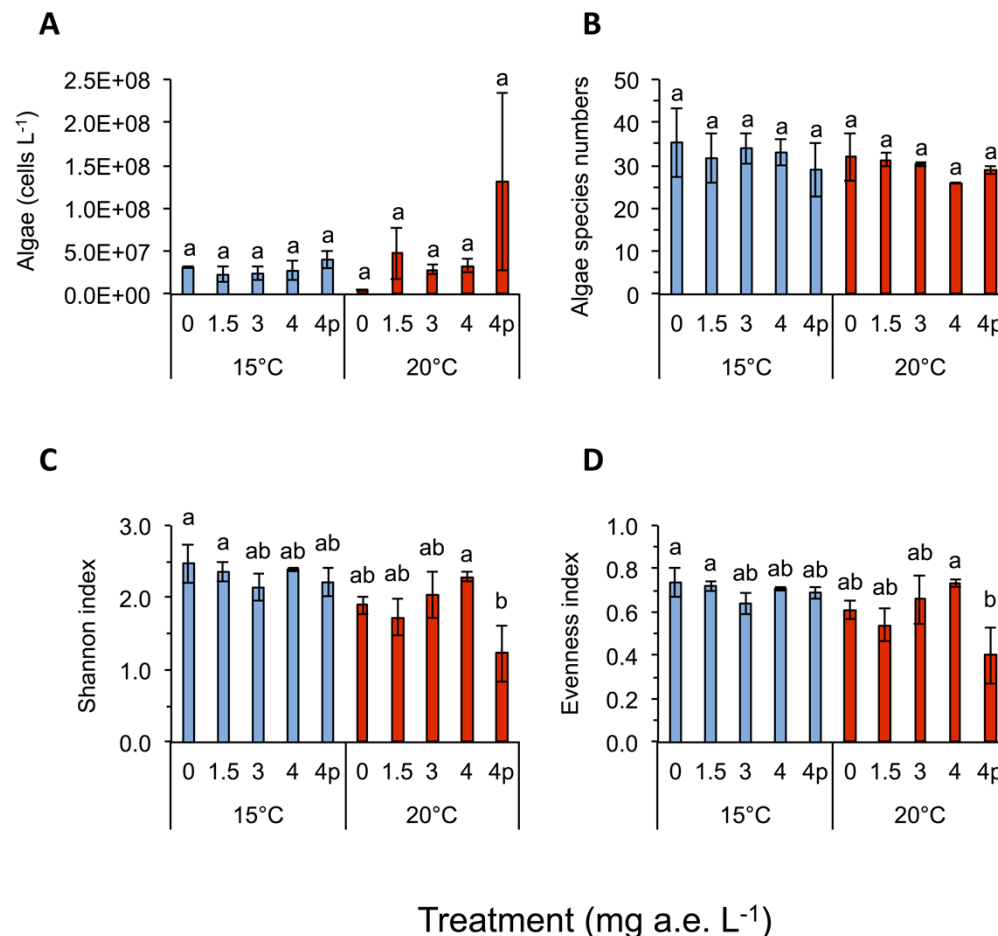
Figure 3: Algae abundance (A), number of species (B) and diversity depicted using Shannon- (C) and evenness-index (D) in response to different herbicide treatments (mg a.e. L^{-1}) at 15°C or 20°C. Mean ± SD, 24 days after experiment start, n = 3. Means with different letters are significantly different (Tukey HSD, p < 0.05).

Interactions between herbicide and temperature significantly affected larval tl, bl, bw, tadpole mortality and occurrence of tail deformations, as well as evenness of algal communities (Figure 2, Figure 3, Table 1). This significant herbicide x temperature interaction led to the lowest tl (9.36 ± 0.64 mm) in the 4 mg a.e. L^{-1} pulse treatment at 15°C and the longest tails (17.79 ± 0.31 mm) at 3 mg a.e. L^{-1} under 20°C (Figure 2A). At 15°C tadpoles in tubs contaminated with the herbicide had averaged across herbicide concentrations 7.8% shorter tails

291   compared to the control treatment (Figure 2A).

292

293   Table 1: ANCOVA results for the effects of herbicide concentrations (0, 1.5, 3, 4, 4p mg a.e. L^{-1}) and
294   temperature (15°C vs. 20°C) on growth stages, body length (bl), body width (bw), tail length, body
295   condition (bl/bw), mortality and occurrence of tail deformations of Common toad larvae and diversity of
296   associated algal communities.

