UNIVERSITI PUTRA MALAYSIA

ALTERATIONS IN GLUTATHLONE S-TRANSFERASE AND ACETYLCHOLINESTERASE ACTIVITIES IN AFRICAN CATFISH (CLARIAS GARIEPINUS) FOLLOWING CHLORPYRIFOS EXPOSURES

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BY

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Thesis submitted in fulfillment of the requirement for the degree of Bachelor Science (Environmental and Occupational Health) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia

ACKNOWLEDGEMENT

Bismillahhirrahmanirrahim. Alhamdulillahi Rabbil Alamin, first of all, I would like to express my gratitude to Allah the Almighty, the most Gracious and the most Merciful, for upon His permission for me to complete my thesis.

I would like to express my heartiest gratitude and appreciation to my supervisor, Dr. Ali Karami Varnamkhasti and my co-supervisor, Dr. Saliza Mohd Elias for their invaluable guidance, support and advice throughout this project. I really appreciate their help as without them, I won't be able to complete this study. Their willingness to motivate me contributed tremendously to my study.

Next, I would like to thank Miss Samaneh Karbalaei, Research Assistant Staff for being supportive and prepare all materials and instruments that used throughout my study. Then, big thanks to post-doctorate students, Dr. Sani for helping and support me continuously since the first day until the end of my study. I will never forget their kindness towards me and I really appreciate it

I also would like to thank Faculty of Medical and Health Science, Universiti Putra Malaysia for giving me the opportunity to do my final year project. Besides, special gratitude to my lab colleague, Wan Muhamad Izzat Farhan that accompany me throughout this study. We have been working together in the lab from the beginning until I finish my study. I am really lucky to have him as lab partners who give lots of help and support during my hard time.

Finally, a very big thank you to everyone that involved either directly or indirectly in this study and giving me support to finish up this final year project. Without helps of the particular that mentioned above, I would face many difficulties throughout this study.

ABSTRACT

ALTERATIONS IN GLUTATHIONE *S*-TRANSFERASE AND ACETYLCHOLINESTERASE ACTIVITIES IN AFRICAN CATFISH (*Clarias gariepinus*) FOLLOWING EXPOSURES TO CHLORPYRIFOS

MOHAMMAD NAZRIN BIN AHMAD

Introduction: Chlorpyrifos (CPF) is commonly used for pest and insect control in agricultural fields and surrounding freshwater reservoirs. Glutahthione *S*-transferase (GST) and acetylcholinesterase (AChE) are fish biomarkers used to measures the cellular, biochemical, molecular, or physiological change in an organism that indicate exposure to or the effects of environmental contaminants. **Objective:** To evaluate the effects of CPF posed to the human by using fish as a vertebrate model. **Method:** In this study, *Clarias gariepinus* were artificially propagated. Thereafter, groups of juvenile *C. gariepinus* been exposed to three graded of chlorpyrifos at concentration (50µg/L, 100µg/L, 150µg/L) for 21 days. Meanwhile, in this research the non-exposed groups was considered as the control group. GST activity was measured in the liver and AChEactivity was measured in the brain using enzyme-linked immunesorbent assay method (ELISA). **Result and discussion**: The finding showed that there were significant differences between the control groups with the exposed groups for AChE activities in the brain and GST activities in the liver after exposure for 21 days. **Conclusion:**These results show that CPF may cause direct cellular injury in the brain and the liver, thus suggest that AChE and GST may be used as a bio indicators to raise awareness about the potential adverse impacts of CPF on human health.

Keywords: Biomarkers, Acetylcholinesterase, Glutathione *S*-transferase, Chlorpyrifos

ABSTRAK

PERUBAHAN AKTIVITI GLUTATHIONE *S*-TRANSFERASE DAN ACETYLCHOLINESTERASE DI DALAM IKAN KELI AFRIKA (*CLARIAS GARIEPINUS)* BERIKUTAN PENDEDAHAN KEPADA CHLORPYRIFOS

MOHAMMAD NAZRIN BIN AHMAD

Pengenalan: Chlorpyrifos (CPF) biasanya digunakan untuk perosak dan serangga kawalan dalam bidang pertanian dan sekitar takungan air tawar. Glutahthione S-transferase (GST) dan acetylcholinesterase (AChE) adalah biomaker aktiviti biologi di dalam ikan yang digunakan untuk mengukur perubahan sel, biokimia, molekul, atau fisiologi dalam organisma yang menunjukkan pendedahan kepada atau kesan-kesan pencemaran alam sekitar. **Objektif:** Untuk menilai kesan CPF ditimbulkan kepada kesihatan manusia dengan menggunakan ikan sebagai model vertebra. **Kaedah**: Dalam kajian ini, *Clarias gariepinus* telah dibiakkan secara buatan. Selepas itu, kumpulan *C. gariepinus* telah terdedah kepada tiga gred chlorpyrifos pada kepekatan (50µg / L, 100µg / L, 150µg / L) selama 21 hari. Sementara itu, dalam kajian ini yang kumpulan tidak terdedah dianggap sebagai kumpulan kawalan. Aktiviti GST diukur di dalam hati dan aktiviti AChE telah diukur di dalam otak menggunakan kaedah immunesorbent assay enzim berkaitan (ELISA). **Keputusan Dan Perbincangan**: Dapatan kajian menunjukkan bahawa terdapat perbezaan yang signifikan antara kumpulan kawalan dengan kumpulan terdedah untuk aktiviti Ache di dalam otak dan GST aktiviti di dalam hati selepas pendedahan selama 21 hari. **Kesimpulan:** Keputusan ini menunjukkan bahawa CPF boleh menyebabkan kecederaan selular langsung dalam otak dan hati, sekali gus mencadangkan bahawa AChE dan GST boleh digunakan sebagai bio indikator untuk meningkatkan kesedaran mengenai kesan buruk kesihatan manusia akibat daripada pendedahan kepada CPF.

Kata kunci: Biomarkers, Acetylcholinesterase, Glutahtione *S*-transferase, Chlorpyrifos

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
CPF	Chlorpyrifos
GSH	Glutathione
GST	Glutathione *S*-tranferase
Clarias gariepinus	*C.gariepinus*
Organophosphate	OP

CHAPTER 1

INTRODUCTION

1.1 Background

Pesticides play an important role in agriculture activities. It is used in agricultures to sustain the agricultural production by protecting all kinds of crops from vector-borne diseases and pest attack. Organophosphate (OP) compounds are one of the most commonly used insecticides in agriculture. Humans can be exposed through multiple routes, including inhalation from spray drift, ingestion of residues on foods, dust, and soil, and dermal absorption from skin contact due to widespread use of OP insecticides (Fortenberry et al., 2014). In Malaysia, the usage of pesticides is controlled under the Pesticides Act of 1994. In addition, the principle legislation for the control of pesticides is implemented by the Pesticides Board which comprises various heads of government agencies, and it is under the jurisdiction of the Department of Agriculture. Pesticides submitted for registration must conform to FAO/WHO specifications. Other specification like Malaysian Standards or even the registrant own specification can be accepted in the absence of FAO/WHO

specifications. The conformation also applies to the contents and levels of impurities of the pesticide

Chlorpyrifos (o,o-diethyl-o-3,5,6-trichlor-2-pyridyl phosphorothioate, CPF) is an organophosphate insecticide, which has been used from last decades to control agricultural and domestic pests. As with other phosphorothioate esters, CPF is rapidly absorbed, metabolized and excreted by mammals following oral administration (Adedara et al., 2016). Mode of transmission is by contact, ingestion and vapour action. It is reported that CPF is metabolized by microsomal mixed function oxidase system into active oxons and thus are likely to produce oxidative stress (Albores et al., 2001). In Malaysia, the fate of CPF in sandy loam soil under tropical condition was studied in a vegetable plot in the Cameron Highlands (Ngan et al., 2005).

