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TOXIC EFFECTS OF COPPER (CU) ON FRESHWATER PLANARIA (*DUGESIA TIGRINA*) REGENERATION

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ABSTRACT

Industrialization and economic development have resulted in copper (Cu) contamination of aquatic environments, resulting in a variety of negative impacts that are still challenging to assess. Invertebrates usually serve as a good model to assess heavy metal toxicity in the natural environment. The freshwater planarian *Dugesia tigrina* is one of the macrobenthic species recognized as important organisms in toxicological studies. Its sensitivity to harmful substances and environmental stressors can be easily evaluated based on its growth, development and unique features such as its regeneration capability endpoints. In this study, the lethal and sub-lethal toxicity of copper to *D. tigrina* was evaluated, particularly in terms of mortality, growth, and development and regeneration performance. The LC50 was 0.751 ppm for 48h exposure, while the final body length, specific growth rate, and regeneration rate were significantly disrupted by copper at a concentration of 0.009 ppm or higher. This study provides evidence that *D. tigrina* shows a sensitive response to copper concentrations. Copper levels of 0.009–0.15 ppm altered individuals' development and regeneration following copper exposure. This study suggests that *D. tigrina* serves as a good indicator for environmental assessment. In addition, its unique regeneration characteristics benefit the assessment of the long-term effects of toxic chemicals on aquatic organisms.

KEY WORDS : Acute toxicity, Copper, Planarian, Regeneration, Indicator, Environmental assessment

INTRODUCTION

Heavy metal contamination in aquatic ecosystems has attracted global attention due to its persistence, toxicity and potential bioaccumulation in the food web (He *et al.*, 2001; Su *et al.*, 2017; Yýlmaz *et al.*, 2017; Ali and Khan, 2018). One of the most toxic heavy metals to aquatic organisms and ecosystems is copper, of which the most bioavailable form is cupric ion (Cu^{2+})(Solomon, 2009). In small amounts, copper is an essential trace element for organisms, taking a role in enzyme catalyzation and carbohydrate metabolism. An increasing amount of copper in the environment can be attributed to natural and anthropogenic factors. However, the major cause of elevated copper concentration is man-made activities such as mining, industrial discharge, and the massive usage of fertilizer and pesticides (Fuchs *et al.*, 1997; Prá *et al.*, 2005; Tang *et al.*, 2014; Yilmaz *et al.*, 2017; Guan *et al.*, 2018). Copper pollution in water bodies as a result of industrialization can affect not only the metabolic processes in aquatic organisms but also the entire aquatic ecosystem(Ribeiro and de Aragão Umbuzeiro, 2014; Leal *et al.*, 2018).

The effects of copper pollution have been broadly examined in microorganisms, invertebrates, fish, bivalves, and macrophytes. Among these, invertebrates usually serve as a model organism to assess chemical toxicity, for example, the daphniids that were recommended by OECD(OECD 2012). However, the use of Platyhelminthes in toxicity testing is relatively scarce. Interestingly, freshwater planarians have received attention over the past three decades as promising test animals for toxicology (Wu and Li, 2018).

Planarians are typical macrobenthos in both lotic and lentic aquatic ecosystems. Macrobenthos, including planarians, is frequently used to assess water quality in streams, since these groups rarely reside in polluted streams (Manenti and Bianchi, 2014). Planarians are slow-moving animals that slide along firm substrate using their self-made mucous. The importance of planarians' presence in aquatic ecosystems relates to the food chain. Planarians are prey for a variety of aquatic animals, including fish, amphibians, and insects, but also a predator of insect larvae, aquatic worms, and crustaceans. Their significant role as a predator of insect larvae, particularly mosquitos, potentially regulates insect population size in some habitats. For experimental purposes and toxicity testing, planarians serve a variety of advantages, such as ease of culture, low cost, and unique aspects derived from their stem-cell system (neoblast) that provide a response that can be used to assess the potential effects of harmful substances (Knakievicz, 2014). In addition, freshwater planarians have been reported to be more susceptible to copper exposure (also mercury (Best and Morita, 1991; Medvedev and Komov, 2005) and cadmium (Calevro *et al.,* 1998)) than to other metals (Wu and Li, 2018). The capability to regenerate provides different endpoints and determines survival competence in a natural ecosystem.