Variable	Herbicide conc.		Temperature		Herbicide x Temp.	
	F	P	F	P	F	P
Tadpoles						
Growth stages (Gosner)	0.642	0.633	150.521	**< 0.001**	0.079	0.989
Body length (mm)	1.200	0.317	837.846	**< 0.001**	4.369	**0.003**
Body width (mm)	0.882	0.478	584.778	**< 0.001**	2.893	**0.027**
Tail length (mm)	4.113	**0.004**	891.085	**< 0.001**	4.851	**0.001**
Body condition (bl/bw)	1.413	0.236	17.040	**< 0.001**	2.303	0.065
Mortality (%)	0.401	0.807	0.760	0.386	2.821	**0.030**
Tail deformation (%)	2.792	**0.040**	5.236	**0.028**	3.077	**0.027**
Algae						
Algal density (cells L^{-1})	2.408	0.090	4.144	0.058	1.632	0.212
Algal diversity (Shannon)	5.022	**0.007**	9.888	**0.006**	2.761	0.062
Algal diversity (Evenness)	4.810	**0.009**	9.562	**0.007**	3.756	**0.023**

297
298

299   Twenty four days after experiment start, tadpoles of the 15°C chamber were on average

300   in Gosner stage (Gs) 29, whereas tadpoles at 20°C were in Gs 35 (Figure 2E). The body

301   condition was highly significantly affected by temperature, being reduced by 4.2% at 15°C

302   compared to 20°C, on average across herbicide concentrations (Figure 2D, Table 1). The highest

303   body condition (1.46 ± 0.05) was found at 3 mg a.e. L^{-1} under 20°C, the lowest bl-to-bw ratio

304   (1.36 ± 0.04) at 0 mg a.e. L^{-1} under 15°C. Tadpole bl and bw also differed significantly under

305   different temperature regimes. Body length at 15°C was lower by 35.4% on average compared to

306   20°C; whereas bw at 15°C was lower by 32.4% on average compared to 20°C (Figure 2C). At

307   15°C bl at 0 mg a.e. L^{-1} (8.0 ± 0.18 mm) was significantly higher compared to the other herbicide

308   treatments (7.67 ± 0.55 mm); at 20°C average bl (11.99 ± 0.39 mm) was unaffected by herbicide

309   concentrations (Figure 2C). At 15°C bw at 4p mg a.e. L^{-1} (5.33 ± 0.33 mm) was significantly

310   lower compared to the other herbicide concentrations (5.67 ± 0.29 mm); at 20°C bw (8.29 ± 0.39

311   mm) was unaffected by herbicide concentrations.

312   At the end of the experiment on average 34.0 ± 14.8% of the tadpoles at 15°C showed

313   deformed tails, while none at 20°C had deformed tails (Figure 2B). Most deformed tadpoles

314   (56.0 ± 26.0%) were found at 1.5 mg a.e. L^{-1}, however, no relation to herbicide concentration

315   was found. No deformed tadpoles were found in the control group without herbicide addition.

316   Whenever deformations occurred the tip of their tails was crooked in one direction (Figure 1C).

317   Mortality was unaffected by herbicide treatment or temperature, but significantly affected

318   by their interaction: on average, 6.4 ± 0.8% of larvae died at 15°C vs. 5.6 ± 0.9% at 20°C (Figure

319   2F, Table 1). The highest mortality rate under 15°C was found at 4p mg a.e. L^{-1} (12.0 ± 1.1%

320   mortality), whereas under 20°C it was found at 0 mg a.e. L^{-1} (8.0 ± 1.1% mortality); mortality of

321   control at 15°C was 4.0 ± 0.9%. No larvae died at 1.5 mg a.e. L^{-1} under 15°C and at 4p mg a.e.

322   L^{-1} under 20°C (Figure 2F).

323   Algal species of 4 different families were observed in the experiment, namely:

324   Chlorophyceae (six spp.), Trebouxiophyceae (two spp.), Xanthophyceae (one spp.) and

325   Bacillariophyceae (34 spp.). Algae Shannon diversity index under 15°C was across treatments

326   20.7% higher than at 20°C (Figure 3C). At 15°C the highest Shannon index was found at 0 mg

327   a.e. L^{-1} (2.48 ± 0.27), whereas under 20°C the highest Shannon index was observed at 4 mg a.e.

