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Baseline

Acute toxicity test of copper pyrithione on Javanese medaka and the behavioural stress symptoms

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ABSTRACT

This study was conducted to investigate the median lethal concentration (LC50) of copper pyrithione (CuPT) at 96-hr exposure on adult Javanese medaka (*Oryzias javanicus*) in revealing toxicological effects of CuPT contamination in the tropical area. Wild stock fishes were acclimatized for 14-days prior analysis. Triplicate of test tanks for seven test concentrations were placed with ten fishes each, this includes two control tanks. The behaviour of the tested fishes was manually observed through a camera. The LC50 of CuPT at 96-h was found to be 16.58 mg/L. Tested fishes swam slowly in vertical movement and swam fast towards food during feeding time as the sign of stress behaviour. Meanwhile, fishes in the two control groups swam actively in a horizontal manner and no excitement during feeding time. No mortality in control groups. Results indicate CuPT to be toxic to Javanese medaka at low concentration and caused behavioural stress.

Medaka fish under genus *Oryzias* is widely known as a biological indicator as they have a diverse adaptability towards different salinity where it can either represent freshwater, brackish water and also seawater condition (Wakamatsu et al., 1994; Inoue and Takei, 2002). In the study comparison of salt water adaptability among four *Oryzias* species, Javanese medaka (*O. javanicus*), Indian medaka (*O. dancena*), Japanese medaka (*O. latipes*) and Marmorated medaka (*O. marmoratus*) inhabiting different osmotic environments. *O. javanicus* which inhabit salt water or brackish water has the full adaptability to both salt water and fresh water at all the lifecycle stages examined. Other species such as *O. dancena* which inhabits brackish water has a limitation of the hatching rate in salt water, *O. marmoratus*, a species inhabit fresh water is low adaptability in salt water for all stages examined. Meanwhile *O. latipes*, another freshwater species shown better adaptability to salt water at most stages examined. Therefore, *O. javanicus* (Javanese medaka also known as Javanese rice fish) has been introduced as the best test organism for toxicity testing. In addition, this species has been confirmed have good adaptability in laboratory control environment with water pH ranging between pH 6–7 as they have low mortality, high in egg production and hatchability (Salleh et al., 2017). Numerous studies have been done in order to enhance more information for it potential as a model organism to assess the toxicity of aquatic systems in tropical freshwater, estuarine, and marine environments (Inoue and Takei, 2002; Inoue and Takei, 2003; Imai et al., 2005; Woo et al., 2006; Yu et al., 2006; Ismail and Yusof, 2011; Khodadoust et al., 2013; Yusof et al., 2014).

Copper pyrithione (CuPT, Copper 2-pyridinethiol-1-oxide) has been developed as new antifouling biocide as a replacement for tributyltin (TBT) due to the severe toxicity of TBT towards marine organisms. As an alternative biocide, CuPT has the ability to be toxic to marine species but its degradation products were assumed to be less toxic due to its photo-degradability (Turley et al., 2000). Even though the neutral Cu–pyrithione complex is thermodynamically more stable than that of Zn (Sun et al., 1964), and in constant darkness, where both pyrithiones are unable to degrade photolytically, transchelation is predicted to occur (Grunnet and Dahllof, 2005). Despite CuPT has been used as marine antifouling paints for replacing TBT, interestingly, CuPT has similar toxicity with TBT in all test species, indeed CuPT was found to be highly toxic to medaka fish larvae comparing to TBT as reported by Bao et al. (2011). In much earlier study done by Mochida et al. (2006), the median lethal concentrations (LC50s) of the pyrithione (PT) antifoulants CuPT and zinc pyrithione (ZnPT) on the basis of actual concentrations to a teleost, red sea bream (*Pagrus major*) were identified at 9.3 and 98.2 μg/L, and a crustacean, toy shrimp (*Heptacarpus futilirostris*) at 2.5 and 120 μg/L, respectively (Mochida et al., 2006). Histological observations on the test organism revealed that CuPT and ZnPT

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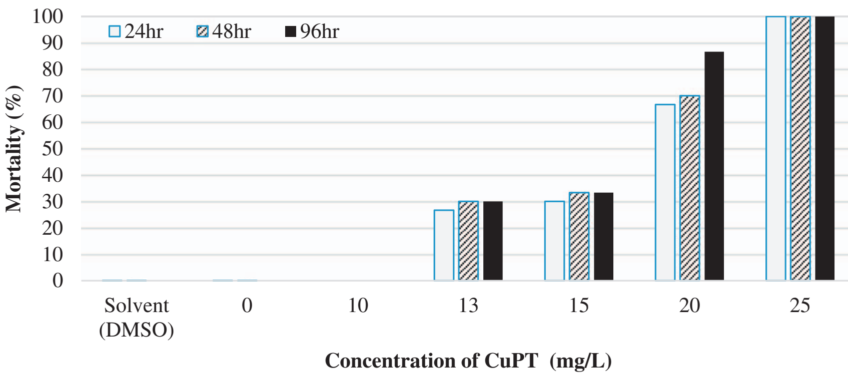

Fig. 1. Mortality of adult Javanese medaka at various time intervals after exposed to CuPT.

have potential to cause fatal hypoxemia among the experimental fish where the secondary lamellae of the gill filaments of the fishes were damaged after exposure to the PTs. The same author also presented data on joint toxicities of the PTs with Cu as CuPT and ZnPT are usually used in combination with Cu. The LC50 values of the joint compounds were 84.4 μg/L for red sea bream and 113 μg/L for toy shrimp, meanwhile results on the joint toxicity of ZnPT and Cu mixture is more than the additive toxicities of CuPT and Cu, especially in toy shrimp respectively (Mochida et al., 2006). The author suggested enhancement of toxicity in the mixture was inferred to be caused by the conversion of ZnPT to the more toxic CuPT in the presence of Cu. Recently, CuPT was also found to affect the developing stage of zebrafish (*Danio rerio*) embryos (Almond and Trombetta, 2016). Thus it is a need to monitor and provide data on the toxic effect of CuPT from other regions in gathering effect of the worldwide use of this compound in the aquatic environment.

