UNIVERSITI PUTRA MALAYSIA

EFFECTS OF SUBLETHAL EXPOSURE OF DIURON ON ADULTS OF JAVANESE MEDAKA (*Oryzias javanicus,* BLEEKER 1854) USING HISTOPATHOLOGY AND IMMUNOCHEMISTRY TECHNIQUES

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By

NUR AMIERA BINTI KAMARUDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfillment of the Requirements for the Degree of Master of Science

March 2019

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DEDICATION

This thesis is especially dedicated to my family,
To my beloved father Kamarudin bin Sidek,
My beloved mother Asimah binti Ayob,
And
Strong supporters from my siblings,
Fatin Raihana binti Kamarudin,
Amirul Aizat bin Kamarudin,
Nurul Yasmin binti Kamarudin,
Farisha Kamilia binti Kamarudin,
Farah Nabila binti Kamarudin,
Nurul Balqis binti Kamarudin,
Kamarul Iskandar bin Kamarudin,
Luqman Hakim bin Kamarudin,
Nur Jannah Safwah binti Kamarudin,
and
Nur Iman Solehah binti Kamarudin

May Allah bless and protect them all.

Abstract of the thesis is presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

EFFECTS OF SUBLETHAL EXPOSURE OF DIURON ON ADULTS OF JAVANESE MEDAKA (*Oryzias javanicus*, BLEEKER 1854) USING HISTOPATHOLOGY AND IMMUNOCHEMISTRY TECHNIQUES

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March 2019

Chairman : Syaizwan Zahmir bin Zulkifli, PhD
Faculty : Science

Diuron has been used around the world either as herbicide or bioactive ingredient in the antifouling paint. However, the knowledge on the impacts of Diuron toxicity that could adversely affect the non-targeted aquatic organisms especially fish is still lacking. Therefore, this study was conducted to observe the toxicity of phenyl-urea herbicide Diuron on adult Javanese medaka (*Oryzias javanicus*) and the possible sublethal effects at the cellular level of the selected vital organ of adults' Javanese medaka which used as a novel model organism. The distinctive characteristic of the Javanese medaka as euryhaline species can be used to represent a wide range of salinity of the contaminated aquatic ecosystems. The methods used to evaluate the effects of Diuron on vital organs through histopathology and immunohistochemistry technique. Histological assessment was conducted by using the haematoxylin and eosin (H and E) staining protocol where the samples were preserved in 10% buffered formalin and section at 5 µm thickness. While, the apoptosis events or cell death can be detected by using Caspase-3 antibody which mediated to the Caspase protein as programmed cell death (apoptosis). Results of acute exposure showed that 100% mortality of Javanese medaka was at 20.0 mg/L of 96-hours of Diuron exposure. The median lethal concentration 50% (LC_{50}-96h) of the acute exposure is 5.9 mg/L. Semi-quantitative analysis from the chronic exposure showed significantly difference ($p<0.05$) from moderate to severe deformities in the liver and kidney tissues exposed at a higher concentration of sublethal level (500 µg/L and 1000 µg/L) as compared to control treatment while mild lesion alterations were already showed even at lowest concentration of

Diuron exposure, 1.0 µg/L. The most evident and observable severity of the lesions in the liver is vacuolation, congestion, and nodule necrosis while, kidney which is the most effected organ showed severity in tubule degeneration, glomerulus shrinkage, and hemosiderin. In addition, Diuron is proven as an endocrine disrupting chemical (EDC) which can interfere with the normal functions of the endocrine system of fish. In addition, Diuron caused the decreasing in gonadal staging and maturity of germ cells in oogenesis and spermatogenesis of female and male Javanese medaka. Therefore, the impairments and destruction in liver and kidney, as well as the modification of gonads in Javanese medaka, can reflect the effect in physiology, life cycle and population in the natural environment. The health of the aquatic ecosystem can also be assessed by using the immunohistochemistry as a biomarker tool. Results showed the significant increment percentages of the apoptotic events as compared to the control of liver, kidney, ovary, and testis; 31.6%, 32.5%, 46.5% and 51.5% respectively at the highest concentration of Diuron exposure ($p<0.05$). Findings of this study will strengthen the documentation in developing Javanese medaka as a novel model organism for tropical region in particular of aquatic toxicological studies. In summation, histopathology study is good enough to be a good biomarker to assess the health quality of aquatic ecosystem through histology of fish's vital organs and for further investigation later on, immunohistochemistry analysis have the ability to visualize the distribution and localization of the specific tissues components with specific antigens and antibodies of stress or death fish tissues from the contaminated aquatic ecosystems. Also, although the concentration of Diuron in Malaysia is still unthreatening, this emerging hazardous chemical can seriously affect the aquatic ecosystems and its living organisms in later years.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Master Sains