The study aims to investigate the acute and sub-acute concentrations of copper for free-living freshwater planarians and the effect on their regeneration competence as a measure of their survival in a natural ecosystem. Among planarians, *Dugesia tigrina* is one of the species most frequently used to examine chemical substances or stressors in the aquatic environment(Debora de Lucca and Smith, 2001; Knakievicz and Ferreira, 2008; Ribeiro and de Aragão Umbuzeiro, 2014; de Oliveira *et al.*, 2018; Wu and Li, 2018). Regeneration in planarians provides an opportunity to observe developmental biomarkers for aquatic pollutants. The results might serve as useful information for ecological risk assessment in the freshwater environment, especially at the nationwide level in Indonesia.

MATERIALS AND METHODS

Test organisms and chemicals

The planaria (*D. tigrina*) were collected from around the mountain area in Tangkuban Perahu, Bandung, Indonesia. Small tree trunks, stones, and litter from the river were collected and placed together in a tray to find the planarians. Individuals were separated and kept in a plastic container together with river water. Once in the laboratory, prior to the beginning of the experiment, the planaria were acclimatized for 4 days to laboratory conditions by holding them in the river water collected during the sampling work. To gain a sufficient number of planaria for the experiment, those with the greatest body length were cut transversally above the pharynx and placed in the aquarium in order to regenerate.

Both the culture and test media for the experiment originated from the sampling site, i.e., the river water of Tangkuban Perahu. The planaria were fed small amounts of raw cow liver twice a week. Culture media was cleaned from the remaining feed and renewed every 2–3 hours after feeding (Pennak, 1989).

A 1-L stock solution was prepared using the river water with 0.393 mg $CuSO_4.5H_2O$ (equal to 100 ppm Cu concentration). The stock solution was diluted with river water to the final Cu concentrations mentioned below. The river water was aerated for 2 hours before making the stock solution. Homogenization of stock solution was carried out using a magnetic stirrer for approximately 15 minutes. To ensure that the condition of the media was suitable for culture and experimentation, the water was renewed regularly and its quality was checked frequently.

Acute toxicity test

An acute toxicity test was first carried out to find a range of test concentrations that would include those that killed 50% of the test organisms. In this test, 10 planaria of similar body size (1.0 ± 0.0 cm) were placed in 200 mL of $CuSO_4.5H_2O$ solution with several dilutions (10^{-2}, 10^{-1}, 10^{0}, 10^{1}, 10^{2}) in quadruplicate. Planaria reared in river water alone with no $CuSO_4.5H_2O$ solution served as a control group. Observation was then carried out every 24 hours to count the dead individuals; this lasted for 48 hours. Following this procedure, the determination of Cu concentration for the LC_{50} toxicity test was then made based on the logarithmic APHA calculation (APHA AWWA WPCF, 1975).

Sub-lethal toxicity test

Once the lethal dose was determined, the sub-lethal toxicity test was then performed. Each planarian (10.0 ± 0.0 mm in length) was then placed in aPetri dishand decapitated above the pharynx. Each posterior (headless) part was immediately placed in 5 different sub-lethal copper concentrations (0, 0.009, 0.018, 0.075, and 0.150 ppm) with 5 replicates for each concentration. Exposure was carried out in 500 mL plastic glass containers containing 200 mL of media. They were exposed to copper for 16 days or through the period of completion of the head formation. For observation purposes, the planaria were placed into Petri dishes equipped with millimeter block paper to facilitate body size measurement. Observationswere madeunder stereomicroscope every two days to follow the regeneration process. The observer recorded information about the planarian's final body length, time (in days) spent to complete the regeneration process, and the specific growth rate until an intact individual developed. The formula used for measuring specific growth rate is described as follows:

$$G = {(\log eL2 - \log eL1)}/{T} \quad .. (1)$$

where G is the specific growth rate, $L1$ and $L2$ are a planarian's body lengths at the initial and final observations, respectively (mm), and T is the observation period (days).