For that purpose, other than the toxicity testing, a study on the behavioural changes among affected animals should be conducted. Example study on behavioural changes in medaka fishes is the stepwise behavioural response (SBR) studied on Japanese medaka after exposure at a low concentration of organophosphorus pesticides by an online monitoring system (OMS) as in Ren et al. (2012). The behavioural changes were observed according to levels of concentrations and some were according to specific chemicals and its concentrations (Ren et al., 2012). In the much earlier behavioural study on Japanese medaka has been done by Chon et al. (2005), animals were after treated with an anticholinesterase and diazinon in semi-natural condition. Eight movement parameters were measured before and after the treatments and it was compared quantitatively. The eight parameters were speed, acceleration, locomotory rate, stop duration, turning rate, meander, Y-position, and Y-max where ANOVA was used to analysed differences between variables (Chon et al., 2005). Based on the available information, limited behavioural study ever conducted on Javanese medaka and not to mention the effect of the CuPT. Therefore, the purpose of this study was to determine the 96-hr median lethal concentrations (LC50) of CuPT towards Javanese medaka in identifying the acute toxicity of CuPT on marine species. In addition, behavioural responses of Javanese medaka were observed to clarify the effects of the compound towards the fish.

The wild stock of Javanese medaka fishes was sampled at Sungai Pelek, Sepang. Fishes were caught using fine-mesh hand nets. They were kept in plastic bottles using the water from the fish habitats. Oxygen stones were provided during the transportation and the fishes were transported in a well-padded container to lessen transfer stress as in Yusof et al. (2013). The fish were acclimatized in the laboratory for two weeks and the water salinity was gradually decreased daily for its adaptability with the water used in the experiment. Fishes were fed with brine shrimp or fish food flakes daily. CuPT were provided by Acros Organic, New Jersey, USA. The stock solutions were prepared by dilution with dimethyl sulfoxide (DMSO, 1.0%) provided by R&M Marketing Essex, UK. The test solutions were made through further dilution of stock solutions with de-chlorinated tap water in glass aquaria. The toxicity tests were generally performed as recommended in the test guidelines of the Organization for Economic Cooperation and Development 203 (OECD, 1992). The test was conducted in a 6-L glass aquaria filled with de-chlorinated tap water. A semi-static test was used to conduct the experiment as suggested in OECD 203 test where the water in the aquaria was changed every 24-h to maintain the appropriate concentration of CuPT in the test solution. The range finding tests were conducted to determine the range for the acute toxicity test. Therefore, the acute toxicity test was conducted by exposing the fish to five different concentration of CuPT (10, 13, 15, 20, 25 mg/L). Ten individuals were used for each exposure test with triplicate for each concentration. Two controls were used in the experiment where the fishes were filled with only dechlorinated tap water and filled with dechlorinated tap water and dimethyl sulfoxide (DMSO). The animals were not fed during the 96-hr test. Mortality was monitored daily. Photoperiod of 14-h: 10-h of light: dark were used during the exposure test. The dead fish were removed when noticed. The water quality parameters were as follows: temperature 26.0 °C ± 2.0; dissolve oxygen > 6.4%; and pH 7.6 ± 0.6. The fish were handled and sacrificed according to the method approved by Institutional Animal Care and Use Committee, Universiti Putra Malaysia (AUP: R006/2016). Behavioural changes of fishes in control and the exposed group were observed manually through camera recording. Few parameters were chosen such as the swimming habit, swimming movement during a normal condition and at the time of feeding and also its sensitivity reaction towards food (Narwaria and Saksena, 2012). Probit analysis was conducted in determining the lethal concentration 50 value using StatPlus 5.9 (AnalystSoft Inc.). Concentrations of CuPT used in this test were plotted against mortality with 95% confidence interval.

Results on mortality of Javanese medaka after exposed to five different CuPT concentration (10, 13, 15, 20, 25 mg/L) at 24, 48, 72 and 96-h are shown in Fig. 1. Findings indicate no mortality observed for control and 10 mg/L of CuPT throughout the experiment (Fig. 1). The mortality rate for tested fishes exposed with 13 and 15 mg/L were stable at 30% after 48, 72 and 96-h (Fig. 1). High mortality rate observed after 24 hr exposure for 20 mg/L with the increment of mortality after 24-hr exposure is relatively higher compared to the increment of after 48, 72 and 96-h. All tested fishes died each time after exposed to 25 mg/L of CuPT (Fig. 1). The median lethal concentration (LC50) of CuPT was determined by probit analysis as shown in Fig. 2. The results showed that the range of toxicity for CuPT with minimum mortality of 0% and the maximum limit of 100% were between 10 mg/L to 25 mg/L. The LC50 value for CuPT was identified at 16.58 mg/L as determined using probit analysis (Fig. 2). Due to limited references, the LC50 of CuPT on wild medaka fishes identified in this study isn't comparable with other study using the same species or other small fishes. A much recent study using wild stock Javanese medaka was done for bioaccumulation of mercury and the exposure effects on gonads (Aziz et al.,