Acute toxicity of glyphosate on various life stages of calanoid copepod, *Pseudodiaptomus annandalei*

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Abstract. Copepods are one of the most important primary producers and biodiversity indicators. They are also highly susceptible to various toxicants. In this study, glyphosate (Roundup[®]), a widely used herbicide was used to investigate the toxicity effect on calanoid copepods, *Pseudodiaptomus annandalei*, focused on their nauplius, copepodid, and adult stages. Different concentrations of glyphosate (i.e. 0 - as control, 0.05, 0.1, 0.4, 1.6, 6.4 and 25.6 mg/L) were used to elucidate the tolerance level of *P. annandalei*. The survival rate of copepod was recorded at the intervals of 24, 48, 72 and 96 h after glyphosate exposure. The analysis was performed using probit test to determine the sub-lethal concentrations. Our results revealed that LC₅₀ of the nauplius stage was recorded as 3.47, 3.02, 1.86 and 1.10 mg/L at 24, 48, 72 and 96 h, respectively. Higher LC₅₀ values were recorded at 4.36 mg/L for 24 h, 3.09 mg/L for 48 h, 2.00 mg/L for 72 h, and 1.12 mg/L for 96 h at the copepodid stage. Generally, adult copepods showed a higher level of tolerance to glyphosate among all stages, whereby at this stage LC₅₀ values were recorded as 11.70 mg/L for 24 h, 10.23 mg/L for 48 h, 7.41 mg/L for 72 h, and 3.61 mg/L for 96 h, respectively. Our results indicated that prolong exposure time of glyphosate could increase the susceptibility of *P. annandalei* to the herbicide. Nauplii are the most sensitive group among all. This study showed that glyphosate could post significant eco-toxicological impact to the non-targeted organism.

Keywords: acute toxicity, copepods, glyphosate, herbicide, sub-lethal concentration

INTRODUCTION

Copepod is a primary food source for fish and other aquatic creatures (Turner, 2004). They are a group of aquatic crustaceans inhabiting in freshwater and marine aquatic bodies. Copepods live dominantly as planktonic organisms. They are generally very diverse and abundant in the water ecosystem. In nature, copepods contribute to the nutrients cycling in the biogeochemical processes of the oceans. They feed on diverse organic items such as phytoplankton, and these planktonic

crustaceans are typically referred to as grazers, detritivores, omnivores, carnivores, or in parasitic forms (Heuschele and Selander, 2014). The ecology of copepods present in the water community has a direct influence on the aquatic environment. In other words, they affect the aquatic ecosystem through the distribution of food resource that could alter, and/or determine the relative quality and quantity of fish population and dynamic in an ecosystem.

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Water pollution is a contemporary issue faced by global communities. Point source and non-point source pollutions are threatening aquatic environment in multiple ways through anthropogenic activities such as agricultural fertilization, industrial discharge, and domestic waste disposal. As a result, the aquatic community by default become the primary target subjects that impacted by anthropogenic pollutants. Like many cladocerans e.g. *Daphnia* and *Moina* spp., copepod is one of the aquatic planktonic organisms highly susceptible to water pollution, particularly to the pesticides. Agricultural pesticides are found to be the main causing agent for the death of the species in the aquatic environment. According to Naqvi *et al.* (1981), 24 h sub-lethal concentrations of copepods *Eucyclops agilis* and *Diaptomus mississippiensis* were recorded at 10 and 205 ppm when exposed to the herbicides - paraquat and metribuzin, respectively. In fact, prolonged exposure to the toxicants could increase the sensitivity of copepods to the chemicals, whereby LC₅₀ values were recorded at 5 and 150 ppm for paraquat and metribuzin after 48 h exposure (Naqvi *et al.*, 1981). A study done by Forget *et al.* (1998), reported the coastal marine copepods, *Tigriopus brevicornis* were highly susceptible to atrazine and carbofuran. Their results showed that 96 h LC₅₀ values for the copepodid stage of *T. brevicornis* were found at the concentrations as low as 124.50 and 35.65 µg/L. This clearly indicates that copepod is highly sensitive to pesticide residues.

Glyphosate is a type of herbicide that most widely used as weed controlling agent in the world. This chemical is often used to manage wild grasses and weeds in agricultural and non-agricultural fields (Steinmann *et al.*, 2012). Glyphosate occupies a major market share among the herbicide products due to the convenience of field application, lower cost, and effective in weed management (Roy, 2004). The use of glyphosate in the United States alone is estimated between 19 and 26 million pounds per annum. In South East Asian (SEA) regions, for instance Thailand, glyphosate isopropylammonium were the heaviest used herbicides accounting for 27 million kg in the year 2013 (Tawatsin, 2015).