KESAN PENDEDAHAN SUBLETHAL DIURON TERHADAP JAVANESE MEDAKA (*Oryzias javanicus*, BLEEKER 1854) DEWASA MENGGUNAKAN TEKNIK HISTOLOGI DAN IMMUNOKIMIA

Oleh

NUR AMIERA BINTI KAMARUDIN

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Diuron telah digunakan secara meluas di seluruh dunia sebagai racun rumput liar dan sebagai bahan aktif di dalam cat antitumbuhan. Namun malangnya, pengetahuan mengenai kesan toksik Diuron yang boleh membawa kesan buruk kepada organisma akuatik yang bukan target seperti ikan masih belum mencukupi. Oleh itu, kajian ini dijalankan untuk melihat kesan racun Diuron terhadap ikan Javanese medaka (*Oryzias javanicus*) dewasa dan kesan-kesan yang tidak melibatkan kematian pada peringkat sel dan tisu organ-organ penting yang terpilih. Ciri-ciri distingtif Javanese medaka sebagai species eurihalin boleh digunakan mewakili pelbagai tahap kemasinan ekosistem akuatik yang tercemar. Kaedah-kaedah yang digunakan untuk mengenalpasti kesan-kesan terhadap organ penting adalah melalui analisis histopatologi dan immunohistokimia. Penilaian histologi telah dijalankan menggunakan protokol warna hematoksilin dan eosin (H dan E) setelah sampel diawetkan di dalam 10% formalin tertimbal dan dipotong pada ketebalan 5 μm. Manakala, kesan apoptosis or kematian sel boleh dikenalpasti mengunakan antibodi Caspase-3 yang mengantarai protein Caspase sebagai program kematian sel (apoptosis). Keputusan daripada pendedahan akut menunjukkan kematian 100% Javanese medaka adalah pada kepekatan Diuron 20.0 mg/L selama 96 jam. Kepekatan median kematian 50% (LC_{50}-96h) adalah pada 5.9 mg/L. Analisis separa-kuantitatif daripada pendedahan kronik menunjukkan perbezaan ketara ($p<0.05$) daripada kecederaan sederhana kepada teruk di dalam hati dan buah pinggang yang didedahkan pada kepekatan yang lebih tinggi (500 μg/L and 1000 μg/L) berbanding dengan rawatan kontrol dan kecederaan ringan juga boleh dilihat pada kepekatan pendedahan Diuron yang paling rendah 1.0

µg/L. Keterukan kecederaan yang jelas dan nyata ialah pembentukan vacuol pada sel, kesesakan pada salur dan nekrosis nodul pada liver, manakala pada buah pinggang adalah organ yang lebih terkesan menunjukkan keterukan kecederaan pada kemerosotan tubul, pengecutan glomerulus dan hemosiderin. Kajian ini juga membuktikan Diuron boleh bertindak sebagai bahan kimia yang mampu menganggu sistem endokrin (EDC) di dalam ikan. Tambahan lagi, Diuron telah menyebabkan penurunan peringkat gonadal dan tahap kedewasaan cel pembiakan di dalam proses oogenesis dan spermatogenesis pada ikan Javanese medaka jantan dan betina. Oleh itu, kecacatan and kerosakkan pada hati dan buah pinggang, juga modifikasi pada organ pembiakkan ikan Javanese medaka boleh memberi kesan pada fisiologi, kitaran hidup dan populasi spesis ini di alam semulajadi jika terdedah kepada pencemaran Diuron walaupun pada kepekatan yang rendah. Tahap kesihatan ekosistem akuatik boleh di ditaksir menggunakan immunohistokimia sebagai alat bio-penanda. Immunohistokimia boleh digunakan sebagai alat yang menyakinkan dengan menggunakan pengesanan antigen terhadap antibodi yang khusus di dalam tisu melalui keratan tisu histologi. Keputusan menunjukkan peningkatan pada peratusan kesan apoptosis berbanding dengan rawatan kontrol pada hati, buah pinggang, ovari dan testis yang masing-masing pada perratusan 31.6%, 32.5%, 46.5% and 51.5% pada kepekatan Diuron yang paling tinggi ($p<0.05$). Oleh itu, kajian ini membantu dalam menguatkan bukti dokumentasi bahawa spesis Javanese medaka mampu menjadi model organisma baharu untuk kawasan tropikal dalam bidang kajian toksikologi. Selain itu, boleh disimpulkan bahawa penggunaan kaedah histologi sebenarnya sudah cukup untuk digunakan sebagai penilaian kepada tahap kesihatan ekosistem akuatik melalui kajian histologi organ-organ penting ikan dan untuk penyiasatan lebih lanjut, kita boleh menggunakan kaedah analisis immunohistokimia yang mampu menggambarkan pembagian dan lokasi tisu khusus dengan menggunakan antigen khusus dan antibodi tekanan atau kematian ikan daripada ekosistem akuatik yang tercemar. Terakhirnya, kami menyimpulkan bahawa walaupun kepekatan Diuron di Malaysia masih pada tahap yang tidak membimbangkan, namun begitu kemunculan bahan kimia yang berbahaya boleh memberi kesan buruk kepada ekosistem akuatik serta organisma hidupannya pada tahun-tahun yang mendatang.