Acetylcholinesterase (AChE) is enzyme that terminates synaptic transmission at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine (ACh). Acetylcholinesterase catalyses the hydrolysis of ACh into choline and acetic acid (Downes & Granato, 2004). Its inhibition is directly linked with the mechanisms of toxic action of organophosphorus and carbamate insecticides, whose use has increased around the world replacing the more persistent organochlorines in agricultural activities (Hernández et al., 1998). Chlorpyrifos was known to inhibit the AChE activities irreversibly and causing convulsions and paralysis and can lead to death (Van Dyk & Pletschke, 2011). In addition, AChE can be found in the bovine blood erythrocytes, the brain of insects and mammals, muscle and brain of fish. The

inhibition of AChE in human has been associated with Alzheimer disease (Bartolini et al., 2003).

Glutathione *S*-transferase (GST) is one of the important detoxication enzymes that catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. Glutathione *S*-transferase is considered among the most controversial biomarkers of water pollutants in fish with little known about factors influencing their activities (Karami & Courtenay, 2015). Chlorpyrifos intoxication was shown to cause a significant decrease in the reduced (GSH) and GST activities (Goel et al., 2005). In human, GST enzyme are a functionally diverse family of soluble enzymes of detoxification that use reduced (GSH) in conjugation and reduction reactions (Whalen & Boyer, 1998) .These enzyme is found primarily in the membranes of the endoplasmic reticulum (microsomes) within liver cells (hepatocytes), as well as many other cell types (Morel et al., 2004). Glutathione *S*-transferase is widely distributed in hepatic and extra-hepatic tissues including those of marine fishes (James et al. 1979).

African sharptooth (*Clarias gariepinus*) catfish is a species dof catfish of the family Clariidae, the air breathing catfishes. African catfish is one of the most abundant and widely distributed fishes in the river and lakes in Malaysia. Besides, in Africa it is also the principal clarid catfish (Okeyo et al., 2004). Pond-reared African catfish is at particular risk of exposure to agricultural chemicals, as they are often farmed in proximity to crop-producing fields using the resulting waste water (James et al., 2008). *C. gariepinus* has been extensively used as a laboratory fish model by

many scientists to monitor microbial, pathological or environmental studies such as (Olaifa et al., 2004).

1.2 Problem Statement

CPF is an organophosphate insecticide and products with CPF are used in agriculture for feed, golf courses and to control and mosquitoes for public purposes. Besides, product containing CPF was also been used to treat wood fences and utility poles. . It is reported that CPF is the second largest selling OP and found to be more toxic to fish than organochlorine compound (Wu & Laird, 2003). The widespread use of CPF in the environment is causing increasing concern about the effects on the health of humans, wildlife and ecosystems. In Malaysia, CPF been used in form of aqueous solution in oil palm plantation (Muhamad et al., 2010). The usage of CPF in the environment will passes via air-drift or surface runoff into natural waters and the river where it is accumulated in different organisms living in water, especially in the fish, thus making it vulnerable to several discernible effects (Bailey et al., 1997). Moreover, study on human enzyme activities after exposure to CPF is unethical. Hence, *C. gariepinus* was selected in this study due to its natural habitat is in the river and this fish is widely been consumed by the local peoples in Malaysia. In addition, this research was concern on the effect of CPF to enzymatic activities of the aquatic life after been exposed in their natural environment by using *C. gariepinus* as a vertebrate model.

1.3 Study Justification

The widely usage of CPF gave adverse health effect to human, birds, mammals and aquatic life. This research helps to determine the potential health effect of CPF exposure in aquatic life which is African catfish. Moreover, the study was carried out in order to enhance better understanding and the potential health effect caused by exposure of CPF to enzymatic activities of the fish. Besides, fish has been generally used as a vertebrate model to assess the quality of aquatic systems as bio-indicators for environmental pollutants (Dautremepuits et al., 2004). Last but not least, the finding may be useful to generate baseline data for future research.

1.4 Objectives

1.4.1 General Objective

To evaluate the effects of CPF posed to the human by using fish as a vertebrate model

