UNIVERSITI PUTRA MALAYSIA

ECOTOXICITY ASSESMENT OF ANTIFOULING BIOCIDE, ZINC PYRITHIONE ON THE JAVANESE MEDAKA (Oryzias javanicus, BLEEKER 1854)

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By

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Javanese medaka (*Oryzias javanicus*) has been proposed as a potential model organism for ecotoxicological studies. In recent years, several emerging pollutants have raised concern among scientists. Among others is zinc pyrithione (ZnPT) which is widely used in personal care products and also in agriculture field as fungicide and bactericide. Recently, ZnPT is used in antifouling paints to replace tributyltin (TBT) to prevent attachment of marine fouling organisms on the ship hull. However, limited studies ZnPT could be harmful to non-targeted and early life stage of organisms. Hence, this study aimed to determine the response on the embryological development of *Oryzias javanicus* and *Danio rerio* towards different concentration on ZnPT and to investigate the ecotoxicological effects in adults *Oryzias javanicus* exposed for 21 days to different sublethal concentration of ZnPT exposure. The effects of ZnPT exposure determined via induction of metallothionen (MT), levels of ionic Zn, histological studies, and quantified apoptotic indices. In the first experiment, *Oryzias javanicus* and zebrafish (*Danio rerio*) eggs were tested with different ZnPT concentrations to determine its effects on egg's hatchability, median lethal concentration (LC50), embryonic development and deformities. Results showed the ecotoxicological tolerance limit LC50 of ZnPT for *Oryzias javanicus* was at 8.392 ppb and *Danio rerio* LC50 was 47.602 ppb. Hatchability of *Oryzias javanicus* ($R = -0.9887$) and *Danio rerio* ($R = -0.9577$) is inversely correlated with the increase of ZnPT concentration. The Fish Embryo Toxicity (FET) test using different ZnPT concentration showed defects during embryological development, including body malformation and death of embryo. ZnPT also induced a delay in the hatching process, deformation and death of newly hatched larvae. It is desirable that the full life cycle of *Oryzias javanicus* and *Danio rerio* to assess the effects of ZnPT chemicals, therefore this studies was a beginning for range finding of NOEC (no observe effect level) and LC50 (median lethal concentration for 50% of sampled animals) in a short-term exposure. Then,

median lethal concentration LC50 of adult Javanese medaka was determined via toxicity test for 96hours; the value was 178.23 µg/L. Furthermore, inductions of metallothioneins (MT) and levels of ZnPT were studied for 21 days exposure of adult fish to different concentration of ZnPT. The correlation between ZnPT and metallothinein (MT) in *Oryzias javanicus* was found statistically significant and positive ($R = 0.9541$); the ZnPT and MT concentrations in *Oryzias javanicus* indicated the increasing in ZnPT levels is followed with the increasing of MT levels. The histological examination of the livers, kidney, and gonad of the fish after 21days exposure showed pathological changes and alterations such as of the liver vacuolation; congestion; nodule necrosis, kidney tubule degeneration; glomerulus shrinkage; hemosiderin. Besides, ZnPT caused non-specific in gonad of exposed organisms such as increasing of the atretic follicle, lobular disintegration, reduction of sperm aggregation and etc. Normal histology and immunoperioxidase shown the apoptosis marker in the exposed groups were significantly different ($P < 0.05$) from the control group. Apoptotic cells were quantified and apoptotic indices were calculated following identification of apoptotic cells by morphological analysis activated caspase-3 immunohistochemistry. Therefore, results from the present study prove that relatively higher concentrations of ZnPT could cause in an increased apoptosis of hepatocytes in the liver, tubular in kidney gonadal germ cells in the testis and ovarian follicles of gonads in *Oryzias javanicus*. In conclusion, ZnPT can cause numerous biological effects to targeted and non-targeted organisms, especially in areas with intensive shipping and industrial activities. This study was to support the evidence for *Oryzias javanicus* as an excellent model organism for ecotoxicological studies in aquatic environment of Malaysia.

