



# Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (*Danio rerio*)

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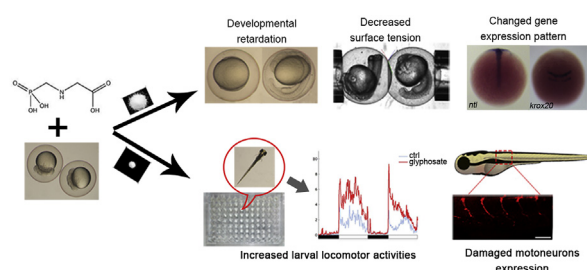
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## HIGHLIGHTS

- High concentrations of glyphosate can induce zebrafish development delay and embryonic death.
- Biomechanics analysis indicates the surface tension of chorion is decreased after glyphosate exposure.
- Embryonic exposure to glyphosate damages embryos CaP axons and induces larval locomotor activities increased.

## GRAPHICAL ABSTRACT



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## ABSTRACT

All of these days, residues of herbicides such as glyphosate are widely distributed in the environment. The ubiquitous use of glyphosate has drawn extensive attention to its toxicity as an organic pollutant. In this study, we employed larval zebrafish as an animal model to evaluate the effect of different concentrations of glyphosate on early development via morphological, biomechanics, behavioral and physiological analyses. Morphological results showed that an obvious delay occurred in the epiboly process and body length, eye and head area were reduced at concentrations higher than 10 mg/L. The expression of *ntl* (*no tail*) shortened and *krox20* (also known as *Egr2b*, *early growth response 2b*) changed as the glyphosate concentration increased, but there was no change in the expression of *shh* (*sonic hedgehog*). In addition, biomechanical analysis of the elasticity of chorion indicated that treated embryos' surface tension was declined. Furthermore, a 48-h locomotion test revealed that embryonic exposure to glyphosate significantly elevated locomotor activities, which is probably attributed to motoneuronal damage. The decreased surface tension of chorion and the increased locomotive activities may contribute to the hatching rates after glyphosate treatment. Our study enriches the researches of evaluating glyphosate toxicity and probably plays a warning role in herbicides used in farming.

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## 1. Introduction

As the active ingredient of more than 750 different broad-

spectrum herbicides, glyphosate is widely used in agriculture because of its effective control of weeds (Mesnage et al., 2015). The glyphosate-based herbicide (common trade name “Roundup”) was first sold to farmers in 1974 (Myers et al., 2016). Since the late 1970s, the volume of glyphosate-based herbicides (GBHs) applied has increased approximately 100-fold (Myers et al., 2016). According to recent findings, more than 600,000 tons of glyphosate have been used globally (Wang et al., 2016). Considering its short half-life and microbial degradation, it will be rapid inactivated in soil (Busse et al., 2001; Mesnage et al., 2015). So glyphosate has been usually thought to be environmentally safe. At sub-agricultural concentrations, glyphosate has little effects on organisms (Bringolf et al., 2007; Gasnier and Dumont, 2009; Akcha et al., 2012; Fan et al., 2013; Jin et al., 2013). Although its mechanism of removing competing vegetation targets an enzyme found only in plants and certain bacteria (Dill, 2005), The toxicity of glyphosate to vertebrates is not fully understood. Previous research has substantially discovered toxicological impacts of glyphosate. Glyphosate-based herbicides produce teratogenic effects on vertebrates -*Xenopus laevis* and chicken (White Leghorn strain) embryos, by impairing retinoic acid signaling (Alejandra Paganelli et al., 2010). Roundup® exposure can increase superoxide dismutase (SOD), glutathione peroxidase (GPx) activity, lipid peroxidation (LPO) and reduce catalase (CAT) activity on Asian clam *Corbicula fluminea* (dos Santos and Martinez, 2014). Glyphosate induced toxic effects in nontarget organisms such as brine shrimp (*A. salina*) nauplii (de Brito Rodrigues et al., 2016) and zebrafish early life stages (Roy et al., 2016b). In addition, breeding zebrafish exposed to 10 mg/L glyphosate during a period of 21 days reduced egg production except fertilization rate, and increased early stage embryo mortalities and premature hatching. Transcript profiling of the gonads revealed 10 mg/L Roundup and glyphosate induced changes in the expression of *cyp19a1* and *esr1* in the ovary and *hsd3b2*, *cat*, and *sod1* in the testis (Uren Webster et al., 2014). One study demonstrated that glyphosate product (Roundup®) might lead to excessive extracellular glutamate levels and consequently to glutamate excitotoxicity and oxidative stress, caused neurotoxicity in hippocampus of immature Wistar rats (Cattani et al., 2014). All in all, several studies have suggested that glyphosate may act as a teratogen, an endocrine disruptor and as a carcinogen (Pandey and Rudraiah, 2015; Claudinei et al., 2016; Myers et al., 2016). In 2005, the Food and Agriculture Organisation (FAO) reported that glyphosate and its major metabolite, aminomethylphosphonic acid (AMPA), are of potential toxicological concern, mainly as a result of accumulation of residues in the food chain (Bai and Ogbourne, 2016). So, this issue should arouse wide concern and deserve our attention.