328   L^{-1} (2.29 ± 0.06; Figure 2). The lowest Shannon index was seen at 3 mg a.e. L^{-1} (2.14 ± 0.18)

329   under 15°C and at 4p mg a.e. L^{-1} (1.23 ± 0.39) under 20°C (Figure 3C). Algal density was

330   unaffected by herbicide concentrations or temperature (Table 1). Averaged across concentrations

331   we observed 33 species and 2.9E+07 ± 7.8E+06 algae cells L^{-1} at 15°C vs. 30 species and

332   4.9E+07 ± 3.0E+07 algae cells L^{-1} at 20°C (Figure 3A and B).

333   Herbicide concentrations affected water pH and water oxygen concentrations,

334   temperature treatment reduced water oxygen concentration (data provided in supplement S1). At

335   15°C, water pH in the control (8.34 ± 0.13) was significantly lower than at treatments with

336   herbicide addition (8.64 ± 0.48), at 20°C control showed the lowest value (8.27 ± 0.07), whereas

337   the pulse treatment had the highest (8.55 ± 0.41). Across temperatures water oxygen

338   concentration increased with increasing herbicide concentrations. The lower temperature

339   treatment showed higher oxygen values throughout all herbicide concentrations, the highest

340   being 13.58 ± 4.50 mg L^{-1} for the 4 mg a.e. L^{-1} treatment at 15°C, whereas the control at 20°C

341   had the lowest average oxygen value with 9.24 ± 0.68 mg L^{-1}. No significant effect of herbicide

342   by temperature interaction on water pH or oxygen was observed. The temperature of the climate

343   chamber had a significant influence on water temperature levels of the tubs, which differed by

344   3.9°C. The mean temperature of the 15°C and 20°C treatment was 14.4 ± 1.3°C and 18.3 ±

345   1.3°C, respectively. Tadpole bw and tl was significantly affected by water oxygen concentration,

346   however not affected by water pH (Table 2). Although bw was significantly affected by water

347   oxygen concentration those parameters were not significantly correlated. Tail deformation was

348   not affected by pH or dissolved oxygen content (Table 2).

349   Neither algal density nor algal diversity showed any significant effects on bl, bw, tl and

350   body condition, although algal density had a marginal effect on tadpole bl (F = 4.096, P = 0.060).

351   Results of glyphosate and AMPA analyses confirmed the indented concentrations at the

352   start of the experiment: < 5.0, 1557.5 ± 33.6, 3002.0 ± 6.3, and 4050.7 ± 300.7 µg glyphosate L^{-1}

353   and generally < 2.5 µg AMPA L^{-1} for the 0, 1.5, 3, 4 mg L-1 one time herbicide application,

354   respectively. Glyphosate and AMPA for the first 1.5 mg L^{-1} addition of the 4p mg L^{-1} treatment

355   was with 1764.3 ± 61.8 µg glyphosate L^{-1} higher than intended. At the end of the experiment

356   measured concentrations across temperature treatments were: < 5.0, 1491.4 ± 148.6, 2923.6 ±

357   124.8, 3979.6 ± 193.7, 6568.3 ± 1490.3 µg glyphosate L^{-1} and < 2.5, 34.6 ± 13.1, 66.0 ± 35.6,

358   130.7 ± 16.2, 201.4 ± 29.8 µg AMPA L^{-1}, for the 0, 1.5, 3, 4 mg L^{-1} one time herbicide and 4 mg

359   L^{-1} pulse treatment, respectively.

360

361   Table 2: ANCOVA results for the effects of water pH and oxygen concentration
362   and time on development, body length, body width, tail length, body condition,
363   mortality and tail deformation of *Bufo bufo* tadpoles and diversity of associated
364   algae communities.