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PENILAIAN EKOTOKSIKOLOGI BIOSIDA ANTITEMPELAN ZINK PYRITHIONE PADA JAVANESE MEDAKA (*Oryzias javanicus,* BLEEKER 1854)

Oleh

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Javanese medaka (*Oryzias javanicus*) telah dicadangkan sebagai model organisma yang berpotensi untuk kajian ekotoksikologikal. Beberapa tahun kebelakangan ini, beberapa bahan pencemar baru yang muncul telah menimbulkan kebimbangan dalam kalangan penyelidik. Diantaranya ialah zink pyrithione yang banyak digunakan dalam produk penjagaan diri dan dalam bidang pertanian sebagai racun kulat dan racun bakteria. Kebelakangan ini, ZnPT digunakan sebagai cat antitempelan untuk menggantikan tributyltin (TBT) untuk menghalang penempelan organisma marin pada permukaan kapal. Walau bagaimanapun kurang kajian yang dilakukan terhadap ZnPT yang boleh menyebabkan kemudaratan terhadap organisma bukan sasaran dan juga peringkat awal organisma. Oleh itu, kajian ini bertujuan untuk menentukan tindak balas terhadap perkembangan embriologi *Oryzias javanicus* dan *Danio rerio* ke arah tumpuan yang berbeza terhadap ZnPT dan untuk mengkaji kesan ekotoksikologi pada *Oryzias javanicus* yang terdedah selama 21 hari ke kepekatan *sublethal* ZnPT yang berbeza. Kesan pendedahan ZnPT ditentukan melalui induksi metallothionen (MT), tahap ionic Zn, kajian histologi, dan indeks apoptosis kuantitatif. Dalam eksperimen pertama, telur ikan *Oryzias javanicus* dan zebrafish (*Danio rerio*) telah diuji dengan ZnPT yang kepekatan berlainan untuk menentukan kesannya terhadap penetasan telur, kepekatan maut median (LC50), perkembangan embrio dan kecacatan. Keputusan menunjukkan batas toleransi ekotoksikologi LC50 ZnPT untuk *Oryzias javanicus* adalah pada 8.392 ppb dan *Danio rerio* LC50 adalah 47,602 ppb. Ketoksikan *Oryzias javanicus* (R = -0.9887) dan *Danio rerio* (R = -0.9577) adalah berkorelasi dengan peningkatan kepekatan ZnPT. Ujian Ketoksikan Embrio Ikan (FET) yang menggunakan kepekatan ZnPT yang berbeza menunjukkan kecacatan semasa perkembangan embriologi, termasuk kecacatan badan dan kematian embrio. ZnPT juga menyebabkan kelewatan dalam proses penetasan, mengubah bentuk dan menyebabkan kematian larva yang baru menetas. Perlu diingatkan bahawa kitaran hidup *Oryzias javanicus* dan *Danio rerio* digunakan untuk menilai kesan bahan kimia ZnPT, oleh itu kajian ini adalah permulaan untuk penemuan pelbagai NOEC (tiada paras kesan pemerhatian) dan LC50 (kepekatan maut median untuk 50% sampel haiwan) dalam pendedahan jangka pendek. Kemudian, kepekatan mematikan median LC50 dari Javanese medaka yang dewasa ditentukan melalui ujian ketoksikan selama 96 jam; nilai itu ialah 178.23 μg / L. Selain itu, induksi metallothionein (MT) dan tahap ZnPT dikaji selama 21 hari pendedahan ikan dewasa kepada kepekatan ZnPT yang berlainan. Hubungan antara ZnPT dan metallothinin (MT) dalam *Oryzias javanicus* didapati secara signifikan dan positif ($R = 0.9541$); kepekatan ZnPT dan MT di *Oryzias javanicus* menunjukkan peningkatan dalam tahap ZnPT diikuti dengan peningkatan tahap MT. Peperiksaan histologi dari hati, buah pinggang, dan gonad ikan selepas pendedahan 21 hari menunjukkan perubahan patologi dan perubahan vaksinasi hati; kesesakan; nodule nekrosis, degenerasi tubule buah pinggang; pengecutan glomerulus; hemosiderin. Selain itu, ZnPT menyebabkan perubahan tisu yang tidak spesifik dan boleh diterbalikkan dalam hati, buah pinggang dan gonad organisme yang terdedah. Histologi dan imunoperioxidase penanda apoptosis pada kumpulan terdedah sangat berbeza ($P < 0.05$) daripada kumpulan kawalan. Sel apoptosis dikira setelah pengenalan sel apoptosis oleh analisis morfologi yang diaktifkan oleh Caspase-3 imunohistokimia. Oleh itu, hasil daripada kajian ini membuktikan bahawa kepekatan ZnPT yang lebih tinggi boleh menyebabkan peningkatan apoptosis hepatosit dalam hati, tiub dalam sel-sel germinal gonadal buah pinggang dalam testis dan folikel ovari gonad di *Oryzias javanicus*. Kesimpulannya, ZnPT boleh menyebabkan banyak kesan biologi kepada organisma yang disasarkan dan begitu juga pada organisma tidak disasarkan, terutamanya dalam bidang perkapalan dan aktiviti perindustrian yang intensif. Kajian ini adalah untuk menyokong bukti untuk *Oryzias javanicus* sebagai model organisma yang sangat baik untuk kajian ekotoksikologi di persekitaran akuatik Malaysia.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
^{o}C	Degree Celsius
±	Plus, minus
AC	Abnormal Curvature
AS	Abnormal Somite
AE	Abdominal Enlargement
APP	Antifouling Paint Particle
BS	Bent of Somite
cm	centimeter
DE	Died Embryo
DMSO	Dimethysulfoxide
DO	Dissolved Oxygen
FET	Fish Embryo Toxicity
F2	Second generation
g	gram
h	hour
IMO	International Maritime Organization
IH	Incomplete Hatching
LC	Lateral Curve
LT	Lack of Tail
LC_{50}	Median Lethal Concentration
mg/L	Milligram per liter
mL	Milliliter
OECD	Organization for Economic Co-operation Development

