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RESEARCH ARTICLE

# Feeding behavior of early life stages of the zebrafish *Danio rerio* is altered by exposure to glyphosate

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## Abstract

Glyphosate levels and the transfer of glyphosate across trophic levels have rarely been studied in zooplankton. The food preferences of zebrafish during the first-feeding stage (which is critical for the survival of organisms), were analyzed because of the requirement for live food. Larval survival begins to be affected when glyphosate intake exceeds 0.3666 µg/larvae/day, in the case that only the food is contaminated; if the medium is also contaminated, the effects on survival start from 0.2456 µg/larvae/day. It was shown that glyphosate was more likely to be incorporated through the medium than through the food (zooplankton), which supports the results of previous studies that have ruled out the potential for biomagnification. The bioconcentration factor (BCF) of glyphosate was determined using an ELISA tests specific to measure glyphosate in the fish *D. rerio*, the rotifers *Brachionus calyciflorus* and *Lecane papuana*, and the cladoceran *Ceriodaphnia dubia*. The experimental design consisted in exposing seven zebrafish adults per replica (four replicates) in three treatments 1, 5, and 10 mg/L of glyphosate for 96 h to obtain bioconcentration factors in the gills, liver, and muscle. These concentrations were selected as potential glyphosate concentrations right after application as double highest reported concentration. Glyphosate levels in zooplankton can represent up to 6.26% of the total weight of rotifers (BFC = 60.35) and in zebrafish adult organs were less than 8 µg/mg of tissue (BCF values < 6). Although glyphosate does not biomagnify, our results suggest that glyphosate affected the dynamics between zooplankton and zebrafish larvae, diminishing survival and feeding rates, given that zooplankton species bioconcentrate glyphosate in large quantities. The BCF values found in this contribution are higher than expected. Glyphosate exposure affected energy metabolism and feeding behavior of zebrafish larvae, which presented high mortality rates at environmentally relevant concentrations.

**Keywords** Energy content · Early life stages · Fish toxicity test · Survival · Pesticide

## Introduction

Glyphosate [N-(phosphonomethyl) glycine] is one of the most commonly used herbicides worldwide and is primarily used to eliminate weeds (Jansons et al. 2018). Glyphoste does not tend to bioaccumulate due to its low affinity to lipids, and biomagnification is also unlikely. Glyphosate

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concentrations in the superficial waters of freshwater systems usually range below 1 mg/L (Székács and Darvas 2018; Annett et al. 2014), although glyphosate concentrations in runoff from agricultural areas 1 day after direct application of glyphosate can reach up to 5.2 mg/L (Edwards et al. 1980).

Although the International Agency for Research on Cancer has classified glyphosate as a human carcinogen (IARC 2017), this herbicide is still used in various countries. In Mexico, for example, glyphosate continues to be heavily used. In 2014, ~ 13,700 metric tons of glyphosate were applied within the country. There is a strong need to generate information on the magnitude of its impacts on both organisms and the environment given that the short- and long-term risks of its use remain unclear (Bejarano 2018).

While the half-life of glyphosate in soil is 1.7–197 days, in aquatic ecosystems, a half-life of 7–142 days has been reported (Annett et al. 2014). However, the constant and excessive application of this herbicide does not allow for its natural degradation, and organisms in the lower trophic levels of aquatic food webs are likely directly affected. Moreover, multiple studies have suggested different toxicological effects in fishes due to exposure to commercial formulations of glyphosate (Uren Webster and Santos 2015; Jofré et al. 2013; Sulukan et al. 2017; Zhang et al. 2017). In all cases, glyphosate was directly taken up from the medium. Nevertheless, the available information on bioconcentration or biomagnification processes within aquatic food webs and glyphosate levels in zooplankton is still limited (Annett et al. 2014).

Glyphosate can elicit negative effects on exposed organisms by altering their somatic growth, reproduction, enzymatic activity (including alteration of the redox balance and producing oxidative stress) (Sulukan et al. 2017). Energy allocation within an organism requires a balance between maintenance, growth, and reproduction; while energy is allocated to growth, it cannot be assigned to reproduction and vice versa. Stressors (abiotic, biotic, and xenobiotics) alter such balance within the organism, increasing the energy demand for mechanisms of defense and repair. Consequently, dealing with environmental stressors can have a high energetic cost for most organisms. Therefore, energy budget assessments constitute a reliable toxicological tool to evaluate organismal responses to pollutants (Arzate-Cárdenas and Martínez-Jerónimo 2012).

*Danio rerio* (Cypriniformes:Cyprinidae) is a worldwide fish used for several purposes and presents several advantages. The genome of *D. rerio* is available and allows in depth studies on the mechanisms of toxicity. Moreover, the database ECOTOX (USEPA) has more than 3,000 records for the assessment of xenobiotics effects with this fish as a model organism. For instance, several publications report the effect of glyphosate on different stages of *D. rerio*. Bioconcentration studies as that of Lanzarin et al. (2019) reported glyphosate concentrations of up to 16.36 µg/L in which zebrafish embryos were exposed to RoundUp® at concentrations of up to 9.30 µg/L of the active ingredient, which represents a bioconcentration factor of up to 2492. However, this study was conducted with the commercial formulation of glyphosate, RoundUp®, which includes several co-adjuvants that increase glyphosate bioavailability and toxicity; thus, the effect of glyphosate was not independently assessed.

Therefore, the present study was aimed to: (1) assess the bioconcentration of glyphosate in two trophic levels [i.e., zooplankton (the rotifers *Brachionus calyciflorus* and *Lecane papuana*, and the cladoceran *C. dubia*) and *D. rerio*], (2) analyze the feeding behavior of zebrafish larvae during the first-feeding stage, (3) analyze the consequences of the altered feeding preferences on the survival of fish larvae, and (4) associate these effects with the energy content of living food (rotifers *B. calyciflorus* and *L. papuana*).

## Materials and methods

### Chemical reagents

Analytical grade glyphosate (99% purity) was obtained from Sigma Aldrich (St Louis, USA; CAS Number 1071–83-6) and used to prepare stock solutions, which were prepared by dissolving 10 mg of glyphosate in 100 mL of distilled water. From these stock solutions, glyphosate concentrations of 1.0 mg/L (0.0059 mM) and 0.8 mg/L (0.0047 mM) were prepared for the experiments.