1.4.2 Specific Objectives

1.4.2.1 To evaluate the effects of CPF on GST activities in the liver of juvenile *C. gariepinus*.

1.4.2.2 To test the effects of CPF on AChE activities in the brain of juvenile *C. gariepinus*

1.5 Hypotheses

1.5.1 There is no significant difference on GST activities between CPF-exposed and unexposed *C. gariepinus*.

1.5.2 There is no significant difference on AChE activities between CPF-exposed and unexposed *C .gariepinus*.

1.6 Conceptual framework

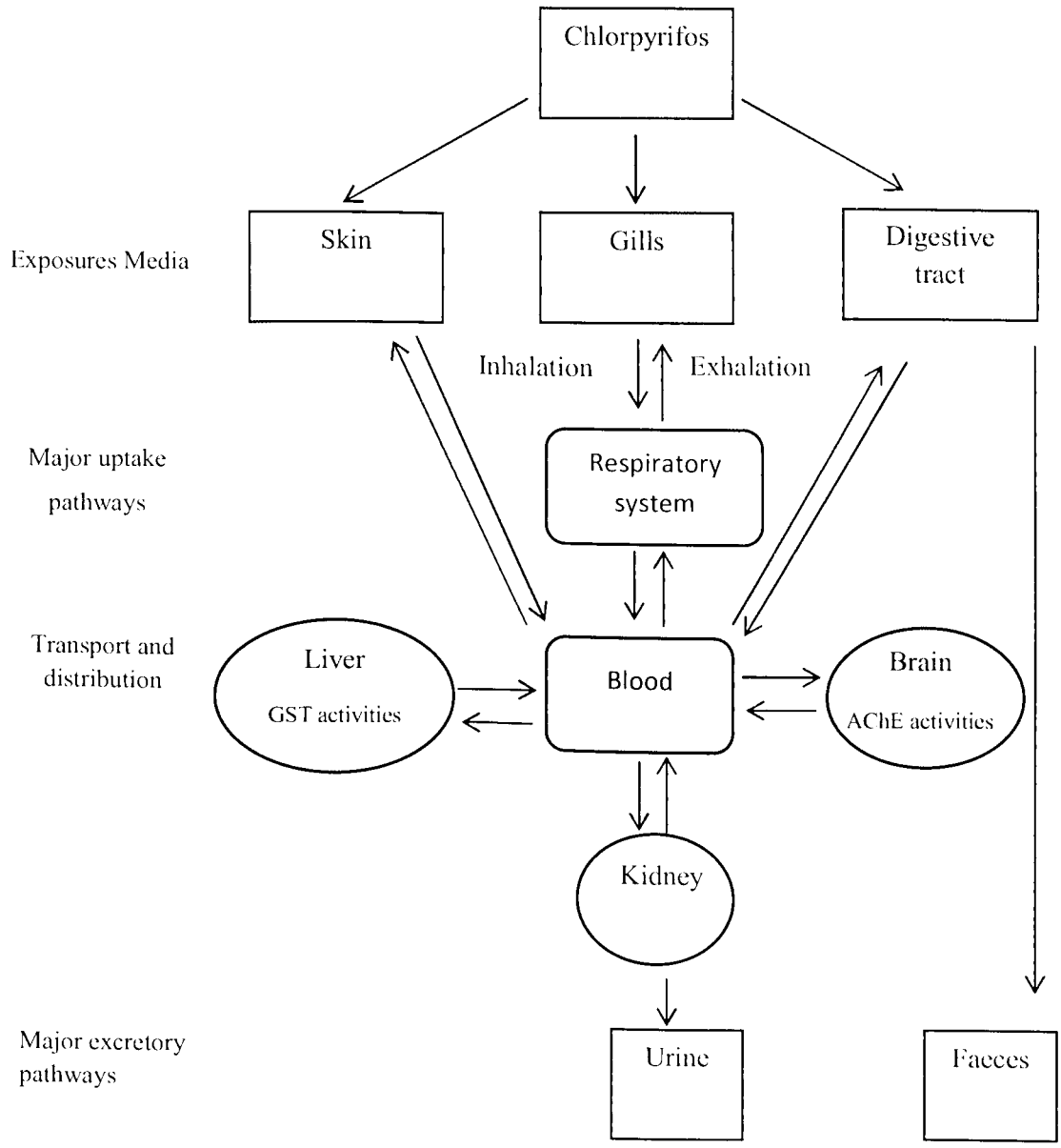

Figure 1.4: Conceptual framework on CPF pathways in the *C.gariepinus*.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquatic toxicology

Aquatic toxicology has been defined as the qualitative and quantitative study of adverse or toxic effects of chemicals and other anthropogenic materials on aquatic organisms. The subject also includes the study of transport, distribution, transformation and ultimate fate of chemicals in the aquatic environment. Within this multidisciplinary field of science, studies of the biochemistry and function of biotransformation enzymes in aquatic organisms hold a central role (Goksøyr & Förlin, 1992). For toxicological and risk assessment studies, *in vitro* Absorption, Distribution, Metabolism, and Excretion (ADME) data in chemical bioaccumulation assessments for fish. The uses of computer-based (*in silico*) modeling tools are widely used to estimate chemical bioaccumulation (Nichols et al., 2007).The conceptual approach of Hugget et al. (2003), or the so called fish plasma model, was invented to estimate potential hazardous compounds and to prioritize experimental testing with higher the risk is that the pharmaceutical elicits an effect in fish. It is

assumed that the closer the estimated fish plasma concentration and the human therapeutic concentration are, the higher the risk is that the pharmaceutical elicits an effect in fish (Huggett et al., 2003).

2.2 Biomaker

Biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Colburn et al., 2001). The World Health Organization (WHO) has stated that a true definition of biomarkers includes almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological and the measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction (WHO, 1993).

Biomarkers are worldwide recognized tools for the assessment of pollution impacts in the marine environment (Mierzejewski et al., 2014). Besides, biomarkers are used to indicate exposure to contaminants and quantify their impact on living organisms, provides a more comprehensive and integrative assessment of biochemical and cellular effects caused by environmental xenobiotic (Cazenave et al., 2009). In ecotoxicology, biomarker has been recently applied in both field and laboratory studies and selection of an appropriate biomarker for use is important (Lam, 2009).

2.3 Glutathione *S*-transferase (GST)

Glutathione *S*-transferase (Figure 2.3) is a multifunctional large family of phase II detoxification/antioxidant enzymes (Rameshthangam & Ramasamy, 2006). They are involved in cellular detoxification thereby protecting the organisms from various endogenous and exogenous molecules which include therapeutic drugs, chemical carcinogens, environmental pollutant and product of oxidative stress by catalyzing the conjugation of GSH (Forman et al., 2009).

These enzymes effectively reduced many organic compounds including reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) by nucleophilic addition of their thiol group (GSH) to a large variety of electrophilic alkylating compounds such as hydroxyalkenals (Roch, 1999). Such catalytic reactions eventually lead to making the end products more water soluble, less toxic and rapidly excerable from cell when compared to non-GSH conjugating substrates, thus protecting the cells from their potential toxic effects (Habig et al., 1974).

Glutathione *S*-transferase has been found in almost all aerobic organisms from insects to plants to mammals, and even in many prokaryotes (Mazari & Mannervik, 2016). On the basis of their amino acid sequences and structural similarities, the numerous soluble mammalian GST (also known as canonical or

cytosolic GST) can be divided into seven different classes designated by their Greek names, alpha, mu, omega, pi, sigma, theta and zeta (Mannervik et al., 2005).

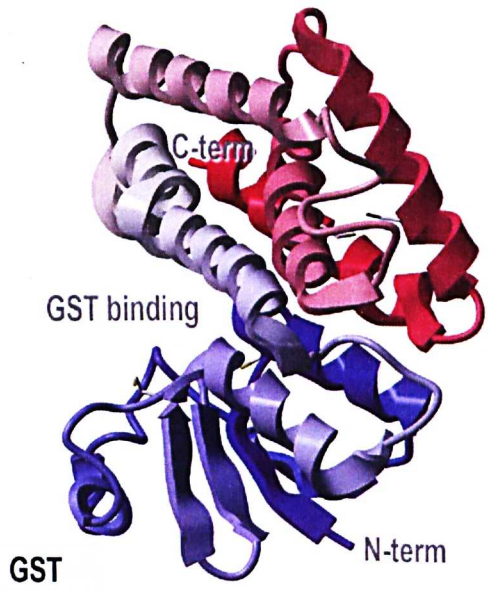

Figure 2.3: Crystallographic structure of GST (Oakley et al., 2005)

2.3.1 Glutathione (GSH)

Reduced glutathione, most commonly named glutathione or GSH, is a relatively small molecule which is ubiquitous in living systems (Dickinson & Forman, 2002). GSH, a linear tripeptide composed by cysteine, glutamic acid and glycine, is an intracellular nucleophile and antioxidant that serves a protective and detoxifying function in the body (Martos-Maldonado et al., 2012). GSH is found in the cytosol of cells where it is in the range of 1–10 mM (Meister, 1988) .Glutathione

is involved in many processes in the body, including tissue building and repair, making chemicals and proteins needed in the body, and for the immune system (Sies, 1999).