pH	potential of Hydrogen
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
TBT	Tributyltin
UE	Undeveloped Embryo
Zn	Zinc
ZnPT	Zinc Pyrithione
µg	Microgram
µL	Microliter

CHAPTER 1

INTRODUCTION

1.1 General

Chemicals usage in daily activities is common to modern society. Various chemical compounds are being used for numerous functions and applications. Appropriately, the increase of their usage is proportionate with their presence in the environment (Stuer-Lauridsen et al., 2000; Jones et al., 2002 Zuccato et al., 2005). Nevertheless, evaluation of chemical effects to the natural system remains tough and largely unknown. The relationship of chemicals usage and environmental consequences, and indeed even the environmental presence of chemicals which have these capabilities had gone either being neglected or unnoticed (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999). Consequently, the task of measuring chemical consequences in natural systems is the ultimate purpose of ecotoxicology. The defined objective of ecotoxicology is the assessment of the effect of chemicals getting into or having effects to enter the natural systems (Rand, 1995). In other words, ecotoxicology is a division of toxicology related to the study of toxic effects, affected by natural or synthetic pollutants to the elements of ecosystems, animal (consisting of human), vegetable, and microbial (Truhaut, 1997). Studies have discovered that since new chemicals are introduced, and chemical uses being altered, ecotoxicology must similarly be adaptive adapt to become aware of the threat from these new sources brings new worries for potential environmental impact.

Zinc pyrithione (ZnPT) is one type of biocides being widely used in this era. Biocides are chemical substances that can prevent or kill microorganisms that may cause the biofouling process (Guardiola et al., 2012). The practice of substituting booster biocides in antifouling paints has been recognized by International Maritime Organization (IMO) because of the recent ban on tributyltin (TBT) (Harino et al., 2009; Abubakar et al., 2015). However, in 1987, the usage of TBT product has been banned for the boats that less than 25m length (Voulvoulis et al., 1999) as it gave negative effects, especially to the marine organisms. The effectiveness of ZnPT against bacteria, fungi, and algae make it as one of the organic biocide substitutions for the organotin compound in antifouling paints. Nonetheless, in the broad spectrum of targeted organisms, many species in coastal marine ecosystems may become accidental targets of the biocide through the antifouling treatment. Besides that, ZnPT also has been broadly used in personal care products such as anti-dandruff shampoos, hair conditioner, medical shampoos (Goka, 1999) and also in cosmetic products which may have side effects to human health.

A model organism is non-human species that is significantly studied to understand specific biological phenomena with the expectancy of discoveries made in the organism's model will provide perception into the workings of other organisms. Many organisms have been used as bioindicator, biomonitoring, and testing organisms in order to assess the environmental quality and toxicological effects of hazardous chemicals. Small fishes such as *Danio rerio* and medaka have been benefitted for the plenty experimental purposes from environmental monitoring agents to toxicity test and other basic science researches. Established zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) as experimental tools are not available in the wild within the whole of Southeast Asia. Therefore, locally existing species can be developed as other model organisms for testing organisms. Hence, Javanese medaka (*Oryzias javanicus*) can be used for similar functions like *Danio rerio* and Japanese medaka (*Oryzias latipes*).

Oryzias javanicus fish is a kind of small bony fish which can be found in a diverse group distributed around Asia. They live in brackish water, freshwater and also the salt water. In the same genus with *Oryzias javanicus*, is Japanese Medaka (*Oryzias latipes*) one of the established fish species in which they are commonly utilized in experimental of vertebral biology for many years (Ismail and Yusof, 2011). Based on Koyama et al. (2008), *Oryzias javanicus* is normally found in estuarine waters of southern to eastern Asia. Nevertheless, Ismail and Yusof (2011) had stated that this species of fish is one of the most appropriate type which can easily be reared in the laboratory. They have a short life cycle and life span with wide geographical range and availability. Then again, the suitable ~~a~~animals used as a bioindicator of pollutant have to be abundant, sufficiently long-lived, reasonable size and are able to adapt well with laboratory surrounding. It was also determined that they have got a fast growth rate, hardy species, easy to be identified and to be cultured. *Oryzias javanicus* is recommended as the excellent fish model, whereby at its early life stage is sensitive to toxicants and endocrine disrupting chemicals (Zha and Wang, 2005).