### Danio rerio maintenance and embryo production

Adult male and female zebrafish were obtained from the fish collection of the Laboratorio de Ecotoxicología of the Benemérita Universidad Autónoma de Aguascalientes (Aguascalientes, Mexico). This laboratory has successfully maintained the controlled cultivation of *D. rerio* for over 2 years. Adults were acclimated in a 100-L pool with cycled water and oxygenation. The fish were kept at 27 °C with a 10:14-h (dark: light) photoperiod. The fish were fed twice a day with Tetramin® Tropical Flakes (Tetra Holding US Inc. Blacksburg, USA). To obtain fertilized eggs, the protocol proposed by The OECD Guideline 236 was followed (OECD 2013). The reproductive adults (2:1, males:females) were placed in 40-L aquaria with a 0.5-cm maternity mesh. The males and females were kept separately and without food the night before mating. The reproductive adults were then allowed

to join at the maternity mesh. Mating, spawning, and fertilization took place within 30 min after the light came on in the morning. The eggs were collected and cleaned in ISO medium (2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM $NaHCO_3$, 0.07 mM KCl, pH 7.8) (International Standard Organization 2012). All eggs were observed under a stereomicroscope; viable embryos were identified and non-viable eggs were discarded, and larvae were used to feeding preferences assay.

## Experimental design

The experimental design consisted in exposing seven zebrafish adults per replica (four replicates) in three treatments 1, 5, and 10 mg/L of glyphosate for 96 h to obtain bioconcentration factors in the gills, liver, and muscle. These concentrations were selected as potential glyphosate concentrations right after application as double highest reported concentration (Edwards et al. 1980). At the same time, three zooplanktonic species (*B. calyciflorus*, *C. dubia*, and *L. papuana*) were exposed to glyphosate for 4, 8, 16, and 24 h to assess the potential bioconcentration at 0.8 and 1.0 mg/L and for the energy reallocation experiments (four replicates). The concentrations in zooplankton correspond to environmental concentrations found in areas of high glyphosate application and rain transport in Argentina (Székács and Darvas 2018). Finally, exposed zooplanktonic species were used for the four treatments of the feeding preferences/bioconcentration of glyphosate through diet experiments (five replicates). We tested whether bioconcentration is achieved through passive uptake or through diet.

## Bioconcentration in adult Danio rerio exposed to glyphosate

Seven adult zebrafish (5-month age) from each of the four replicate groups were exposed to 1, 5, and 10 mg/L of glyphosate for 96 h; and concentrations in the gills, liver, and muscle tissue were determined.

The organisms were euthanized according to international ethical guidelines (National Research Council 2011), and the OECD Guideline 203 was followed (OECD 2019). Briefly, after the exposure period, the organisms were dissected, and their tissues were washed five times with EPA medium (96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4 \cdot 7H_2O$, and 4 mg KCl in 1 L of distilled water) at pH 7.5 (USEPA 2002).

The samples were stored in a DW-86L338 ultra-freezer (Haier Biomedical, Qingdao, China) until further use. The samples were homogenized in 1 mL of phosphate buffer solution (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$ in deionized water, pH 7.4). Homogenized tissue suspensions were centrifuged at 15,000 *g* for 15 min. The supernatant was collected and used for glyphosate determination through commercially available ELISA test kit (Eurofins Abraxis, Warminster, USA; PN 500,086). The kit consists of a direct competitive ELISA based on the recognition of glyphosate by polyclonal antibodies. The sample to be tested is derivatized and then added to microtiter wells coated with goat anti-rabbit antibodies. A rabbit anti-glyphosate antibody solution is added to the wells with the derivatized samples and allowed to incubate for 30 min. The glyphosate enzyme conjugate is then added, and a competitive reaction occurs between the glyphosate, which may be present in the sample, and the enzyme-labeled glyphosate for the binding sites of the rabbit anti-glyphosate antibodies bound by the goat anti-rabbit antibodies are immobilized on the microtiter plate. The reaction is allowed to continue for 60 min. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of glyphosate present in the sample. The color reaction is stopped after 30 min and the color is evaluated using an ELISA reader. The concentrations of the samples were determined by interpolation using the standard curve constructed with each run. The Glyphosate ELISA Kit was stored in the refrigerator (4–8 °C). Solutions were allowed to reach room temperature (20–25 °C) before use.

## Bioconcentration of glyphosate in zooplanktonic species

Zooplanktonic organisms (*B. calyciflorus*, *C. dubia*, and *L. papuana*) were exposed to glyphosate for 4, 8, 16, and 24 h to assess the potential bioconcentration. The 0.8 and 1.0 mg/L exposure concentrations were selected as environmentally relevant concentrations.

A stock solution of glyphosate was prepared in distilled water. A total of 320 *B. calyciflorus* (BC) individuals per replicate were exposed to 0.8 mg/L glyphosate, whereas 320 *L. papuana* (LP) individuals per replicate were exposed to 0.8 mg/L glyphosate. Finally, 320 individuals of *C. dubia* (CD) per replicate were exposed to 1 mg/L glyphosate. Each treatment consisted of four replicates. After the exposure period, the organisms were washed five times with EPA medium (pH 7.5), and glyphosate quantification was performed as reported for adult *D. rerio*.

## Energy reallocation of proteins, carbohydrates, and lipids

Three zooplanktonic species were exposed to glyphosate as previously described in quadruplicate. After 24-h exposure, organisms were collected and washed five times with EPA medium, rapidly frozen, and stored at − 80 °C until further

use. Each sample was homogenized in 1 mL of PBS, and energy content was determined in terms of carbohydrates, lipids, and protein content.

Total protein quantification was conducted with the Bradford method according to the procedures of Carbajal-Hernández et al. (2017) using serum bovine albumin as the standard. One milliliter of Bradford reagent was added to 100 µL of homogenized sample tissue. Then, the mixture was incubated at 25 °C for 5 min, and absorbance was read at 595 nm with a GENESYS 10S UV–VIS spectrophotometer (Thermo Scientific, Waltham, USA). Results are expressed as content of protein per dry weight.

Total carbohydrates determination was performed using the phenol–sulfuric acid method which is based on the formation of yellow-orange color as a result of the reaction between carbohydrates and phenol in the acidic medium. Absorbance was read against a standard curve of glucose (Carbajal-Hernández et al. 2017). Briefly, a total of 100 µL of the homogenized sample was used; 300 µL of sulfuric acid and 60 µL of phenol (5%) were added while kept within an ice bath to avoid overheating. Then, samples and standard curve test tubes were incubated at 90 °C for 10 min. Once at room temperature, absorbance was read at 530 nm with the GENESYS 10S UV–VIS spectrophotometer (Thermo Scientific). Results are expressed as the content of carbohydrates per dry weight.