2.3.2 Glutathione *S*-transferase mechanism

Glutathione *S*-transferase catalyzes the formation of the thiol group of glutathione to electrophilic xenobiotics. It utilizes glutathione to scavenge potentially toxic compounds including those produced as a result of oxidative stress and is part of the defence mechanism against the mutagenic, carcinogenic and toxic effects of such compounds (Armstrong, 1991).

Glutathione *S*-transferase catalyze nucleophilic attacks by glutathione on compounds containing electrophilic centers. The presumed mechanism is through deprotonation of reduced (GSH) to form GS^- by a tyrosinate ion in the active site, which enhances the nucleophilicity and reactivity of GSH (Atkins et al., 1993).

The assay described by Akerboom & Sies (1993)was based upon the GST-catalyzed reaction between GSH and GST substrate, 1-chloro-2, 4-dinitrobenzene (CDNB), which has the broadest range of isozyme detect ability (e.g. alpha-, mu-, pi- and other GST isoforms). Under certain conditions, the interaction between glutathione and CDNB is totally dependent on the presence of active GST. The GST

enzyme catalyzed formation of GS-DNB to produces a dinitrophenyl thioether which can be detected by ELISA plate reader at340 nm (Akerboom & Sies, 1981).

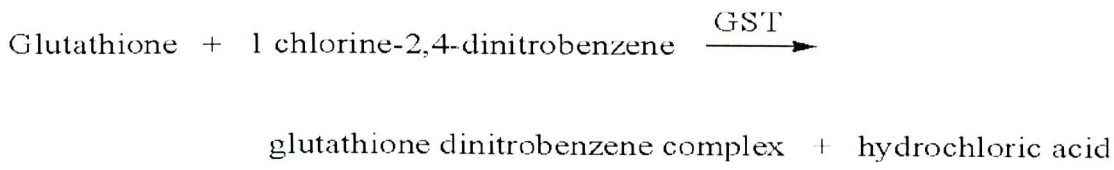

Figure 3: Oxidation reaction of GST (Chen et al., 2010)

2.3.2 Glutathione *S*-transferase activities in human

Study on GST activity in human was done by Chow et al. (2007). He used total of 42 healthy volunteers underwent a 4-week washout period by refraining from tea or tea-related products. At the end of the washout period, a fasting blood sample was collected, and plasma and lymphocytes were isolated for assessment of GST activity and level. The finding shows that there were differential effects on GST activity and level based on baseline enzyme activity, with GST activity and GST-pi level increased significantly in individuals with low baseline enzyme activity. This suggests that green tea polyphenol intervention may enhance the detoxification of carcinogens in individuals with low baseline detoxification capacity (Chow et al., 2007).

Besides, research on GST mediated detoxification and bioactivation of xenobiotics during early human pregnancy was conducted by Datta (1994). The study was conducted by using human intrauterine conceptal tissues (HICT) at 6–10 weeks of gestation. The result obtained show GST specific activity decreased with an increase in the gestational. Covalent binding of DNA and protein was greater in the presence of HICT-GST at 6 weeks gestation than that at 10 weeks gestation. These results suggest that HICT possess a significant amount of GST capable of detoxification or activation of xenobiotics during the critical organogenesis period (Datta et al., 1994).

Study on biochemical characterization of herbicide toward GST activities from human and rice was done by Cho & Kong (2007). In this study, the genes of the plant specific phi and tau class GST enzymes from *Oryza sativa* (*Os*GST) and human pi class GST enzyme (hGSTP1-1) were cloned and expressed in *Escherichia coli* with the pET and pKK vector systems, respectively. The hGSTP1-1 showed very high specific activity toward atrazine. On the other hand, the phi class *Os*GST enzymes showed high specific activity toward chloroacetanilide herbicides, acetochlor, alachlor and metolachlor. From the finding, they conclude that the phi and the tau class GST enzymes show herbicide specificities and also they play an important role in the detoxification reaction of plant toward herbicides (Cho & Kong, 2007)

2.4 Acetylcholinesterase (AChE)

Acetylcholinesterase (Figure 2.4) is an esterase member of the superfamily of proteins termed α, β-hydrolase-fold family and it is endogenous neurotransmitter at cholinergic synapses and at neuroeffector junctions in the central and peripheral nervous systems (Taylor et al., 2009). Their function is to terminate neurotransmission by rapidly hydrolyzing ACh released by the motor neurons thus recycling the neurotransmitter so that acetylcholine does not accumulate in the neurotransmitter (Rotundo, 2009). AChE can be found in the nervous system, liver and muscle tissues in vertebrate and invertebrates (Cousin et al., 1996). All species varies according to the number of genes coding for cholinesterase; one in insect, two in vertebrates and three in nematodes (Massoulié et al., 1993).

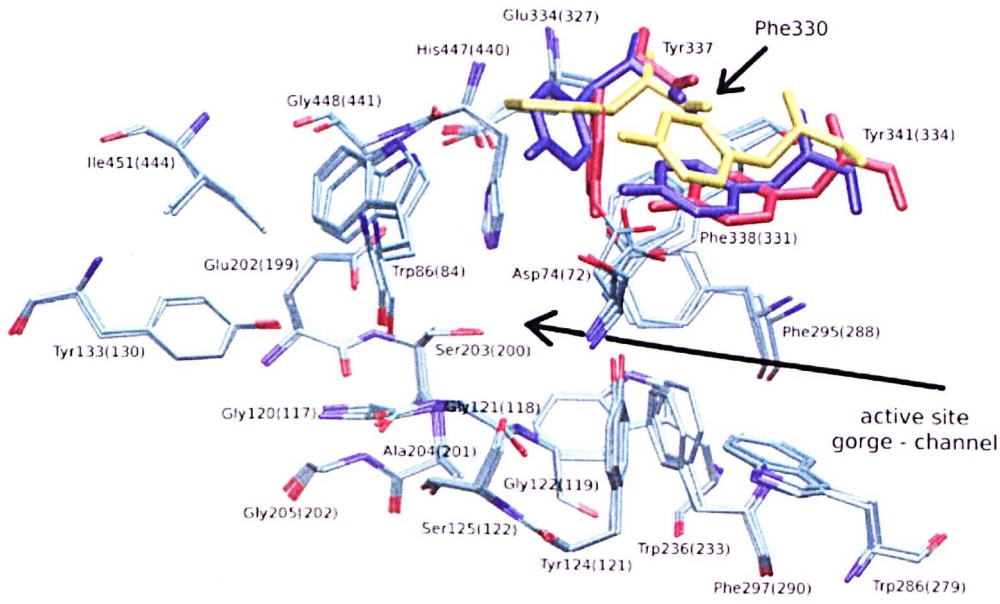

Figure 2.4: Acitve site of AChE (Wiesner et al., 2005)

2.4.1 Acetylcholinesterase hydrolysis mechanism

AChE is an essential enzyme in the transmission of the nerve impulse that degrades ACh to choline and acetic acid in the synaptic gap of cholinergic synapses and neuromuscular junctions. AChE activity is unselectively inhibited by metals, and selectively inhibited by organophosphate and carbamate pesticides, leading to severe physiological impairment in marine organisms (Tsangaris et al., 2010).