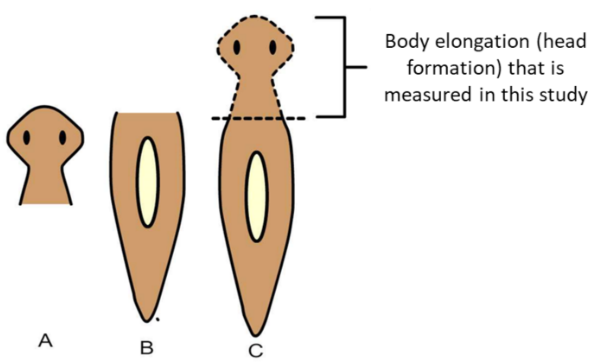

Fig. 1. Transversal decapitation of *D. tigrina*: A. Anterior part (for culture purposes); B. Posterior part (for sub-lethal toxicity test); C. The completion of the regeneration process.

Statistical analysis

The LC_{50}values at 24 h and 48 h were obtained by Probit analysis at a 95% confidence interval using the EPA Probit Analysis Program version 1.5. The effect of copper exposure on sub-lethal endpoints was assessed by one-way ANOVA followed by Duncan's post-hoc test to confirm differences between the treatment groups. Statistical analysis was performed using version R-3.6.1 of the '*R*' software environment for Microsoft Windows.

RESULTS AND DISCUSSION

Acute toxicity test of Cu

The toxicity test showed that there was no mortality in the control group during the acute toxicity test. In contrast, surviving individuals declined with increasing copper concentrations as presented in Figure 1. The 24-h and 48-h LC_{50} values were 1.342 and 0.751 ppm, respectively (Table 1).

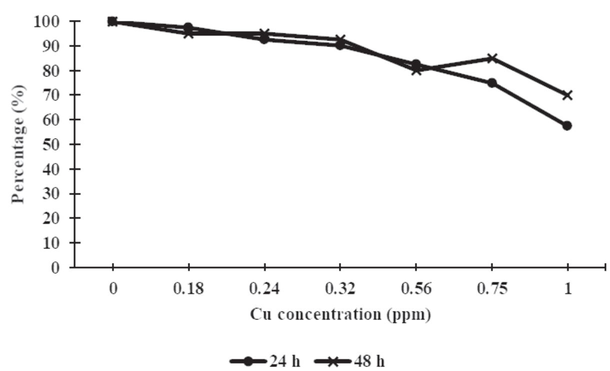

Fig. 2. Survivorship of the planaria exposed to different copper concentrations during the acute toxicity test.

Table 1. LC_{50} of planarians exposed to different Cu concentrations.

Replicate	LC_{50} (ppm)	
	24 h	48 h
I	1.648	0.637
II	1.013	0.769
III	1.225	0.832
IV	1.483	0.769
Mean LC_{50} ± SD	1.342 ± 0.280^a	0.751 ± 0.082^b

Note : LC_{50}: median lethal concentration that killed 50% of test organisms. Each unit of replication consisted of 10 planaria. Mean LC_{50} values with different superscript letters indicate a significant difference (p< 0.05) according to the independent sample t-test.

Cu effects on body length, growth specific rate and regeneration time

The exposure of the planaria to copper significantly reduced their body size (Figure 2, $p < 0.01$). Reduction of body size was dose-dependent. The mean body sizes of the planaria in the control group and those exposed to copper were, respectively, 8.00 ± 0.00 mm and 6.00 ± 0.00 to 7.40 ± 0.55.

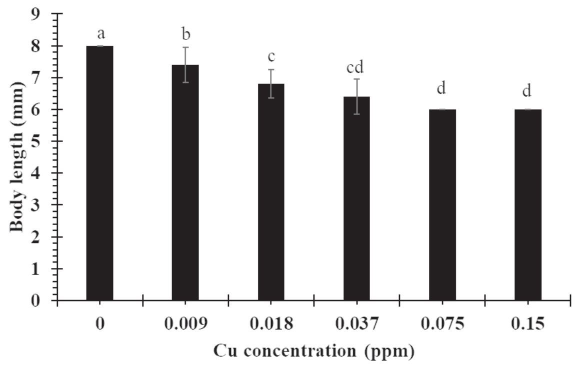

Fig. 3. Average final body length of planaria exposed to different copper concentrations during the regeneration process. Data presented as mean ± SD. Different letters indicate differences among treatment groups.