Total lipids determination was performed by the sulfo-phospho-vanillin method (Carbajal-Hernández et al. (2017). Lipids extraction started by adding 500 µL of methanol/chloroform (2:1, v/v) to 100 µL of the homogenized sample tissues. The mixture was then vortexed for 30 s. Then, 100 µL of deionized water was added, and two phases separated (organic and water phases). The organic phase was recovered in separated test tubes. Extraction was performed twice more. The organic phase was heated to dryness at 80 °C. After which, 500 µL of concentrated sulfuric acid and 100 µL of water were added, and the mixture was heated to 80 °C for 5 min. Finally, 600 µL of vanillin reagent (9 mM dissolved in concentrated phosphoric acid) was added and allowed to form the colored complex. The mixture was cooled on ice. Absorbance was read at 525 nm using cholesterol as the standard. Results are expressed as content of lipids per dry weight.

The energy content in all species was estimated through multiplying the content of carbohydrates, lipids, and protein by their combustion factors: 4.11 cal/mg, 9.45 cal/mg, and 6.65 cal/mg, respectively (Arzate-Cárdenas and Martínez-Jerónimo 2012).

## Feeding preferences during the first-feeding stage of Danio rerio larvae

Four treatments were included in this study: (A) control, neither larvae of *D. rerio* nor zooplankton were exposed to glyphosate, (B) fish larvae were exposed to glyphosate at 0.8 mg/L and fed with LP-BC, (C) fish larvae free cultured in glyphosate-free ISO medium and fed with LP-BC, which were previously exposed to 0.8 mg/L of glyphosate in ISO medium, and (D) fish larvae were exposed to glyphosate at 0.8 mg/L and fed with zooplankton (LP-BC), which were previously exposed to 0.8 mg/L of glyphosate. The experiment began when larvae reached 8 days postfertilization (dpf) and continued for seven more days until fish reached 15 dpf. Each treatment consisted of a microplate with 24 larvae, one for each well. The initial ratio of rotifers consisted of 12 rotifers (8 LP and 4 BC) on the first day. The number of rotifers increased accordingly to fish daily requirements. The number of individuals consumed by *D. rerio* was determined experimentally. The CD cladoceran was not used for this experiment because CD is too big, and the exposed organisms did not reach the appropriate size for consumption.

The experiment was conducted with five replicas ($n = 20$). Observations were conducted daily to record feeding and survivorship. At the end of the test, the concentration of glyphosate in fish larvae was determined through ELISA (Eurofins Abraxis; PN 500,086). In addition, the quantification of biomolecules (carbohydrates, lipids, and proteins) in the survivor fish larvae was conducted with four replicates per treatment ($n = 16$).

## Estimation of the bioconcentration factor (BCF)

The BCF was calculated from the difference in the glyphosate concentrations found in zooplankton (dry weight) and the concentration values of the ISO medium. In the case of zebrafish, adult BCF was calculated from the difference in the glyphosate concentration found in the organ tissues (dry weight) and the concentration values of the ISO medium (Harangi et al. 2017). The BCF in zebrafish larvae was calculated from the relationship between the glyphosate concentration in tissues and the concentration of the zooplanktonic species consumed plus the concentration values of the ISO medium.

## Statistical analysis

All data was analyzed with the software GraphPad Prism 6 for Windows 10 (www.graphpad.com). Normality was checked with the Kolmogorov–Smirnov test (Gaussian distribution) and homoscedasticity with the Brown-Forsythe test. The data that meet these assumptions were tested through one-way analysis of variance (ANOVA) and multiple comparison tests of Tukey HSD; otherwise, the non-parametric comparisons by ranks of Kruskal–Wallis tests were performed and Dunn's

multiple comparisons. Energy reallocation for each three zooplanktonic species between control and treated groups was evaluated with paired *t* tests. The feeding preferences were tested through two-way analysis of variance (treatment and time) for each rotifer species. The log-rank test was applied for comparison between the survival curves. Significant differences were established when $p < 0.05$. In addition, we conducted a power test to assess the suitability of four replicas for statistical inferences. The power test indicated that four replicates were adequate to compare BCF values in zebrafish larvae and adults ($p > 0.98$), as well as in zooplankton species ($p > 0.80$).

## Results

### Bioconcentration factor in adults of Danio rerio

We determined the real concentrations of glyphosate: (a) 0.8 mg/L was $0.730 \pm 0.016$; $N = 3$, (b) 1 mg/L was $0.818 \pm 0.046$; $N = 3$, (c) 5 mg/L was $4.812 \pm 0.011$; $N = 3$, (d) 10 mg/L was $11.057 \pm 0.022$; $N = 3$. The BCF for the tissues of adult *D. rerio* was higher within those organisms exposed to glyphosate at 1 mg/L. Organisms exposed to 10 mg/L exhibited BCF values that were significantly lower in comparison to the group exposed to 1 mg/L (Fig. 1a–c). In addition, a qualitative inspection