Glyphosate is highly toxic to the environment. Small crustaceans e.g. shrimp and some cladocerans. A study showed that 72 h and 96 h

LC₅₀ of the decapod shrimp *Caridina nilotica* was recorded at 107.53 and 60.97 µg/L respectively after the exposure to glyphosate (Ashoka Deepananda *et al.*, 2011). Cladocerans e.g. *Daphnia magna* showed 48 h EC₅₀ at 75.2 µM after treated with glyphosate (Hansen and Roslev, 2016). Alteration of this primary producer in the aquatic food web could be a disastrous consequence to the upper hierarchy in the ecosystem. Therefore, this study is conducted to investigate glyphosate toxicity effect to various life stages of the primary producer - the copepods, *Pseudodiaptomus annandalei* which is also a common free-living calanoid copepod perennially found in coastal, estuarine and brackish water in the SEA regions. In the present study, sub-lethal concentrations of glyphosate were determined to elucidate the tolerance level of copepods against the herbicide at different life stages of their life span. This information is important to understand how glyphosate impacts the organisms in various life stages. This bioassay data also provides important baseline data for environmental conservation and protection.

MATERIALS AND METHODS

Stock culture and maintenance of *P. annandalei*. Diatom *Skeletonema Costatum* was maintained in the Environmental Lab, at University of Malaya, Kuala Lumpur. The diatom was used as the culture medium for *Pseudodiaptomus annandalei* in this experiment. To culture the diatom, 0.22 µm-filtered de-chlorinated artificial seawater at 30 ppt was added to fill the tank [33.0 (H) x 15.2 (W) x 20.3 cm (D)]. For sub-culture, 150 mL of diatom culture stock was transferred to two 1 L conical flasks. Subsequently, 4.5 mL of Guillard's (F/2) marine enrichment medium (Sigma) was added to each of the conical flasks using a measuring cylinder. The culture was aerated continuously under the illumination of 12 h of light and 12 h of dark. A sub-culturing process was repeated at 2-day intervals followed the procedure mentioned above.

The copepods culture was collected from wild habitat (Matang river - Kuala Sepetang, Malaysia) and maintained at laboratory condition (28±1°C,

8.4-9.2 mg/L of dissolved oxygen, pH 7.3 - 7.8 and salinity at 20 ppt) in the University of Malaya. Feeding of copepods with diatom was performed every two days. To perform the feeding, sub-cultured diatoms were transferred to a plastic tank. The diatom was diluted to approximately 20 ppt of salinity with de-chlorinated filtered-salt water. Approximately 700 mL of diluted diatom at 10^2 to 10^5 cells/mL was added to the copepods culture tank for the feeding purpose.

Isolation of different life cycle stage of *P. annandalei*. The isolation of different stage of

copepods, the body size and the development of appendages of copepods were observed under the microscope. Copepods were categorized into nauplius (Figure 1a), copepodid (Figure 1b) and adult copepods (Figure 1c). The copepodid was identified when the organism was measured with a shorter body and the length of appendages compared to adult stages (Figure 1b and 1c). Sorting was performed carefully using sterile pipette in Petri dishes under a dissecting microscope to avoid physical stress to the testing organisms.

Figure 1. (a) Nauplius stage of *P. annandalei* under 20× magnification. (b) Copepodid stage of *P. annandalei* under 20× magnification. (c) The adult stage of *P. annandalei* under 20× magnification.

Preparation of glyphosate. Glyphosate (Roundup®, Monsanto (M) Sdn. Bhd., Malaysia) stock solution (25.6 mg/L) was prepared by adding 14.44 µL of the glyphosate-isopropylammonium (41% w/w or equivalent to 360 g/L) to a 200mL of volumetric flask containing sterile artificial seawater (15 -18 ppt). Serial dilution was performed to obtain testing concentrations i.e. 0.05, 0.1, 0.4, 1.6, 6.4 25.6 mg/L of glyphosate. A total of 7 treatments including a control (0 mg/L of glyphosate) were

prepared for the bioassay. All testing solution was freshly prepared prior to the experiment.