365

Variable	Water pH		Water oxygen	
	F	P	F	P
Tadpoles				
Growth Stage (Gosner)	2.264	0.133	0.005	0.942
Body length (mm)	0.640	0.426	3.689	0.058
Body width (mm)	0.365	0.548	5.063	**0.027**
Tail length (mm)	0.017	0.898	14.450	**< 0.001**
Body condition (bl/bw)	2.388	0.126	0.044	0.835
Mortality (%)	0.118	0.732	0.288	0.592
Tail deformation (%)	0.109	0.744	0.302	0.586
Algae				
Algal density (cells L^{-1})	1.352	0.261	1.635	0.218
Algal diversity (Shannon)	0.361	0.556	0.375	0.548
Algal diversity (Evenness)	0.837	0.373	1.185	0.291

373

374

Discussion

376   Our study showed for the first time for Common toads that potential contaminations of spawning

377   ponds with the glyphosate-based herbicide Roundup PowerFlex can reduce the tail growth of

378   toad larvae, lead to tail deformations and reduce the diversity of associated algal communities.

379   Increased water temperature accelerated tadpole development and growth and reduced algal

380   diversity. Moreover, significant interactions between herbicide contamination and temperature

381   affected most morphological parameters (body length, body width, tail length, body condition,

382   mortality, tail deformation) and algal diversity (evenness-index), suggesting that the herbicide

383   effects are temperature-specific. However, we found no clear indications of a relation between

384   herbicide concentration and tadpole growth parameters or algae diversity or density. However,

385   the presence of glyphosate was decisive for an effect, suggesting that the lowest observed effect

386   concentration (LOEC) was 1.5 mg a.e. glyphosate L^{-1} water.

387   Overall, our findings for a European amphibian species corroborate former studies

388   showing effects of different Roundup formulations on the growth of mainly North-American

389   amphibian species (Edge et al. 2014; Howe et al. 2004; Lanctôt et al. 2014; Navarro-Martín et al.

390   2014; Relyea 2004; Relyea 2012; Wojtaszek et al. 2004). Our results of 4.2% decrease in tail

391   length of *B. bufo* tadpoles in tubs containing Roundup PowerFlex (15°C) compared to the control

392   is in line with findings of a previous study where we tested effects of Roundup LB Plus on *B.*

393   *bufo* development (Baier et al. 2016). However, the current findings are in contrast to findings of

394   elongated tails of *Rana sylvaticus* treated with 2.9 mg a.e. L^{-1} VisionMax (Navarro-Martín et al.

395   2014). In any case it appeared that these glyphosate-based herbicides interfere with molecular

396   signaling processes or growth factors influencing tail growth. Clearly, detailed studies are needed

397   to investigate the underlying mechanisms. Another explanation for a reduced tadpole growth

398   after herbicide addition could be an herbicide-induced decrease of algae diversity that are an

399   important food source for tadpoles (Wojtaszek et al. 2004). Also in our current experiment algae

400   were the preferred food for tadpoles although they were offered *ad libitum* ground fish food. Our

401   finding that herbicide concentrations affected tadpole growth only at 15°C but not at 20°C

402   suggests that increasing temperature did not significantly enhance the toxicity of the tested

403   herbicide. This observation may also indicate heat hardening in the 20°C groups, perhaps caused

404   by an induction of chaperones and following stabilization of developmental processes (Díaz-

405   Villanueva et al. 2015). Overall, our findings indicate that temperature stress and herbicide stress

406   do not necessarily point in the same direction (Baier et al. 2016; Rohr et al. 2011). Not much is

407   known on ecological consequences of reduced tail length for tadpoles. However, it has been

408   suggested that shorter tailed tadpoles can escape less easily from predators than longer tailed

409   ones (Hoff & Wassersug 2000; Wilbur & Semlitsch 1990). Also, tadpole tails are important

410   energy and calcium reservoirs for metamorphosis eventually resulting in smaller toads (Vitt &

411   Caldwell 2013).

412   As expected, and in accordance with other studies (Derakhshan & Nokhbatolfoghahai

413   2015; Sanuy et al. 2008), we found that tadpoles at 20°C developed and grew much faster than at

414   15°C, with tail length being 1.6, body length 1.4 and body width 1.4 times higher on average.