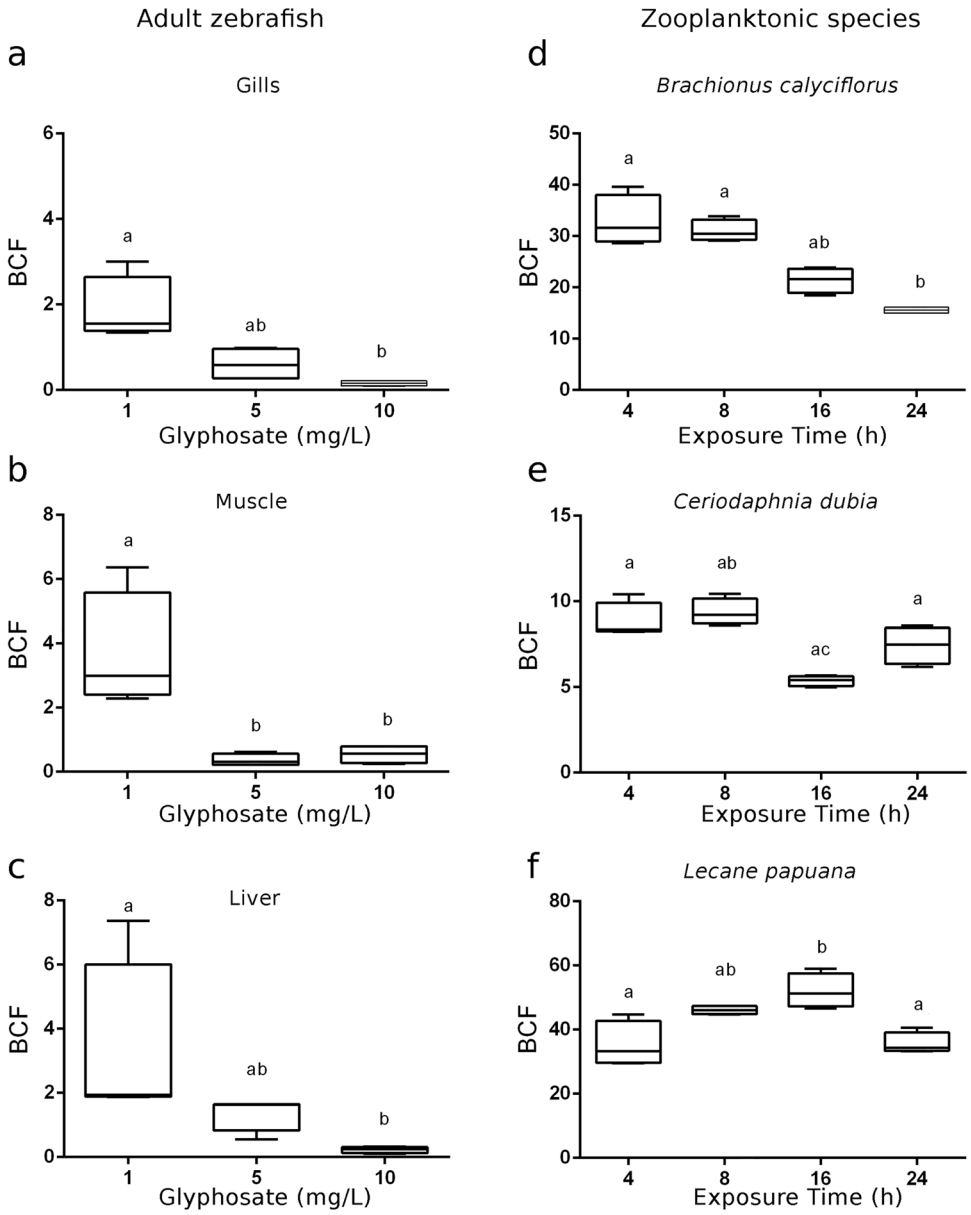


**Fig. 1** Bioconcentration factor (BCF) of organisms exposed to glyphosate. **a**–**c** Adult zebrafish exposed for 96 h ($n = 12$). **d**–**f** Zooplanktonic species ($n = 12$). **a** Gills. **b** Muscle. **c** Liver. **d** *Brachionus calyciflorus* exposed to glyphosate at 0.8 mg/L. **e** *Ceriodaphnia dubia* exposed to 1 mg/L glyphosate. **f** *Lecane papuana* exposed to glyphosate at 0.8 mg/L. Different letters correspond to significant differences at $p < 0.05$, using Kruskal–Wallis and Dunn's multiple comparisons. Box plots show a central line (mean), box limits (first and third quartile), and error bars (minimum and maximum values)

revealed the formation of a red coloration in the abdomen and the production of gray granules in the coloration lines in *D. rerio* exposed to 1 mg/L of glyphosate. No apparent physical changes were observed when fishes were exposed to 5 mg/L except for light red coloration. A loss of coloration was observed in organisms exposed to glyphosate at 10 mg/L. In the liver, at increasing concentration of glyphosate in the media, lack of color and turgor were observed along with an increase in the number of capillary vessels (Figure s1).

## Effects of glyphosate in zooplankton species

### Bioconcentration factor in zooplankton

We analyzed the BCF in zooplankton to determine the most suitable species for further experiments and the shortest period to ensure rotifers or cladocerans accumulate glyphosate (Fig. 1d–f), and serve as "contaminated food source" for further assays of feeding behavior of *D. rerio*.

The BCF values for zooplankton ranged from 5 to 60.35 (Fig. 1d–f) when organisms were exposed to 0.8 mg/L. In BC, the highest BCF was observed at 4 and 8 h of exposure; after this time, the concentration of glyphosate decreased but the BCF remained above 10 (Fig. 1d). For CD, the BCF varied less compared to that of the rotifers, but the highest BCF for cladocerans was below the lower limit for that of the rotifers (Fig. 1e). The highest BCF was found in LP exposed to 0.8 mg/L (BCF = 60.35 at 16 h of exposure) (Fig. 1f).

### Caloric content of zooplankton exposed to glyphosate

The protein content decreased in BC and CD (Fig. 2a) but no significant differences were observed in the case of LP, which were the rotifers that experienced decreased content of carbohydrates due to glyphosate exposure (Fig. 2b). The content of carbohydrates in CD increased after the exposure to glyphosate (Fig. 2b). CD showed significant decrease of the lipid content (Fig. 2c). The rotifers exposed to 0.8 mg/L showed no significant changes of lipids content. It is worth mentioning that CD control organisms presented higher content of lipids in contrast to those of the BC and LP controls (Fig. 2c). Once the content of carbohydrates, lipids, and protein was transformed to calories, the energy content in CD and LP species was diminished compared to their respective controls (Fig. 2d).

## Effects of glyphosate during the first-feeding stage of Danio rerio larvae

### Feeding preferences

Due to their potential to bioconcentrate glyphosate and because of their relatively small size, we selected the rotifers species BC and LP to feed fish larvae. Thus, it allowed assessing the effect of "contaminated food source" in the feeding behavior and survival of fish during their first-feeding stage (about 7 days after hatching).

In the four treatments, zebrafish larvae fed firstly on LP and then switched to BC since the second day (Fig. 3a–b).