The pattern of body size reduction was in line with the specific growth rate of the planaria, as indicated in Figure 4a ($p<0.05$). The planaria exposed to copper tended to have a slower growth rate in comparison to those in the control group. Without copper contamination, the planaria could increase their body length as much as 0.07 ± 0.01 mm/day. The slowest growth rate was recorded in the planaria exposed to the highest copper concentration (i.e., 0.15 ppm) at 0.012 ± 0.001 mm per day.

In the present study, the completion of the regeneration process was indicated by head formation, two eyespots (ocelli) and auricles. The period necessary to complete the regeneration process was significantly longer in the planaria exposed to copper (Figure 4b, $p<0.01$), i.e., it ranged from 8.00 ± 0.00 to 15.20 ± 1.09 days compared to 6.80 ± 1.09 days for the control group. Copper exposure caused the delayed completion of the regeneration process. However, no malformations or abnormalities were observed in the regenerated tissue in the copper exposure groups.

DISCUSSION

The present study provides valuable information for toxicity assessments of any chemical substances for aquatic organisms. The sensitivity of planaria to copper contamination suggests that this species is reliable as a test organism employed in toxicity testing. For the lethal level, *D. tigrina* exhibited a good response to a low concentration of copper, demonstrating that the species satisfies the recommended requirement. In addition, the results of this study also indicate that copper exposure negatively affects the regeneration and the growth rate of planaria, a response that is dose-dependent. There are two pathways for planaria to bioaccumulate copper: through penetration of the cutaneous layer through diffusion (Kapu and Schaeffer, 1991) and through food consumption. Copper accumulation in the tissues through either of these mechanisms would later interfere with the physiology and behavior of *D. tigrina*.

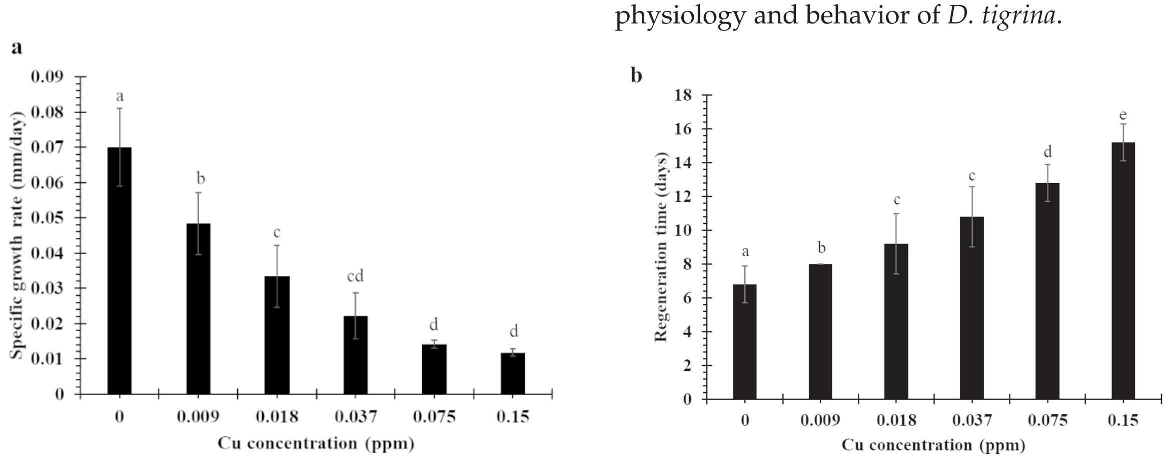

Fig. 4 (a). Average specific growth rate and (b) average time taken to complete the regeneration process for the planaria exposed to different copper concentrations. Data presented as mean ± SD. Different letters indicate differences among the treatment groups.