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This thesis was submitted to Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
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(LC50) mammals, birds, fish and aquatic organisms. (WSDOT Roadside Vegetation Management Herbicide Fact Sheet, 2017)

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
°C	Degree Celcius
/	Per
±	Plus, Minus
µg	Micro-Gram
µL	Micro-Litre
cm	Centimetre
DMSO	Dimethysulfoxide
DO	Dissolved Oxygen
EPA	Environmental Protection Agency
et al	*et-Alia* –And Other
g	Gram
h	Hour
L	Liter
LC_{50}	Median Lethal Concentration
m	Meter
min	Minute
mg/L	Milligram Per Liter
ml	Milliter
N	Number Of Sample
OECD	Organization For Economic Co-Operation Development
pH	Potential of Hydrogen
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Present issues concerning the environmental degradation are the largest threats happen in the world now days. Deterioration of the environment occurred as a results from human activities such as urbanization, industrialization and agricultural activities. Environmental issues from the agricultural activities are one of the factors that seriously impact the aquatic environments specifically through the usage of herbicides (Rohila and Duhan, 2017). Globally, herbicides are the phytotoxic chemicals that had played relatively a pivotal role in agriculture sectors by eliminating or destroy unwanted vegetation in crops plantation areas. Since the 1960's, Malaysia had been a country with agricultural economic basis with various plantation of crops such as oil palms and rubber plantations (Istikoma and Rahman, 2015). These agricultural developments rise along with the used of herbicides. A study conducted by De et al. (2014), statistically showed that the worldwide used of herbicide is almost 48% of the total pesticides usage and it is represented as the most rapidly growing section in the past three decades in the pesticides industry. Although the use of herbicides is focusing on the killing of weeds in the agriculture industry, it unintentionally affects aquatic organisms, since it can be found in water bodies as a toxic pollutant.

The concern herbicide in this study is Diuron as one of the most heavily used herbicides around the world as it is easily taken up by the plant through root system from the soil solution (Moncada, 2013). Also, Giacomazzi and Cochet, (2004) and Ferell et al. (2004) supported that Diuron is mostly used to control the annual grassy weeds and broad leaf weeds in pre-emergent and post-emergent. In addition, the study by Ali et al. (2014) reported that the other sources of Diuron found in the coastal areas of Malaysia are from the antifouling paint applied underneath the boats and ships and it is contaminated the coastal areas of Klang, Johor, and Kemaman. The runoff of sedimentation, leaching, spring drift or accidental spills of Diuron from the plantation areas into ecosystem such as rivers can indirectly affect freshwater vertebrates such as tilapia and goldfish (Saglio and Trijasse, 1999) and invertebrates such as water flea and freshwater snail (Lopez-Doval et al., 2014) as reported. Also, the level of toxicity reported for fish and aquatic invertebrate is considered moderately toxic as the Diuron acute LC_{50} is between 1.0-10.0 mg/L based on the Wildlife Toxicity Category.

The toxic effects of Diuron exposure on the aquatic organism can be studied by using the local model for test organism from genus *Oryzias*; Javanese medaka (*Oryzias javanicus*) or commonly known as rice fish. Javanese medaka originated from the genus of *Oryzias* (rice fishes) can be used as a potential alternative model organism for toxicity testing in the field of Ecotoxicology instead of using the establish models due to its unique characteristics. The distribution of this species of medaka can be found from Peninsular Malaysia, Singapore, Indonesia, Thailand, and Western Borneo (Yusof et al., 2013). By representing the tropical South-east Asia area, Javanese medaka can be a substituted model organism demonstrating the toxicants present around these areas for toxicology studies. This species of medaka is closely related to the establish laboratory fish, Japanese medaka (*Oryzias latipes*) in term of morphology, physiology and its behaviour which have been used for over than 50 years (Padilla et al., 2009). Similarly, the adult size is small (2-4 cm), highly tolerant to temperature and a wide range of salinity which make them able to survive in the new freshwater environment. Furthermore, Javanese medaka is hardy, and rarely threatens by diseases in laboratory cultivating condition (Inoue and Takei, 2002). The fact of small fish used in the laboratory toxicity testing is expedient and conclusive as experimental and investigation tools in the ecotoxicological field.