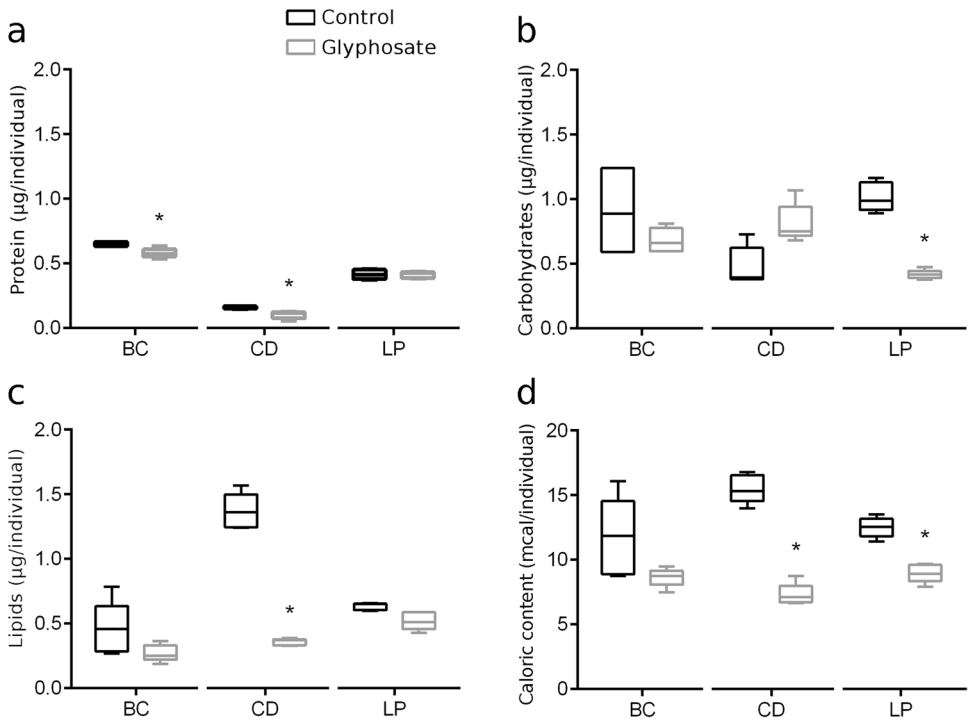


**Fig. 2** Energy allocation in zooplanktonic species after 24-h exposure. **a**–**d** *Brachionus calyciflorus* (BC) exposed to 0.8 mg/L glyphosate, *Ceriodaphnia dubia* (CD) exposed to 1 mg/L glyphosate, *Lecane papuana* (LP) exposed to 0.8 mg/L glyphosate. **a** Proteins. **b** Carbohydrates. **c** Lipids. **d** Caloric content. *Significant differences at $p < 0.05$, $n = 4$ for each species, using pairwise comparisons. Box plots show a central line (mean), box limits (first and third quartile), and error bars (minimum and maximum values)

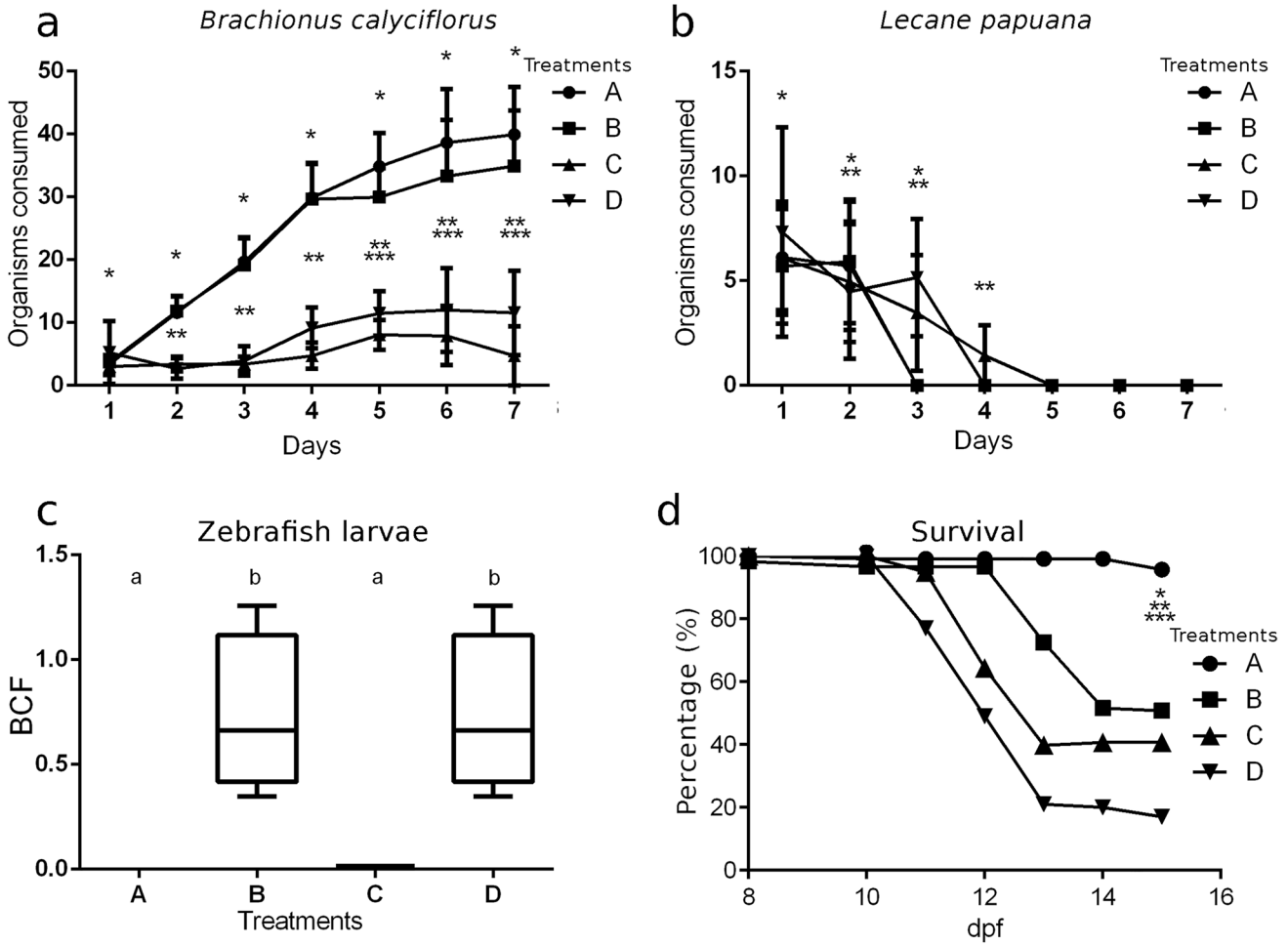


**Fig. 3** Effects of glyphosate during the first-feeding stage in zebrafish larvae. **a** *Brachionus calyciflorus* consumption. **b** *Lecane papuana* consumption. **c** Bioconcentration factor (BCF) of fish larvae feed on treatments A–D. **d** Survivorship, as percentage within every treatment. Treatment A, control (totally free of glyphosate); Treatment B, fish larvae and rotifers in ISO medium with 0.8 mg/L of glyphosate; Treatment C, rotifers previously exposed for 24 h to 0.8 mg/L of glyphosate and fish larvae in ISO medium (free of glyphosate); and Treatment D, rotifers previously exposed to 0.8 mg/L of glyphosate during 24 h and larvae in ISO medium with glyphosate at 0.8 mg/L. *** indicates differences between A and B treatments, ** indicates differences between A and C, * indicates differences between A and D treatments, and different letters show significant differences, at $p < 0.05$, using two-way ANOVA in consumption plot (**a** and **b**, $n = 20$), Kruskal–Wallis and Dunn's multiple comparisons in BCF plot (**c**, $n = 16$); and log-rank test in survival plot (**d**, $n = 20$). Box plots show a central line (mean), box limits (first and third quartile), and error bars (minimum and maximum)