In vivo toxicity bioassay. Acute toxicity assay was conducted based on APHA standard (2005). Three stages of *P. annandalei* i.e. nauplii, copepodids and adults were isolated from the stock culture using a stereomicroscope (Leica, Germany) as mentioned early. Fifteen individuals of nauplius, copepodid and adult individuals were carefully pipetted and transferred into different

Petri dishes contained different glyphosate concentrations (i.e. control – 0 mg/L; 0.05, 0.1, 0.4, 1.6, 6.4 and 25.6 mg/L). Artificial seawater (15–18 ppt) without glyphosate was served as a control. Approximately 2 mL of diatom (10^5 cells/mL) was added to each Petri dish in every two days. Occasionally, the Petri dishes were gently swirled to ensure the testing solutions were well oxygenated. Mortality of the testing organisms was observed and recorded every 24 hours (i.e. 24, 48, 72 and 96 h) after exposed to glyphosate at different concentrations. The mortality rate of *P. annandalei* in percentage was determined after the experiment. Mortality was confirmed through observation under a microscope, subsequently dead individuals were transferred to new Petri dishes. To confirm the fatality, an individual is considered dead when no sign of heartbeat was observed and the individual did not show any life responses towards teasing using a tip (Ashoka Deepananda *et al.*, 2011). All treatments were performed in triplicate.

Statistical analysis. The data were tested for normality and variance heterogeneity before analyses. Data of LC_{50} and P-value were calculated and analyzed using the Probit Test. The significant differences in all analyses were accepted at $p \leq 0.05$.

RESULTS

LC_{50} of nauplius decreased conversely as the time of exposure towards glyphosate prolonged. After a 24 h exposure, LC_{50} was recorded at 3.47 mg/L in nauplius, and yet, the value started to decrease gradually until 96 hours. After 96 h of exposure, LC_{50} of the nauplius was reported at 1.10 mg/L of glyphosate (Table 1). Likewise, a similar trend of LC_{50} was observed in copepodid. LC_{50} of copepodid was recorded at 4.36 mg/L in first 24 h (Fig. 2), despite having a similar trend, it was however higher than those reported in nauplius (3.47 mg/L) (Table 1). After 96 h of exposure, LC_{50} value in copepodid was recorded at 1.12 mg/L with an implication of higher susceptibility of copepodid to the chemical as a prolonged time of exposure (Table 1; Figure 5). Table 1 summarized the LC_{50} values of various life stages

of *P. annandalei* across the exposure times. Copepodid demonstrated moderate tolerance level compared to adult copepods, while nauplius showed the highest sensitivity to the herbicide. In the adult stage of copepods, LC_{50} shared a similar trend between the nauplius and copepodid. However, 24 h LC_{50} in adult stage was found significantly higher ($P = 0.008$) at 11.7 mg/L. Unlike those in the younger stage of copepod, LC_{50} values continued to decrease until the 96 h (6.31 mg/L) after exposed to glyphosate. It is worth noting that, all the LC_{50} values in adult copepods were higher compared to the LC_{50} of nauplius and copepodid (Table 1). The 48 h LC_{50} at different life stages were found to be at 3.03 mg/L (nauplius), 3.09 mg/L (copepodid) and 10.23 mg/L (adult copepods); and 72 h LC_{50} at 1.86 mg/L (nauplius), 2.00 mg/L (copepodid) and 7.41 mg/L (adult copepods), respectively. In general, nauplius showed lower LC_{50} and tolerance level against glyphosate. Complete mortality was observed even at 6.4 mg/L of glyphosate after 24 h of exposure time. On the other hand, adult copepod was found to have higher LC_{50} and tolerance level against the chemical, 60% of survival rate was observed at 6.4 mg/L after 96 h of exposure time. Our results showed the survivorship of nauplius, copepodid and adult copepods decreased as the concentration of glyphosate and exposure time increased (Figure 2 – 5; Table 1).

Figure 2. The survival rate of *P. annandalei* at 24 h after exposure to various concentrations of glyphosate.

Figure 3. The survival rate of *P. annandalei* at 48 h after exposure to various concentrations of glyphosate.

Figure 4. The survival rate of *P. annandalei* at 72 h after exposure to various concentrations of glyphosate.

Figure 5. The survival rate of *P. annandalei* at 96 h after exposure to various concentrations of glyphosate.