415   Higher temperature also accelerated tadpole development with tadpoles at 20°C reaching Gs 35,

416   whereas individuals at 15°C reached on average Gs 29 until the experiment was terminated; body

417   condition (i.e. body length to body width ratio) was slightly increased by 5% at higher

418   temperatures. Temperature also had a significant effect on the occurrence of deformed tails, as

419   they were only observed on herbicide treated specimens at 15°C but absent at 20°C. Although

420   climate change models predict higher temperatures during breeding periods (Laloe et al. 2014),

421   breeding and hibernation phenologies of ectotherms might shift to earlier dates during warmer

422   springs for compensating the warmer climate (Benard 2015; Gao et al. 2015; Klaus & Lougheed

423   2013). In some cases, the phenological shifts following warmer springs and winters even resulted

424   in lower temperatures for the developing tadpoles (Benard 2015). Overall there is still a great

425   lack of data regarding seasonal variations in the water temperature of aquatic bodies used as

426   spawning bonds and to what extent global climate change might affect these.

427 The ecologically most significant results of the current study are perhaps the interactive

428 effects between herbicide and temperature on various parameters regarding tadpoles and algae.

429 To our knowledge it is the first time that such herbicide-temperature interactions for a

430 glyphosate-based herbicide was studied on European toad species and their associated algae. In

431 the herbicide treated tubs at 15°C 34% of all tadpoles had deformed tails, whereas no deformed

432 tails occurred in the herbicide-free control at 15°C or in all herbicide concentrations at 20°C.

433 Generally, deformation rates >5% are considered unnatural and are therefore implemented

434 amphibian monitoring guidelines (Wagner et al. 2014). However, in our experiment tail

435 deformations were not related to herbicide concentrations; to what extent other principles than a

436 dose-response relationship might be effective would need further studies perhaps by also using a

437 greater sample size (Vandenberg et al. 2012). Substantial tail deformations by 50-90% due to

438 glyphosate-based herbicides have also been found for the tropical frog species *Scinax nasicus*

439 when exposed to 3.0-7.5 mg L^{-1} of Glyfos (Lajmanovich et al. 2003). Also herbicides containing

440 other active ingredients than glyphosate, e.g. isoproturon, have also been reported causing

441 deformations in larvae of *Bombina variegata* and *Rana arvalis* at rather low concentrations (0.1-

442 100 µg L^{-1}) (Greulich 2004). Interestingly, we did not observe tail deformations on tadpole

443 larvae in a previous experiment testing non-target effects of Roundup LB Plus on *B. bufo* (Baier

444 et al. 2016). This putative discrepancy of effects might be due to the different active ingredients

445 of the tested herbicide products, isopropylamin salt in Roundup LB Plus and potassium salt in

446 Roundup PowerFlex which have been shown to evoke different non-target effects (Cuhra et al.

447 2016) or be the result of different, not-declared adjuvants in the products (Cox & Surgan 2006;

448 Cuhra et al. 2013; Mullin et al. 2015; Relyea 2005b).

449   Interactive effects between herbicide and temperature resulted in a herbicide-induced

450   decrease of body length, body width and tail length by 2-10% at 15°C, but the same parameters

451   seemed to be (not significantly) increased by 2-8% at 20°C for tadpoles in herbicide treated tubs

452   compared to non-treated tubs. Overall, very little is known about combined effects of pesticides

453   and temperature on aquatic organisms, especially amphibians (Holmstrup et al. 2010; Rohr et al.

454   2011; Wagner & Lötters 2013), as most studies investigated herbicide effects at one temperature

455   level only. A study using Roundup Original MAX found effects on tail depth and body depth but

456   not on the body length of *Rana pipiens* and *R. sylvaticus* tadpoles at 12°C (Relyea 2012). Similar

457   to our findings snout-vent length of *Rana pipiens* larvae decreased when treated with 0.6 and 1.8

458   mg a.e. L^{-1} Roundup Original and Roundup Transorb at 20°C (Howe et al. 2004). In contrast

459   with our study, growth rates of *R. clamitans* tadpoles treated with 1.43 mg a.e. L^{-1} Vision

460   herbicide at 20°C increased (Li et al. 2013). Again, an explanation for these contrasting findings

461   could be that these glyhosate-based herbicides usually differ considerably in the formulation of

462   their active ingredients (Cuhra et al. 2016) their not-declared adjuvants that have been shown to

463   be as detrimental to non-target species as the active ingredient (Mann & Bidwell 2001; Relyea

464   2005a; Wagner et al. 2013). To what extent spawning ponds used by amphibians are

465   contaminated with glyphosate-based herbicides is very much dependent on regional

466   circumstances, e.g. how careful herbicide application was conducted following regulations and

467   distances to water bodies. Generally, most studies reporting glyphosate concentrations of surface

468   waters considered larger lakes or streams, while many amphibians are using small, shallow and

469   non-flowing water bodies for spawning. Nevertheless, reported glyphosate concentrations found

470   in surface waters shortly after application scored between 0.27 - 3.10 mg a.e. L^{-1} (Wagner et al.