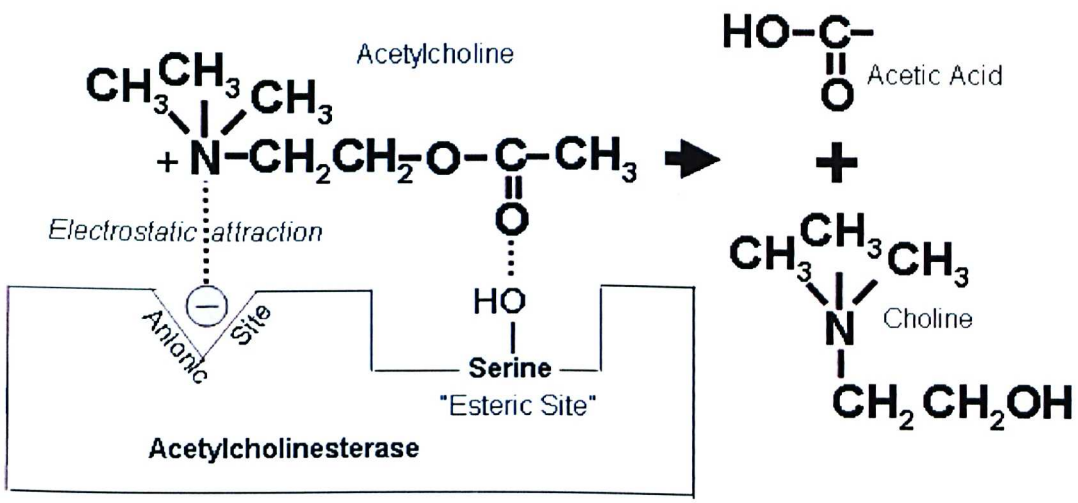

Figure 2.4.2: Normal hydrolysis of AChE (Heide, 2012)

The acute toxicity of OP nerve agents and pesticides is based on the irreversible inhibition of the serine esterase AChE. The failure of inhibited AChE to hydrolyze the neurotransmitter ACh results in an endogenous ACh overflow followed by an over-stimulation of muscarinic and nicotinic ACh receptors in the autonomic, peripheral and central nervous system, disturbance of numerous body functions and finally respiratory arrest and death (Holmstedt, 1959).

2.4.2 Acetylcholinesterase activities in human

Low-level pesticide exposures are important concern in pregnant women and young children. Fetal and infant brains are rapidly developing, leaving them highly vulnerable to potentially long-lasting effects of pesticide exposure, such as adding to concerns for fetal exposure, pesticides are able to cross the placenta (Bradman et al., 2003) and fetuses tend to have lower levels of detoxifying enzymes (Eskenazi et al., 2008) both of which are thought to increase fetal susceptibility. Low-level pesticide exposures during pregnancy or childhood have been found to be associated with neurodevelopmental deficits in the brain such as lower IQ, disorders such as autism, attention deficit-hyperactivity disorder, pervasive developmental disorder due to AChE activities inhibition in the brain (Bouchard et al., 2011; Eskenazi et al., 2007; Marks et al., 2010; Rauh et al., 2011).

Very little is known about the effects of early-life pesticide exposure on the auditory pathways of the brain or other sensory systems in human due to disturbance

of AChE activities in the brain. There have been some reports of hearing loss and ototoxicity following pesticide exposure, but most of the evidence comes from animal or occupational case studies where high-level exposures are the norm (Gatto et al., 2014). One recent study found deficits in cochlear status in children exposed to organochlorine pesticides (Sisto et al., 2015). Regarding visual sensory function, a recent study in an Arctic population with high DDE exposure found that both pre- and postnatal DDE exposure were associated with visual processing impairment at school age (Cartier et al., 2014).

2.5 Pesticides

Due to population growth and the resulting requirement to improve crop yields, pesticide use has risen considerably in recent years in order to protect the crops. The global market value for pesticides stood at US$54.8 billion in 2014 and is projected to reach US$81.8 billion by 2020 as described by (Matamoros & Rodríguez, 2016). Agricultural run-off of these compounds from crops after a rainfall event is reported to be the main source of their presence in the aquatic environment (Schäfer et al., 2007).

Synthetic pesticides are toxic to biological systems by design. Many act by disrupting signaling mechanisms in the central nervous system (CNS) thereby inhibiting neurological function. Evidence from animal studies and adult occupational poisonings has demonstrated that these insecticides act via similar

neurotoxic mechanisms in mammals following high-dose exposure (Abdollahi & Karami-Mohajeri, 2012; Yang & Deng, 2007). Less is known about the mechanisms of neurotoxicity at low-level exposures that are relevant to the general population.

Non-occupational pesticide exposure is most likely to occur via consumption of contaminated food. Additional exposure may also occur via contaminated drinking water, dust, and spray drift, especially in rural, farming communities, as well as from the use of residential pesticides in the home or yard (Ashman et al., 2009).

2.5.1 Insecticides

Insecticides are chemicals used to control insects by killing them or preventing them from engaging in behaviors deemed undesirable or destructive (Whitacre et al., 2004).They are classified based on their structure and mode of action. Many insecticides act upon the nervous system of the insect (e.g., Cholinesterase (ChE) inhibition) while others act as growth regulators or endotoxins (Spencer et al., 2009).

The use of pesticides has been informally reported since 1000 B.C., but insect chemical control began in World War II, when the concept of insect control became established, opening a new era of synthetic organic insecticides, of which DDT was the first to be applied (Whitacre et al., 2004).The mid-20th Century saw the

development of many pesticides and organophosphates,insecticides based on phosphorus; their development was also hastened during World War II, when they were tested to replace nicotine, mainly in Germany(Thacker, 2002). Because of the high toxicity of this pesticide it has been not recommended since 1990

2.5.2 Chlorpyrifos

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichlor-2-pyridyl phosphorothioate) is a broad spectrum organophosphate insecticide (OP) that is commercially used to control foliar insects that affect agricultural crops (Rusyniak & Nañagas, 2004). CPF was first introduced into the marketplace in 1965 and has been used globally as an insecticide to control pests agriculturally and in the home (Venkateswara Rao et al., 2005). It is the second largest selling OP and found to be more toxic to fish than organochlorine compounds (Tilak et al., 2001).