There are several studies related to Diuron that can cause mortality at higher concentration, but at a lower concentration of exposure are more reflective to natural environment causes more toxicity effects in whole body system in a longer period of time. This sublethal effect can affect on the neurotransmitter, immune response, physiological, morphological and reproduction (Lopez-Doval et al., 2014). The endpoint of this study is to observe the impairment and abnormalities in the cell structures and reproduction of rice fish, *O. javanicus*. The low concentration of the toxicants exposure has environmental relevance as in the concentration in natural environment and the selected endpoints have the ecological relevance. Furthermore, the selected endpoints of biocide Diuron exposure in medaka have not been studied recently and to date.

Histopathological and Immunohistochemistry (IHC) analyses are the bioassay techniques used intended to reveal the impairment in the tissues of Javanese medaka after exposure to the herbicide Diuron. The histopathological technique provided analysis of microscopic study between normal healthy tissues and disrupted tissues. While, Immunohistochemistry (IHC) technique may provide more details results in the cell death (apoptosis) and cell stress using specific antibodies. It is stressing on the demonstrating the presence and location of the protein in the intact tissues

and its staining accomplished with highly specific antibodies that can be recognized the target protein.

In summation, the findings from this study may contribute in the interpretation of the biological effects of herbicide Diuron which had been extensively used around the globe. The contamination of this herbicide in the aquatic ecosystem can be bio-monitored with practical bioassays methods of histopathology and immunohistochemistry techniques. These applicable methods can be used as promising biomarker tools to observe the early effects and long terms exposure of herbicides or any other pollutants on the cellular and tissues level which been a very basic structure and components for every biological organization. Also, the LC_{50} value is important in assisting to develop the proper guidelines for the appropriate safety used of the herbicide Diuron in the aquatic ecosystem by the environmental protection authority. The LC_{50} value can be as one of the sources of the toxicity information of the any new chemicals introduced. Furthermore, the lower concentrations and longer time of of exposure of chronic toxicity can provide additional information on toxic effects of the test organisms. Thus, this study may contribute in strengthening the documentary evidence in developing Javanese medaka (*O. javanicus*) as a novel model organism in tropical areas for aquatic toxicological studies.

1.2 Objectives

The objectives of this study are:

i. To determine the LC_{50} value of Diuron by acute exposure on the adults of Javanese medaka (*O. javanicus*).
ii. To determine the effect of sublethal chronic Diuron exposure using histopathological technique on liver, kidney and gonads of Javanese medaka (*O. javanicus*).
iii. To determine the apoptotic event of chronic Diuron exposure using immunohistochemistry technique on liver, kidney and gonads of Javanese medaka (*O. javanicus*).

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BIODATA OF STUDENT

Nur Amiera binti Kamarudin was born in October 1992, at Hospital Besar Melaka. She received her early education at Sekolah Kebangsaan Telok Mas from 1999 to 2004 and Sekolah Menengah Kebangsaan Telok Mas, Melaka from 2005 to 2009. She obtained 6As and 4Bs in Malaysian Certificate of Education (SPM), a national examination set by Government of Malaysia. After finishing secondary school in 2009, she continued her studies at Malacca Matriculation College (KMM) for one year 2010-2011. She finished her matriculation with CGPA of 3.675.

Next, she chose to continue her Bachelor degree at Universiti Putra Malaysia (UPM) from 2011 to 2015. She was also a recipient of Jabatan Perkhidmatan Awam Scholarship from Ministry of Education (MOE) of Malaysia from 2011 to 2015. She received her Bachelor of Science Majoring in Biology (Honours) in November 2015 with Second Class Upper Degree; CGPA 3.678.

In 2015, she was decided to further her study in ecology field of research as a Master student at the Ecotoxicology Laboratory in Department of Biology, UPM. She also received a scholarship from Ministry of Higher Education (MyBrainSc) and this study was supported by the Putra Grant-Putra Graduate Initiative (IPS) (Project Number: GP-IPS/2017/9577500) from Universiti Putra Malaysia and was performed in relation to the JSPS Core-to-Core Program, B. Asia-Africa Science Platforms. Also, the other activity that she involved during her Master degree is organizing the Malaysia Ecology Seminar in year 2016, 2017, and 2018 as a committee member. Findings of the research also have been presented in several local and international scientific conferences.

LIST OF PUBLICATIONS

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