Zebrafish have several inherent advantages for experiments. They are small, inexpensive to maintain and easily bred in large numbers – a single spawning produces 100–200 eggs. In addition, administration of drugs is simple. Zebrafish larvae absorb small molecules diluted in the surrounding water through their skin and gills. As a predictive animal model, zebrafish has been used in a variety of assays for assessing cardiotoxicity, hepatotoxicity, neurotoxicity and developmental toxicity (McGrath and Li, 2008; de Esch et al., 2012). Researchers used the rest/wake behaviour of larval zebrafish to study psychoactive drugs (Rihel et al., 2010). Someone assessed the toxicity of compounds according to the behavioral profiling (Li et al., 2014; Wang et al., 2015). Previous research on the mechanical properties of the chorion focused on the change in shape and elastic modulus of the zebrafish chorion under perturbation by an external force. The chorion is described as viscoelastic sphere (Kim et al., 2004; Lu et al., 2009; Nam et al., 2010). Here, we focused on the surface tension of chorion to detect the damage of chorion.

It is reported that annual farm-sector glyphosate usage reached to approximately 240 million pounds (~108.8 million kilograms) by 2014 (Myers et al., 2016). Worldwide, glyphosate concentrations can reach up to ~10–15 µg/L averagely in more diluting bodies of water, like rivers (Uren Webster et al., 2014; Roy et al., 2016a). The maximum concentration of glyphosate in surface water reached to 40.8 µg/L in the Orge watershed (France). Maximum concentrations (75–90 µg/L) were detected during application period and rainfall event in storm sewer and in wastewater sewer due to overflows (Botta et al., 2009). Levels of glyphosate in waters ranged from 0.10 to 0.70 mg/L, while in sediments and soils values were between 0.5 and 5.0 mg/Kg in the field from a transgenic soybean cultivation area located in the north of the Province of Buenos Aires, Argentina (Peruzzo et al., 2008). Glyphosate is easy to contaminate the surface runoff, groundwater and farmland because of its high solubility in water (12 g/L at 25 °C) and high sorption (>1.000 L/kg) to soil particles (Jin et al., 2013). Although the concentrations in the normal environment are very low, the concentrations of glyphosate from farmland orchards and ponds are possibly higher due to the glyphosate application and lack of water flow. With regard to the highest concentration, it mainly associated with direct aquatic application and in isolated environments (Uren Webster et al., 2014). Furthermore, if farmers and children were exposed to glyphosate herbicide directly, they would suffer serious damages. Previous studies proved that environmentally relevant concentrations of glyphosate have induced different damages to organisms in some respects. The treatment concentrations in our study included both the environmental relevant concentrations and impossible concentrations in extreme cases. Here, we added higher concentrations of glyphosate to explore its toxicity. Zebrafish was chosen as the laboratory animal model to access the toxicity of glyphosate on organisms under the comprehensive concentrations. Initially, developmental, morphological and genetic effects of glyphosate on zebrafish embryos from 3 hpf (hours post fertilization) to 96 hpf were evaluated. Subsequently, biomechanical impacts induced by glyphosate were examined by quantifying the surface tension of embryos exposed to different concentrations of glyphosate at 3, 10 and 24 hpf. Furthermore, the automatic, high-throughput video-tracking system was introduced to observe the behavioral effect caused by glyphosate via continuously monitoring the locomotion of zebrafish over 48 h. Finally, locomotion increase was probably attributed to the alteration of larval motoneuron at 28 hpf by immunofluorescence assay.

## 2. Materials and methods

### 2.1. Zebrafish maintenance

The zebrafish (AB strain) were raised at a constant temperature of 28.5 °C on a constant light cycle (14 h light/10 h dark). The fish-farming system was equipped with circulating water to maintain a standard water system (KCl 0.05 g/L, NaHCO<sub>3</sub> 0.025 g/L, NaCl 3.5 g/L, and CaCl<sub>2</sub> 0.1 g/L, with 1 µg/mL methylene blue, pH 7.0–7.3). The zebrafish were fed fresh brine shrimp twice every day. In the night before experiment, one male and one female zebrafish were transferred to a breeding tank with a sliding door. The next morning, the sliding door was removed to allow the male to pursue the female. Sufficient eggs were collected in about 15 min after fertilization. The eggs were washed to remove the unfertilized eggs and debris. The normal fertilized eggs were used for subsequent experiments. All experimental protocols and procedures involving zebrafish were approved by the Committee for Animal Experimentation of the College of Life Sciences at Nankai University (no. 2008) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (no. 8023, revised in 1996).

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