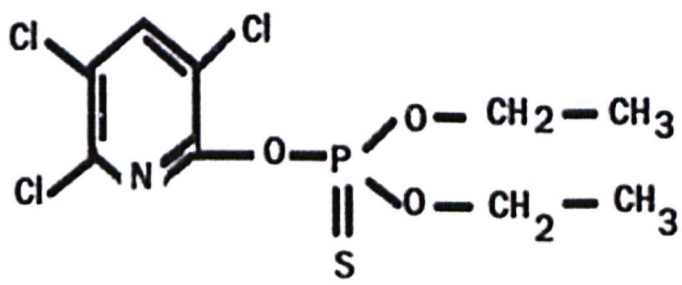

Figure 2.5.2: Chlorpyrifos structure (Tilak et al., 2001).

In Malaysia, the usage of CPF is within the recommend dosage. However, the environmental fate of CPF has recently attracted much attention because of its potential to pollute the environment. In the oil palm plantation, it was observed that no CPF residue was found and has very low persistence in the soil and therefore may have low impact on the environment as it is used within the recommend dosage (M. Halimah et al., 2016; M Halimah et al., 2011). Meanwhile, the study conducted in Cameron Highland show that CPF was dissipated rapidly under the climatic conditions of the Cameron Highlands in Malaysia.

2.5.3 The Effects of Chlorpyrifos

Physiology of the fish was disturbed due to CPF-ethyl exposure. The toxicant caused haematologically disturbances which could lead to impairment of the fish ability to combat disease, reduce its change for survival and potential for growth and reproduction (Mahmud et al., 2007).

The recovery effect of CPF on antioxidant enzymes, locomotor behaviour and the target enzyme AChE interaction were studied after exposure in mosquito fish, *Gambusia affinis*. The results showed that the organophosphate CPF besides its inhibitory effect on target enzyme AChE, it also inhibits antioxidant enzymes, which can be used as biomarkers in the pesticide-contaminated aquatic stream (Kavitha & Rao, 2008).

Developmental CPF exposure decreased dopamine and serotonin levels and increased transmitter turnover in developing zebra fish (Eddins et al., 2010). The AChE of larva zebra fish was inhibited after exposure to CPF and zebra fish provides a complementary model for studying the neurotoxicity of very early developmental exposures (Jerry Yen et al., 2011). CPF exposure during early development caused clear behavioral impairments detectable during the post hatching periods and also caused behavioral alterations in zebra fish, which lasted throughout adulthood (Levin et al., 2004).

Besides, the study of the effects of CPF was conducted on selected immune system functions in male Fisher 344 rats. In contrast, CPF impaired T-lymphocyte blastogenesis induced by concanavalin A and phytohemagglutinin, but did not alter B-lymphocyte blastogenesis induced by lipopolysaccharide/dextran. Humoral immunity (anti-sheep red blood cell), a T-lymphocyte macrophage-dependent response, was also reduced (Blakley et al., 1999).

Moreover, there was a study on effect of subchronic oral CPF administration on hematological and serum biochemical indices, and the possible ameliorating effect of vitamin C on the indices in mice. The study by demonstrated that pretreatment of CPF-administered mice with vitamin C significantly altered some important hematological and serum biochemical parameters, revealing the protective action of the vitamin against some organ damage induced by CPF (Griffin et al., 1999).

Far from our concern, there is no study of CPF exposure to human enzymatic activities due to unethical and study on human exposure quite limited. But there is some study related to CPF exposure to human which indicated that daily occupational exposure through dermal contact may result in accumulation of CPF and its metabolites, possibly resulting in adverse effects (Griffin et al., 1999; Meuling et al., 2005).

2.5.4 Chlorpyrifos fate in Humans and Animals

In humans, CPF and its principal metabolites are eliminated relatively rapidly following a single dose (Nolan et al., 1984). It is readily absorbed into the bloodstream through the gastrointestinal tract if it is ingested, through the lungs if it is inhaled, or through the skin if there is dermal exposure (Pesticide Management Education Program, 1984). After a single oral dose, its half-life in the blood appears to be about one day (New York State Department of Environmental Conservation, 1986). CPF was found in its original form in the blood, brain and liver of a 61-year old man who lived only one day after accidentally eating this material (Hayes, 1982).

CPF is eliminated primarily through the kidneys in urine (Giesy et al., 1999). Following oral intake of chlorpyrifos by rats, 90% was removed in the urine and 10% was excreted in the feces (Guha et al., 1997). It is detoxified quickly in rats, dogs and other animals (Worthing et al., 1987). Following intake, some CPF becomes stored

in fat tissues. It is eventually moved out of the fat tissue and eliminated from the body, with a half-life of about 62 hours (Hayes, 1982).

CHAPTER 3

Materials and Methodology

3.1 Specimen

The experiment was conducted by using juvenile *C.gariepinus*. Briefly, milt and eggs were obtained from Ovaprim®-injected sexually mature male and female fish, respectively. Then, it was transferred to a fiber glass tank filled with 500 L UV-sterilized water. Fishes were fed at a rate of 5–10% of body weight per day (the rate started with 10% and gradually reduced to 5%) three times a day and reared for 14 weeks in 2000 L fiber glass tanks prior to the start of the experiment. No signs of sexual differentiation were observed among the fish up to the end of the experiment.

3.2 Exposures to CPF

Every three days, stock solutions of CPF (analytical standard grade, Sigma-Aldrich, USA) were prepared using HPLC-grade acetone (Fisher Scientific). Juvenile

fish was exposed to three nominal concentrations of CPF (50, 100, and 150) µg/L for 21 days. Working solutions were obtained by diluting the stock solution with UV-treated and filtered water in 324 L glass aquaria (120L × 60H × 45Wcm; glass plates were bound to each other using silicone aquarium sealant). To minimize stress on the fish, every morning 70% of the water was siphoned from the bottom of the aquariums, and another 30% was siphoned 10 h later in the evening. Five fishes per treatment were placed in each aquarium (one aquarium/treatment). Aquaria were gently aerated with air stone bubblers. Mean (SD) (n = 42) water parameters throughout the experiments were as follows: temperature 27.08 (0.73) °C, pH 6.69 (0.24), dissolved oxygen 6.44 (0.61) mg/L, alkalinity 39.85 (6.67) mg CaCo3, hardness 60.65 (5.98) mg $CaCO_3$, and salinity b1 mg/L. A negative control group and a solvent (acetone) control group were included. The highest concentration of acetone used in the test solutions was below 0.001% (V/V). The experiment was performed on a 12/12 light/dark cycle. During the exposure, fishes were fed *ad-libitum* once daily. No mortality was recorded in any of the treatments throughout the study. At the end of the experiment, fishes were sacrificed by an overdose of clove oil. The liver and brains were dissected out and transferred into cryovials. The vials were immediately snap frozen in liquid nitrogen and stored at−80 °C freezer

3.3 Experimental Group Designs

The layout of this experiment was shown on Table 1. Basically, juvenile *C.gariepinus* was divided equally into 5 groups. There were group for normal *C. gariepinus*, solvent control group (vehicle), and 3 treatments groups of CPF

treatment with particular concentrations (50µg/L, 100µg/L, and 150µg/L). Five fishes were allocated for each particular group with n=5.