A clear preference for BC over LP was observed when the rotifers were not previously exposed to glyphosate (treatments A and B, Fig. 3a), even when glyphosate was present in the medium (treatment B). In all cases, LP consumption ended on the fourth day of the experiment (Fig. 3b). It is important to point out that BC is a limnetic species and spends more time in the water column, while LP is a littoral rotifer that can be mainly found on surfaces.

When feeding on the rotifers previously exposed to glyphosate, zebrafish larvae preferred BC since the second day (Fig. 3). The patterns of food ingestion in the different treatments indicated that when glyphosate is found in the medium, feeding on zooplankton is affected. However, when zooplankton species were previously exposed to glyphosate, then, fishes avoided consuming those organisms. During the test, we observed that *D. rerio* ingested the rotifers of the previously exposed to glyphosate group and then expelled them from their mouth, which were often found dead within the microplates.

### Bioconcentration factor and survivorship

Larvae from treatments B (freshwater medium + glyphosate, feeding on BC-LP) and D (freshwater medium + glyphosate, feeding on glyphosate-exposed BC-LP) registered BCF values between 0.36 and 1.30 in contrast to those of treatment C, which registered the lowest BCF values overall (Fig. 3c). Fish larvae fed on previously exposed rotifers did

not accumulate glyphosate (Fig. 3c), but the larvae exposed to glyphosate through the medium (independently on the food source) exhibited low BCF values (less than 1, in average). Thus, bioconcentration did not occur through the diet but because of exposure of dissolved glyphosate in the water.

The survival percentages indicate that the presence of glyphosate-exposed zooplankton resulted in higher mortality rates of *D. rerio* larvae, given that glyphosate-contaminated food was rejected by the larvae, which induced death. Larval survival begins to be affected when glyphosate intake exceeds 0.3666 µg/larvae/day, in the case that only the food is contaminated; if the medium is also contaminated, the effects on survival rate start from 0.2456 µg/larvae/day. The presence of glyphosate alone in the medium decreased the survival of fish larvae by 45%. The treatment that contained contaminated rotifers decreased survival by 55%, and the combination of glyphosate within the medium and food decreased survival by 79% (Fig. 3d).

### Energy content in zebrafish larvae exposed to glyphosate through diet

The content of total protein in fish larvae significantly decreased in treatments C (freshwater medium, feeding on glyphosate-exposed BC-LP) and D (freshwater medium + glyphosate, feeding on glyphosate-exposed BC-LP). For the treatment B (freshwater medium + glyphosate, feeding on BC-LP), the content of protein was significantly higher than the control. All treatments, B–D showed significantly lower contents of carbohydrates compared to the control. The content of lipids decreased in the following order: B > C > D. Thus, the combined exposure through the medium and the diet elicited the higher effect on the lipid content, which is responsible for the higher content of energy. As a result, the energy content followed the same pattern as the lipid content of zebrafish larvae (Fig. 4).

## Discussion

Our results show that glyphosate was easily taken up from the medium and incorporated by organisms. The ease with which glyphosate was taken up from the medium was consistent with recent reports of the presence of glyphosate in many fluids, grains, and organisms worldwide (Bus 2015; Pérez et al. 2017; Tarazona et al. 2017; Jansons et al. 2018). The BCF of glyphosate has been rarely reported in the literature. Considering chemical characteristics according to their octanol–water partition (log Pow), then, BCF values should be lower than 0.01 (Contardo-Jara et al. 2009); nevertheless, the BCF found in this contribution was higher than expected (Fig. 1), which is in agreement with previous reports on the incorporation of glyphosate within tissues of animals (Contardo-Jara et al. 2009; Dey et al. 2016).

The BCF values of glyphosate in three different zooplanktonic organisms (used in this study) were one order of magnitude higher (up to 60.35) than the highest BCF values found in the different organs and tissues of adult zebrafish. Literature reports that BCF for glyphosate in different organisms can reach values from 1.4 to 5.89 in *Lumbriculus variegatus*, the California blackworm (Contardo-Jara et al. 2009), and up to 20–2080 in *Ludwigia peploides*, the floating primrose willow (Pérez et al. 2017). Therefore, our results are within the values previously reported for other species.

Differences on the zooplankton uptake of glyphosate from the media might be related to the intrinsic characteristics of the species selected for this study. Some authors like Hernández-Ruiz et al. (2016) and Garza-León et al. (2017) considered that hard loricated rotifers could present a natural barrier to avoid the entrance of xenobiotics. Nevertheless, LP presented the highest BCF values in this study. Therefore, glyphosate comprised up to 6.26% of the total dry weight of the LP rotifers exposed to 0.8 mg/L of glyphosate. Our results indicate that the zooplankton species tested could bioconcentrate higher amounts in comparison to other organisms like the lichen *Xanthoria parietana*, in which glyphosate represented only 1.1–1.2% of the total dry weight (Vannini et al. 2016). The intrinsic factors that enable LP to bioconcentrate glyphosate to that extend are beyond the interest of this study, although it opens the opportunity to do further research in the area of bioremediation or water treatment as species of this genus have been tested for the improvement of biological processes (Aidan Al-Hussieny et al. 2014; Fiałkowska et al. 2016).

The highest BCF values in the liver, muscle, and gills of adult *D. rerio* were obtained when fishes were exposed to glyphosate at 1 mg/L, which might be too low to activate detoxification/excretion process (Ferrario et al. 2018). On the other side, Braz-Mota et al. (2015) stated that at sublethal glyphosate concentrations, defense mechanisms arise in the gills, such as epithelium lifting, hypertrophy, and hyperplasia, which prevent the absorption of xenobiotics, thus, avoiding the glyphosate to pass through the gills.