Present study was designed with the observation that, to this point in time; little evidence is made available on the ecotoxicology effects of ZnPT in the aquatic ecosystems. Accordingly, the objectives of this study have become a comprehensive assessment of the potential effect of ZnPT on freshwater aquatic organisms using *Oryzias javanicus* as a model organism. It is required that the entire life cycle of *Oryzias javanicus* to be evaluated towards the consequences of ZnPT. Thus, this study is a starting point for range finding of NOEC (no observed effect level) and LC50 (median lethal concentration for 50% of sampled animals) in a short-term exposure. Hence, this study shall deepen our understanding of ZnPT toxicity by evaluating its acute and chronic effects using the histological approach and evaluated quantification of apoptosis in tissues using antibodies that specially identified the activated Caspase 3. This study is a part of the fundamental researches to determine the suitability of *Oryzias javanicus* to be promoted as an important test organims.

1.2 Objectives of the Study

The studies of *Oryzias javanicus* are still at the infancy stage particularly in the enhancement of a culturing system and the tolerance limit of this species to selected pollutants. Therefore, to promote this species as a model organism, some studies (e.g.: histopathological, immunological, biochemical) needed to be developed. The target of this study is to strengthen the evidence for *Oryzias javanicus* as a beneficial model organism for ecotoxicological studies in the aquatic environment of Malaysia. The specific objectives of the study are:

i. To determine the ecotoxicological tolerance limit and the response of embryological development of *Oryzias javanicus* and *Danio rerio* towards different concentrations of ZnPT.
ii. To determine the biochemical response of *Oryzias javanicus* towards zinc pyrithione concentration by measuring the induction of metallothionein and Zn concentration.
iii. To investigate the ecotoxicological effects of ZnPT on *Oryzias javanicus* under sublethal level and the effects of ZnPT exposure on the liver, kidney, and gonad via histological studies.
iv. To determine the ecotoxicological effects of ZnPT exposure by means of quantification of apoptosis in tissue sections using antibodies that specifically recognize the activated Caspase 3 effect on *Oryzias javanicus* in sublethal concentration exposure.

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Fatin Zahidah Binti Abdul Aziz was born on September 07, 1990, at Hospital Tanjong Karang, Selangor Darul Ehsan, Malaysia. She started her primary school at Sekolah Kebangsaan Khir Johari, Sungai Sumun, Perak from 1997 to 2002. She continued her secondary education at Sekolah Menengah Kebangsaan Khir Johari, Sungai Sumun, Perak from 2003 to 2007. She obtained 5A1, 2A2 and 3B3 in the Malaysian Certificate of Education (SPM), a national examination set and examined by the Malaysian Examinations Syndicate (Lembaga Peperiksaan Malaysia). Once finished her secondary school, she continued her study in matriculation in Life Science track at the Kolej Matrikulasi Penang (KMPP) for one year; 2008-2009. She finished her matriculation with CGPA 3.44.

Fatin Zahidah chose to further her Bachelor study at Universiti Putra Malaysia (UPM) from 2009 to 2013. She was also a recipient of Jabatan Perkhidmatan Awam Scholarship from the Ministry of Education (MOE) of Malaysia from 2011 till 2013. Throughout her Bachelor degree years, she actively participated in Kelab Bimbingan Mahasiswa (Treasurer) and Biology Club, Faculty of Science. In October 2013, she graduated with Bachelor of Science (Hons.) – Major Biology with First Class Honours; CGPA 3.75.

With a strong interest in ecology research, she decided to continue her study in Doctoral of Philosophy (Ph.D.) level (full research mode) in the field of Ecotoxicology at Faculty of Sciences, UPM, starting from September 2013. While doing her Ph.D. degree, she grabbed the opportunity to get involve with Ecotoxicology and Wildlife Ecology Research Group at the Faculty of Science UPM and was involved as Committee Member in organizing the Malaysia Ecology Seminar Year 2016 and Year 2017. Findings of her research have been presented in several local and international conferences. In fact, she managed to publish one article in ISI/CIJ journal and currently writing on few more papers.

LIST OF PUBLICATIONS

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