Table 3.3: Experimental group design for CPF exposure

Treatment	**Juvenile *C. gariepinus* (n=25)**
Negative Control	5
Solvent Control (Acetone)	5
50µg/L	5
100µg/L	5
150µg/L	5

3.5 Tissues Preparations

For AChE, the brain tissues were homogenized using a homogenizer in 500µL of potassium phosphate buffer (0.1M, pH7.2), and the supernatants obtained after centrifugation (4°C, 3800g, 3 min) were removed and proceed to enzymatic analysis.

While for GST, the liver tissues were homogenized using a homogenizer in 1 mL K-Phosphate 0.1 M buffer, pH 7.4. The remaining tissues homogenate (700µL) were centrifuged at 10,000 g for 20 min (4°C) to isolate the Post-Mitochondrial

Supernatant (PMS). All microtubes were stored at -80 °C until further enzymatic analysis.

3.6 Protein Determination

The method described by (Bradford, 1976) can be used for quantitative determination using bovine serum albumin (BSA) as the protein standard. This method is adapted to be used with a microplate reader. For each microplate well, 280 µL of Bradford's reagent (phosphoric acid, methanol, and Coomassie brilliant blue) is added to 100 µL of 10 times dilution of the sample. A protein standard curve was prepared each time the assay is performed by diluting lyophilized bovine serum albumin with distilled water. Dilutions of the concentrated Bradford's reagent (commercially available) with distilled water must be utilized within two weeks. The colour development is stable for one hour. Absorbance is read at 595 nm and the sample concentration is calculated from the standard curve.

3.7 Acetylcholinesterase assay

In this study, the activity of AChE was measured according to the method of Ellman (Ellman et al., 1961) with modification for microassay (Guilhermino et al., 1996) using standard 96 well micro plate. Activity of AChE is expressed as the amount of thiocholine produced from ATC per minute under the specified

conditions. In a 96 well microplate, 250 µL of the reaction solution was added to 50 µL of the sample and the absorbance was read at 414 nm, after 10, 15 and 20 min. The reaction solution had 1 mL of 5, 50-dithiosbis-2-nitrobenzoic acid (DTNB) 10 mM solution, 1.280 mL of 0.075M acetycholine iodide solution and 28.920 mL of 0.1M phosphate buffer. The enzymatic activity was expressed as µmol/min/mg of protein. The enzyme activities were calculated as described by (Yin et al., 2001).

Enzyme Activity = Unit (U)

Unit (U) = µmol/min

$$\mu mol/min = \left(\frac{\Delta 450nm}{1.36 \times 10^{4} M^{-1} cm^{-1}.min} \right) \div \text{Protein concentration (mg)}$$

Where;

Δ Absorbance = Wavelength at 450 nm after 20 minutes of incubation

Final – Initial

$0.136\ mM^{-1}.cm^{-1}$ = Extinction coefficient of DTNB at 450 nm

3.8 Glutathione *S*-transferase assay

The juvenile *C. gariepinus* GST activity in the liver was determined based on method described by Habig (Habig et al., 1974a) adapted to 96- well microplate by (Frasco & Guilhermino, 2002). 100 µL of sample was mixed in 200 µL of a

reaction solution. The reaction solution was a mixture of 4.95 mL K-phosphate buffer 0.1 M (pH 6.5) with 900 µL of L-glutathione reduced (GSH) 10 mM, and 150 µL 1 –chloro-2,4-dinitrobenzene (CDNB) 10 mM and it was measured at 340 nm. GST activity was calculated using the formula by (Boyland & Chasseaud, 1969).

$$\text{GST activity} = \frac{[(\Delta 340/\text{min})/\ 0.\ 0096\ \mu\text{M-1/cm}] \times (350\ \mu\text{l}\ /100\ \mu\text{l}) \times 10\ \text{sample dilution}}{\text{Protein concentration (mg)}}$$

3.9 Statistical Analysis

Data were log-transformed when required to meet assumptions of normality (Shapiro-Wilks test). Prior to ANOVA, responses on negative control and vehicle (acetone) control groups were compared by Student's t-test. One-way ANOVAs was used to compare AChE and GST activities cross CPF treatments. All of the data obtained were expressed as means ± standard deviation (S.D). Tukey *post hoc* test was performed to compare each treatment group with negative control group and also to find out the significance difference cross treatment concentration.

3.10 Quality Control

In order to ensure ensure the precise data, chemicals were purchased from manufacturers who guaranteed the purity and suitability for the desired application.

Laboratory equipment were periodically controlled and maintained according to the specific recommendation of the suppliers. The experimental study was conducted according to Guidelines for the Use of Fish in Research by (Jenkins et al., 2014). To ensure the quality of the result, five numbers of fishes were used similar for each treatment and these numbers of fish are similar with previous study (Gobas et al., 1999; Havel et al., 2015; Poléo et al., 1997). Meanwhile, three replicates were used during enzymatic assay to ensure the quality control of the result.

3.10 Ethics Statement

Animal ethic approval was obtained from the Animal Ethics Committee, Universiti Putra Malaysia. This study was supported by the Universiti Putra Malaysia's Research University Grant Scheme (Project number: UPM/IACUC/AUP-R076/2013).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Acetylcholinesterase activity in the brain

The comparison between normal control and solvent control showed significant difference with $p < 0.05$ in independent t-test (Table 4.1). Thus, the normal control was excluded from further analysis and solvent control has been used as control group. Activities of AChE in the brain of juvenile exposed to doses CPF using Tukey *post hoc* test are shown in Figure 4.1. In all treated groups, decreased in AChE activity was observed in a dose-dependent manner. From the result, there was a significant difference between AChE activity between control group and CPF exposed groups after exposure to 21 days. The exposures of CPF at concentration 50, 100, 150) µg/L showed decreased in the AChE activities in the brain of the fish. However there was no significance difference between three graded CPF exposed groups. The finding showed that the lowest concentration used in this study was able to inhibit the AChE activities in the liver. The inhibiton of the AChE activities irreversibly can cause convulsions and paralysis which can lead to death (Van Dyk &

Pletschke, 2011). Thus, indicated the toxicity of CPF to *C. gariepinus* brain and may pose similar result to human brain if been exposed at this concentration.