DISCUSSION

Our results showed that LC_{50} values were comparatively lower in the stage of nauplius (1.10–3.70 mg/L) than the stage of copepodid (1.12–4.36 mg/L) after 24 to 96 h exposure to

glyphosate (Roundup®). Higher tolerance level was found at the adult stage of *P. annandalei* (6.31–11.70mg/L) (Table 1). In general, this study indicated that the adult stage of *P. annandalei* showed higher tolerance against glyphosate compared to their younger stages. Glyphosate killed copepods by altering their mitochondrial activity, presumably through uncoupling of oxidative phosphorylation in the process of cellular respiration (Mensah *et al.*, 2015). According to Tsui and Chu (2003), LC_{50} values of marine copepods *Acartia tonsa* was recorded at 49.3 mg/L after exposure to glyphosate. In fact, the toxicity of glyphosate is usually lower than the formulated herbicide, but the toxicological synergy occurs between POEA (a surfactant mixture) and glyphosate greatly increased the toxicity of the product. In a study conducted by Chen *et al* (2004), a commercial formulation of glyphosate - Vision® (containing POEA) can cause a 100% of mortality rate to the female cladoceran *Simocephalus vetulus*, and causing a 50% reduction of total neonates in the bioassay. Several reports on the impact of the chemical to other invertebrate were also available. For example, LC_{50} of the amphipod *Hyaella Azteca* was recorded at 1.5 mg/L (Tsui and Chu, 2004), and *Gammarus pseudolimnaeu*, was found to be 62.1 mg/L (Folmar *et al.*, 1979) after treated with glyphosate. While in the aquatic teleost, 620 mg/L of LC_{50} was recorded in the carp *Cyprinus carpio* (Neškovic *et al.*, 1996), and fathead minnows *Pimephales promelas* was found at 2.3 mg/L (Folmar *et al.*, 1979). Susceptibility to pesticide is dosage-, genotype-, species- and life stage-dependant, however, different commercial preparation of the herbicide can cause a different level of toxicity. For example, channel catfish *Ictalurus punctatus* showed 50% mortality at 130 mg/L in glyphosate treatments (Folmar *et al.*, 1979), which has lower susceptibility compared to the finding reported by Abdelghani *et al.* (1997). The authors claimed that LC_{50} of *I. punctatus* was recorded at 14.5 mg/L when the commercial grade of glyphosate was used.

Population dynamics of zooplankton can be affected by pesticides through a life-cycle breakdown, which in turn, significantly influences the efficacy of energy transfer from the bottom of the food chain to the predators (Chuah *et al.*, 2007). There are extensive works of literature

focus on the toxicity of heavy metals to copepod's life-cycle. In a study conducted by O'Brien *et al.* (1988), 96 h LC₅₀ of the harpacticoid copepods *Tigropus californicus*'s egg stage was reported at 3.6×10^{-9} M treated with copper. The value increased to 4.8×10^{-9} M at the nauplius stage, while copepodid showed the least susceptibility towards copper at 12.0×10^{-9} M (96 h LC₅₀) of copper. Similar findings were observed in another harpacticoid copepod, *Tisbe holothuria* when exposed to copper and cadmium. One-day-old nauplii showed 50% mortality at 0.3142 mg of Cu/L and 0.5384 mg Cd/L after 48 h of exposure. As in accordance to the report, 5-day old nauplii exhibited slightly better tolerance to copper (48 h LC₅₀; 0.3415 mg Cu/L) and cadmium (48 h LC₅₀; 0.645 mg Cd/L), older stage of nauplius such as 10 day-olds showed even higher tolerance against the heavy metals (48 h LC₅₀; 0.5289 mg Cu/L, and 0.9061 mg Cd/L, respectively) (Verriopoulos and Moraitou-Apostolopoulou, 1982). Likewise,

similar toxicity threshold was also observed in the present findings, whereby 96 h LC₅₀ (Table 1) increased from nauplius (1.10 mg/L) to copepodid (1.12 mg/L) and followed by the adult stage (6.31 mg/L). Like many fish and invertebrates, younger stages are more vulnerable to toxicants compared to their adults (Hutchinson *et al.*, 1998; Chuah *et al.*, 2007; Herkovits *et al.*, 2009; Mohammed *et al.*, 2009). Generally, the survival rate of *P. annandalei* decreased with the increment of glyphosate concentration and exposure time. Early stages of copepods showed more sensitive to toxicants compared to fully developed life stages (Mohammed *et al.*, 2009). Lethal concentration (100% mortality rate) of glyphosate was reported as 6.4 mg/L for nauplius and copepodid in this study. This finding is in an agreement with Bustos-Obregon and Vargas (2010). The authors showed that a similar group of organophosphates - diazinon could kill 100% *Artemia salina* nauplii at 9 mg/L.