471   2013).

472   Algae diversity (Shannon- and evenness-index) in our experiment was significantly

473   reduced by herbicides and increased temperatures, whereas herbicide effects were more

474   pronounced at higher temperature than at lower temperature (herbicide x temperature

475   interaction). Reductions of algal cells after herbicides application were lower as expected by a

476   pesticide supposed to kill plants. Possible effects of glyphosate-based herbicide formulations on

477   aquatic algae communities have rarely been investigated and our study appears to be among the

478   few addressing this topic for algae communities. Growth of green algae (*Scenedesmus acutus, S.*

479   *quadricauda*) was inhibited by technical grade glyphosate and Ron-do, a glyphosate formulation

480   used in aquatic environments (Saenz et al. 1997). In toxicity tests analyzing the effect of

481   glyphosate based herbicides on single species of diatoms and green algae, stronger negative

482   effects on the diatom species (*Skeletonema costatum)* were observed (Tsui & Chu 2003). Other

483   studies found growth of *S. quadricauda* was inhibited when treated with an unspecified

484   glyphosate-based formulation (Wong 2000) or by treating four freshwater phytoplankton species

485   with technical grade glyphosate (Vendrell et al. 2009). Since algae are able to produce

486   mycosporine-like amino acids when stressed and this process can depend on the shikimate

487   pathway (Shick & Dunlap 2002), it is possible, that glyphosate-based herbicides lead to a

488   decrease of the build up of these amino acids (Shick et al. 1999), probably also increasing the

489   effects of temperature stress on algal communities. As expected in our study, dissolved oxygen

490   level was negatively correlated to water temperature. This means that the colder the water, the

491   higher the oxygen content becomes (Wetzel 2001). Although values of water oxygen

492   concentrations varied broadly across temperature and herbicide treatments, we ruled out a lack of

493   oxygen, as the lowest value measured being 8.2 mg L^{-1}, which is considered to be sufficient for

494   proper development of tadpoles (Ultsch et al. 1999). As a consequence of increased temperature

495 dissolved oxygen levels decreased, which also affected tadpole growth. Contrary to our study,

496 others found decreasing water pH levels with increasing Roundup concentrations (Tsui & Chu

497 2003), however this could also be due to unknown adjuvants in the different formulations used.

498

Conclusions

500 Our findings might contribute several additional aspects to the broad discussion on non-target

501 effects of pesticides. First, as extreme temperature events will most likely become more frequent

502 due to human-induced climate change (IPCC 2013), it seems imperative to address non-target

503 effects of pesticides also in a climate-change context (Köhler & Triebskorn 2013; Rohr & Palmer

504 2013). The herbicide-temperature interactions in the current and a previous (Baier et al. 2016)

505 experiment suggest that interspecific and intraspecific relationships in aquatic ecosystems will

506 most likely be affected when taxa respond differently to these environmental stressors. Second,

507 chronically sub-lethal effects in the morphology and body deformations as well as changes in

508 algae communities such as observed in the current study and their ecological consequences

509 should attract more attention. Third, our study also suggests that ecotoxicological protocols

510 assessing non-target effects of pesticides conducted under constant temperature levels might

511 underestimate real world conditions, especially for poikilothermic vertebrates.

512

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518

Supplemental Information

520 The raw data was submitted as Data S1.

521

Additional Information and Declarations

523

Competing Interests

525 The authors declare there are no competing interests.

526

Author Contributions

528 F.B., E.G. and J.G.Z. conceived and conducted the experiment and analyzed the data; B.S.

529 helped with statistics. T.H., E.B.-K. and I.M. analyzed the algal samples. C.B. provided advice

530 throughout the experiment. All authors wrote on the manuscript.

531

Data Availability

533 The following information was supplied regarding data availability:

534 The raw data was submitted as Data S1.

535

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