Previous studies have shown generalized toxic effects of pure glyphosate in adult zebrafish after prolonged exposure at high concentrations. Evidence of moderate changes in the liver histoarchitecture has also been found to occur after 30 days of exposure to glyphosate at 18 mg/L, suggesting the presence of hyperemia (Jofré et al. 2016). However, after 24 h of exposure, apoptosis and a reduction in glutathione S-transferase activity in a zebrafish hepatocyte

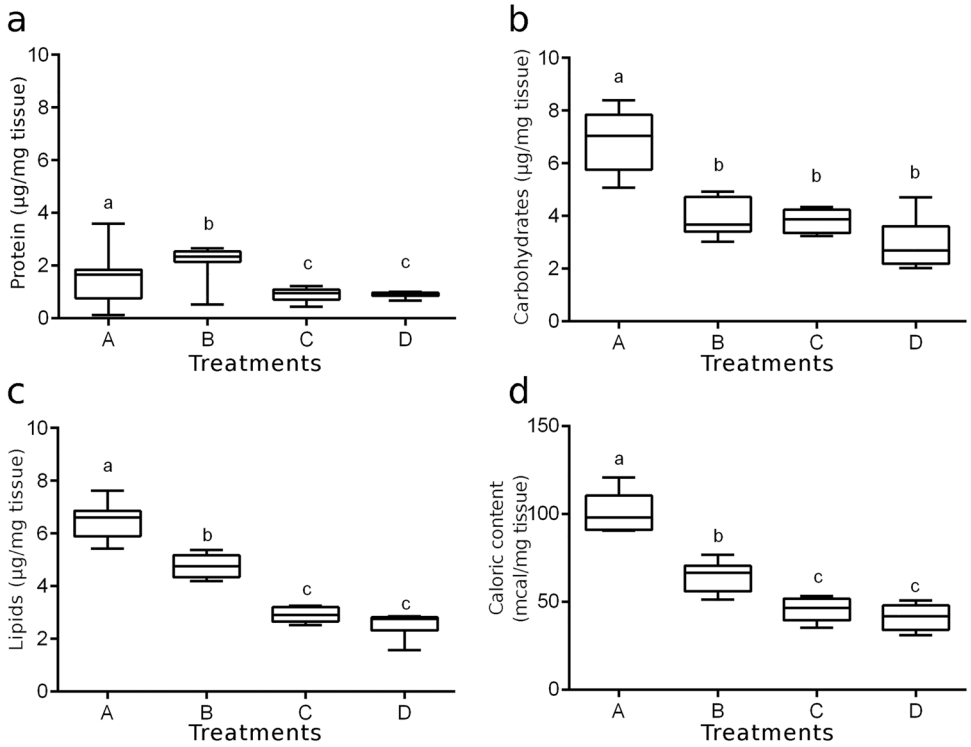


**Fig. 4** Energy content in zebrafish larvae after 7-day feeding on rotifers. **a** Total protein. **b** Total carbohydrates. **c** Total lipids. **d** Caloric content. **a** Treatment A, Control (totally free of glyphosate). **b** Treatment B, fish larvae and rotifers in ISO medium with 0.8 mg/L of glyphosate. **c** Treatment C, rotifers previously exposed for 24 h to 0.8 mg/L of glyphosate and fish larvae in ISO medium (free of glyphosate). **d** Treatment D, rotifers previously exposed to 0.8 mg/L of glyphosate during 24 h and larvae in ISO medium with glyphosate at 0.8 mg/L. Different letters show significant differences at $p < 0.05$, using ANOVA, $n = 16$. Box plots show a central line (mean), box limits (first and third quartile), and error bars (minimum and maximum values)

cell line have been found (Moreira et al. 2018). Swimming behavior changes have also been recorded at a glyphosate concentration of 0.5 mg/L (Bridi et al. 2017), which demonstrates that even short exposure periods can affect zebrafish. Such effects were observed in this study in the physical appearance of the zebrafish tissues. In the case of *D. rerio* larvae, the BCF values were not significantly greater than one, either by direct exposure within the freshwater medium or by feeding on glyphosate-contaminated zooplankton. Thus, in zebrafish larvae at 8 dpf, bioconcentration (from the media) and biomagnification (from the diet) did not take place.

The main energy component for cladocerans is lipids, which actively participate in reproduction, but in case of exposure to xenobiotics, that energy is reallocated to detoxification processes (Sancho et al. 1996). In the case of CD, lipid content decreased as a consequence of the exposure to glyphosate at 1 mg/L, which might be due to energy reallocation to cope with the glyphosate-induced stress, as reported for *Daphnia magna* exposed to tebuconazole (fungicide) (Sancho et al. 1996), for *D. schoedleri* exposed to α-cypermethrin (Martínez-Jerónimo et al. 2013), and the chydorid *Alona guttata* (Osorio-Treviño et al. 2019).

In CD, we found a higher production of carbohydrates when the organisms were exposed to glyphosate, which was probably linked to compensation mechanisms or adjustments in the metabolism to deal with the glyphosate-induced stress. Although Li et al. (2017) reported glucose increase in the fish *Carassius auratis* exposed to a commercial formulation with 30% glyphosate and discussed that the effect might be linked to alterations on the activity of the pyruvate kinase (an enzyme that regulates the speed of glycolysis), the loss of activity in this enzyme provoked the accumulation of glucose and decreased the synthesis of ATP, which could explain the increased amount of carbohydrates in glyphosate-exposed zooplankters.

The first-feeding stage is crucial for the growth and survival of fishes. The first prey items must be accessible; so fishes do not have high energy cost to capture their prey (Lawrence 2019). In this study, the assays with fish larvae at their first-feeding stage showed that glyphosate altered zebrafish feeding behavior, which had consequences for both energy reallocation and survival.

Feeding preferences changed when zooplanktonic species were previously exposed to glyphosate. Under control conditions during the first 3 days, zebrafish larvae consumed LP, which is a benthic species and does not require a high energetic cost to be captured (larvae need only expand their buccal cavity and suck) (Pekkan et al. 2016). After this time, the larvae began to feed on BC, which constantly swim during the day (Castro-Barrera 2003). This switch in feeding preference occurred even when larvae were exposed to dissolved glyphosate in the freshwater medium, but rotifers were not previously exposed to glyphosate. Thus, exposure to dissolved glyphosate did not change the feeding preferences although the number of prey diminished during time, which could be linked to differences in the locomotion of fish larvae (Bridi et al. 2017; Zhang et al. 2017).