Table 1. Acute toxicity of glyphosate to various life stages of the copepod, *P. annandalei*.

Stages of copepods, <i>pseudodiaptomus</i> <i>annandalei</i>	Exposure time (hours)	LC ₅₀ (mg/L)	P-value
Nauplius	24	3.47	0.055
	48	3.02	0.068
	72	1.86	0.238
	96	1.10	0.347
Copepodid	24	4.36	0.010*
	48	3.09	0.012*
	72	2.00	0.122
	96	1.12	0.262
Adult	24	11.70	0.008*
	48	10.23	0.072
	72	7.41	0.093
	96	6.31	0.349

LC₅₀ of *P. annandalei* at the various time was calculated using Probit Test ($n = 15$ /replicate). Three replicates were performed for each concentration group. * Indicate that the data is significantly different ($P < 0.05$).

So far, this is the first report documented the effect of glyphosate on different life stages of *P. annandalei*. The variability in the susceptibility between various life stages may be attributed to several factors; these factors often take into consideration which may be different between life

stages, for examples differences on the morphological, physiological, behavioral and biochemical characteristics. The organs systems of younger aquatic organisms are underdeveloped, and thus very sensitive to toxicants, but once it was fully developed, they

may be less vulnerable to stressors (Ozoh, 1979; Bentivegna and Piatkowski, 1998). Or, it might be due to the smaller size of early life stage, specific time to be taken to reach and damage the target organs by the toxicant may be shorter compared to older stages. This could be one of the possible reasons that cause the variation of sensitivity in different life stages of copepods towards toxicant. The younger copepods might have an underdeveloped nervous system; thus, they have high sensitivity towards toxicant compared to the fully developed copepods. The assumption is supported as most embryos and larval forms have poor or incomplete development of organs such as functional nerve system, gills, and liver. Their skin also poorly functions as a protective barrier, which acts as a primary method of ionic regulation in the early life stages (Florkin and Scheer, 1974). Permeable skin could render to a larger surface area for toxicants to be uptake to the body. As a result, toxicants increased above the threshold of the susceptibility of the larva (Arufe *et al.*, 2004).

Unlike heavy metals, glyphosate is a systemic herbicide. It inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate biosynthetic pathway. EPSPS is an important enzyme to catalyze the transformation of phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P) in the biosynthetic pathway (Amrhein *et al.*, 1980). Inhibition of EPSPS enzyme production can be fatal to plants and animals. Ashoka Deepananda *et al.* (2011) showed that 24 h exposure of glyphosate can kill 50% of *Phyllodiaptomus annae* as low as 1.059 mg/L. The present results showed that adult stage of *P. annandalei* (24 h LC₅₀; 11.70 mg/L Gly) demonstrated slightly better tolerant to glyphosate compared to the adult stage of *Phyllodiaptomus annae* as reported previously. This could be attributed to variation in many biotic and abiotic factors such as geographical distribution, species, innate immune response etc. For instance, *P. annae* was collected from the rivers of Sri Lanka (Ashoka Deepananda *et al.*, 2011), while *P. annandalei* in this study was collected from an estuary in Matang river, Kuala Sepetang, Perak, Malaysia. The climate and variation in abiotic environmental factors such as temperature, salinity, and diet preference could be direct or indirectly influence the biological reaction, metabolism and physiological changes of the

copepods. These environmental factors are of particularly important in terms of survival, maturation time, the number of ovigerous females and fecundity in copepods (Hall and Burns, 2001; Rhyne *et al.*, 2009).

CONCLUSION

The present findings showed the concentration as low as 1.10 mg/L in glyphosate could cause a significant deleterious effect on the survivorship of *P. annandalei*. Early stage of copepods (i.e. nauplius) is very sensitive towards glyphosate (96 h LC₅₀; 1.10mg/L Gly) compared to copepodid (96 h LC₅₀; 1.12mg/L Gly). While adult copepod could tolerant slightly better than their younger stages (96 h LC₅₀; 6.31mg/L Gly). The present study provides a useful ecotoxicological dataset on the tolerance level of *P. annandalei* against glyphosate.

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