From the results of the acute toxicity test, the 24h and 48h LC50 values of the *D. tigrina* examined in this study were not markedly different from those reported by Knakievicz and Ferreira (2008) using a similar species in the adult phase (0.50 ppm for 48h LC50). Similar results were also found in the study of Guecheva *et al*. (2003) using *Dugesia schubarti* (where LC50 was 1.23 ppm), and in Zhang *et al.* (2014) using *Dugesia japonica* (where LC50 was 1.563 ppm). Although the 24h LC50 in this study was far above the value of 0.026 ppm Cu^{2+} reported for sensitive species *Daphnia magna* (Mastin and Rodgers, 2000; Mohammed, 2007) and for other macrobenthos such as the worm *Lumbriculus variegates* (O'Gara *et al*., 2004), *D. tigrina* clearly displayed a sensitive response as a test organism. The sensitivity of the planarians to chemical substances indeed varies among species. However, Wu and Li (2018) have documented that this is still in the range of sensitivities of other invertebrates and issome what similar to those commonly used as test organisms.

This study also demonstrates an inhibitory effect of copper exposure on the regeneration process of the planaria. Higher copper exposure prolonged the regeneration process in test planaria compared to the control group. In Cu-exposed groups, the regeneration process took place over a longer period, with no abnormalities found in the regenerated head. These results are in agreement with Knakievicz and Ferreira's (2008) findings that copper has cytotoxicity effects on *D. tigrina*. Teratogenic effects of heavy metals that result in planarian abnormalities and delayed regeneration have also been recorded for exposure to other metals, for example, chromium (Calevro *et al.,* 1998; Knakievicz and Ferreira, 2008) and aluminum (Calevro *et al*., 1998). Regeneration is one of the unique features of the planarians, and it provides good biomarkers for copper exposure (Knakievicz and Ferreira, 2008). Tissue regeneration in amputated fragments of planarians, particularly head formation, is a useful indicator with which to evaluate the toxicity of environmental chemicals (Best and Morita, 1991; Calevro *et al*., 1998).

The growth and regeneration capabilities of planarians are crucial for the survival of the animals. This includes tissue and organ maintenance and healing from injuries. This also applies to planarians' extraordinary ability to regenerate as part of their reproductive strategy (Vila-farré and Rink, 2018). Interference in the growth rate and regeneration process of planarians may have ecological implications. Reductions in growth and regeneration rates make them less likely to survive in a natural environment. In addition, planarians have an important role as both prey and predator in the aquatic food chain. The impaired growth rate and regeneration process will reduce the availability of food for the higher taxa, such as certain types of fish and shrimp, the planarians' predators. This condition also applies to planarians' prey, such as larval insects. Growth interference may also restrict the mobility and movement of planarians, making it difficult for them to escape from unfavorable conditions. At the population level, a further implication of copper intoxication of planarians could be the loss of the ability to regenerate and/or reproduce, leading to an unbalanced aquatic ecosystem.

Overall, this study enhances toxicological knowledge concerning the effects of copper on aquatic organisms. Moreover, the considerable unique characteristics of planaria position them as useful bioindicators for the aquatic environment. The result supports the use of freshwater planarians as model organisms for assessing heavy metal contamination. The sensitivity of the study species to low levels of copper contamination meets the requirement for a test organism in toxicological assessment. In addition, for the purpose of sub lethal evaluation, its growth and unique regeneration characteristics facilitate the assessment of the long-term effects of toxic chemicals on aquatic organisms. Due to the massive contamination of freshwater ecosystems with copper because of inappropriate wastewater disposal into surface water, especially in downstream areas, further research into the survival and regeneration of *D. tigrina* in water bodies contaminated with copper is a priority to pursue, especially to determine the interactions of copper and other substances in this species.

CONCLUSION

To conclude, as it was exhibited in the results of copper exposure on acute, growth and regeneration effects of *D. tigrina*, this species serves as a good indicator for environmental assessment. The species is able to be used as a model organism both in lethal and sublethal toxicity testing.

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