In contrast, when rotifers were exposed to glyphosate and offered to fish, larvae preferences changed and LP preys were consumed for 2 more days (Fig. 3c). The switch to feeding on BC was delayed, and only few preys were consumed in comparison to the groups fed on non-exposed rotifers. Fish larvae in the first-feeding stage present an olfactory system that is functional at this stage, and it helps fishes find and detect food (Miyasaka et al. 2013); taste also plays an important role in prey selection (Lawrence 2019). Therefore, glyphosate might change palatability or odor of rotifers as they were captured but fish larvae expelled them. We consider that BC were captured as a result of extreme necessity and due to the minimum capture effort required. Some brain electrophysiological alterations of the midbrain with reduced locomotor activity have been recorded in zebrafish larvae after 5 days of glyphosate exposure at 1 mg/L (Forner-Piquer et al. 2021). However, in larvae and adult zebrafish exposed to food contaminated with deoxynivalenol, neither food rejection nor adverse effects on growth were reported (Sanden et al. 2012). Nonetheless, studies on glyphosate effects on feeding behavior in aquatic food webs are scarce; therefore, this work presents information of high relevance as it describes alterations on feeding preferences of fish larvae, which in fact negatively affected fish survival.

Regarding the reallocation of energy in *D. rerio* larvae, the concentrations of macromolecules decreased in a similar way to what has been observed in juvenile fish *Rhamdia quelen* (Persch et al. 2017), in which the amount of lipids was found to be the most affected, as lipids constitute the macromolecule that is most used by larvae (Lawrence 2019). Lipids are also the most active macromolecules in detoxification processes, as observed by Rodríguez-Estrada et al. (2016) who exposed *D. rerio* to α-cypermethrin.

The treatments that most affected the energy allocation and survival fish larvae were those in which zooplankton was previously exposed to glyphosate. From the time of the first exogenous feeding (8 dpf), the available energy resources, which were obtained from the vitelline sack, are used in detoxification, in detriment of growth, which has been observed in *D. rerio* exposed to other pesticides Rodríguez-Estrada et al. (2016). In addition, contaminated live food does not contain the optimum amount of nutrients (as they require more energy for their survival in a stressful medium, decreasing algae consumption and increasing lipids and protein consumption), which increases the associated energy expense. That effect could limit the energy allocation to growth, and when demand surpassed the available energy, then, survival was no longer possible. Panetto et al. (2019) assessed the effects of glyphosate commercial formulation and found no significant effects in *D. rerio* embryos when evaluating the content of protein, glucose, glycogen, and triglycerides. Nevertheless, they reported decreased activity of hexokinase, which diminishes the metabolism of carbohydrates (explaining the increased amount in glyphosate-exposed organism) and the incapacity of zebrafish to inflate their swim bladder, thus, limiting their locomotion and acquisition of food, and as consequence, compromising their survival.

The results herein presented show high mortality rates (> 40%) with exposure of fish larvae to relatively low concentration of glyphosate during the first feeding stage. It is known that the developmental stage of the organisms constitutes a key factor for their susceptibility; for instance, fish embryos are protected by the outer membranes of the corion and toxic compounds might not affect them (Hallare et al. 2006). High mortality rates have been reported in older fish larvae exposed to different toxic compounds (Burkhardt-Holm et al. 1999; Kristofco et al. 2016). It has also been demonstrated the transgenerational effect of glyphosate. In this case, mortality of fish larvae increased when progenitors were exposed to glyphosate during gametogenesis; mortality of direct exposure of larvae was lower at concentrations of 10 mg/L (Uren Webster et al. 2014). Therefore, the age of zebrafish larvae is an important factor to be considered for the assessment of the toxic effects of chemicals (Kristofco et al. 2016).

Our exposure scenario focused on the first-feeding stage and included the crucial transition from endogenous to exogenous feeding when high mortality occurs. In our experimental design, the use of live food like rotifers and artemia nauplii in the first-feeding stage improved larvae survival as shown in previous reports (Lawrence 2019; Best et al. 2010).

The tested glyphosate concentration in the medium was 0.8 mg/L, which was considered to fall within the concentration interval most likely to be found in the environment (< 1 mg/L). Mortality rate in treatment B (freshwater medium + glyphosate at 0.8 mg/L) exceeded the values found in scientific reports that employed similar concentrations (Uren Webster et al. 2014; Fiorino et al. 2018). This mortality was also found to increase when exposure to glyphosate in the medium was combined with dietary exposure through contaminated rotifers. Thereafter, the performance of fish larvae is subjected to high stress levels as medium and food are contaminated and the transference of glyphosate may take place, affecting their energy metabolism (increased energy demand to cope with stress and less energy available in food with poor energy content), their feeding behavior, and their locomotion (diminishing the capture of rotifers as food); and finally, the lack of energy and glyphosate-promoted stress increase mortality rates of fish larvae.

## Conclusions

In this study, we observed a bioconcentration of glyphosate in zooplankton of up to 6.26% of the total dry weight of rotifers (BFC = 60.35). Our results of BCF values differed from those from other studies with zebrafish larvae and suggest that no biomagnification occurs in *D. rerio* larvae given that the values obtained were not significantly greater than one. Furthermore, exposure to glyphosate in the medium was more important for its incorporation into zebrafish larvae than consuming contaminated rotifers. We also found the organ-related bioconcentration of glyphosate, with the liver bioconcentrating the most glyphosate. The BCF values found in this contribution were higher than expected. Glyphosate exposure affected energy metabolism and feeding behavior of zebrafish larvae, which presented high mortality rates at environmentally relevant concentrations. Finally, because of pressure by the media and contaminated food, cumulative long-term effects may be present in aquatic ecosystems.

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**Author contribution** GBAS: conceptualization, investigation, writing-original draft, formal analysis, data curation, methology, writing—review and editing; MSB: conceptualization, writing—review and editing; MAAC: investigation, formal analysis, writing—review and editing; ALCH: investigation, methodology, writing—review and editing; BYR: conceptualization, funding acquisition, writing—review and editing, supervision; RRM: conceptualization, funding acquisition, writing—review and editing, supervision. All authors read and approved the final manuscript.

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**Data availability and materials** The database generated in the present study are available from corresponding authors on reasonable request.

**Code availability** N/A.

## Declarations

**Ethics approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. In the case of *Dario rerio* methodology, the Bioethical Committee of the Universidad Autónoma de Aguascalientes approved these procedures.

**Consent to participate** N/A

**Consent for publication** N/A

**Competing interests** The authors declare no competing interests.

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