Table 4.1: Independent t-test for negative control group and solvent control group

	t-test for Equality of Means		
	t	df	Sig. (2-tailed)
Acetone concentration	14.51	4	0.001

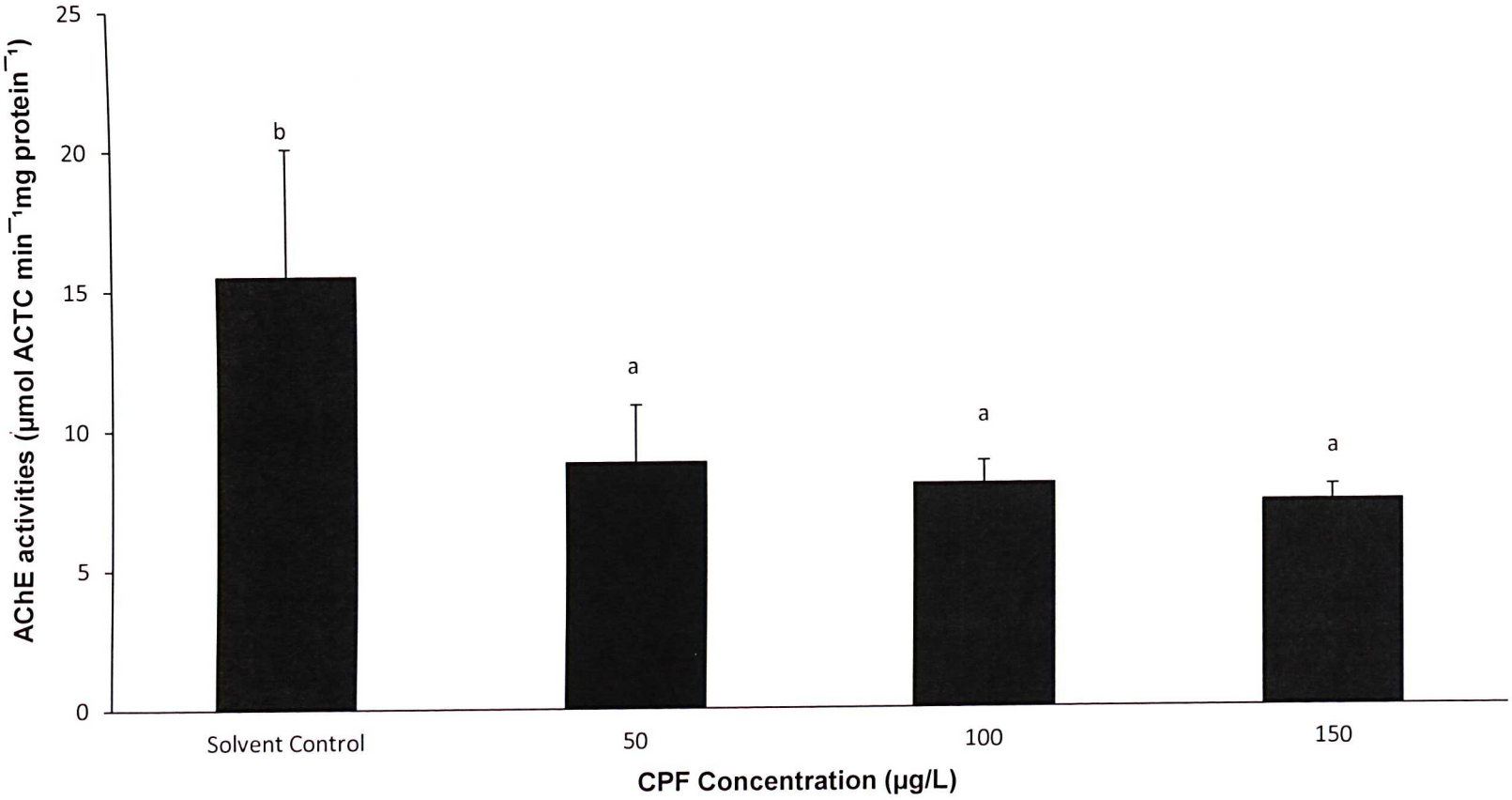

Figure 4.1: AChE activity across CPF treatments. Treatments labelled with different letters are significantly different ($P<0.05$ Tukey's multiple-range test)

The finding result indicated that CPF exposure caused AChE activity inhibition as indicated in the previous studies, despite these being extensively reported in other fish species. For example, (Xing et al., 2014) indicated that CPF induced AChE inhibition in the brain of common carp. In another study, CPF caused a significant decrease in brain AChE activity in Oryzias latipes (Khalil et al., 2013). Finally, (J. Yen et al., 2011) has tested CPF on larval zebrafish, observing an AChE inhibition in the fish brains.

In addition, study done by White (1977) has showed that the brains of patients with Alzheimer Disoder (AD) were deficient in acetylcholine (ACh), one of the main neurotransmitters of the central nervous system that serves to facilitate in attention and learning (White et al., 1977). One of the most consistently reported brain deficits in AD is in the cholinergic system, thus many treatment regimens have aimed at enhancing cholinergic transmission (Quirion et al., 1990). This discovery led to the development of the cholinergic hypothesis, which states that cognitive, functional and behavioural dysfunction associated with AD may be caused by an inability to transmit neurologic impulses across cholinergic synapses (Ferreira et al., 2006).

4.2 Glutathione *S*-tranferase activity in the liver.

CPF exposure to the liver of *C. gariepinus* showed there was significant difference between normal control groups with solvent control group in independent t-test (Table 4). Hence, the solvent control group was taken as control group. From the Tukey *post hoc* test (Figure 2) showed that there was a significant difference between control groups with CPF exposed group for concentration 100µg/L and 150µg/L. Nevertheless there was no significance difference between solvent groups with CPF 50 µg/L. It was shown that the juvenile *C.gariepinus* may be resistant to environmental pollutant as it showed no response in the GST activities after exposed to 50 µg/L CPF. However, juvenile exposed CPF group with 100 µg/L, and 150 µg/L concentration showed response to the CPF toxicity as xenobiotic compound in their body and reacting to detoxifies the CPF that enter into its body.

The level of induction in GST-CDNB activity in juvenile *C .gariepinus* liver reported here was consistent with previous studies of GST induction in other aquatic species. For example, treatment of brown bullhead with ethoxyquin.ranged from 5.6 to 10.4 mg L (-1) resulted in 1.2-fold to 1.5-fold increases of GST activity toward CDNB (Henson et al., 2001). Treatment of rainbow trout (*Oncorhynchus mykiss*) with various dosages of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,2-bis(p-chlorophenyl)-1,1-dichloroethane (p,p'-DDE) resulted in 2-fold inductions of GST-CDNB activity (Celander et al., 1993).

Table 4.2: Independent t-test for negative control group and solvent control group

	t-test for Equality of Means		
	t	df	Sig. (2-tailed)
Acetone concentration	-3.734	4	0.01

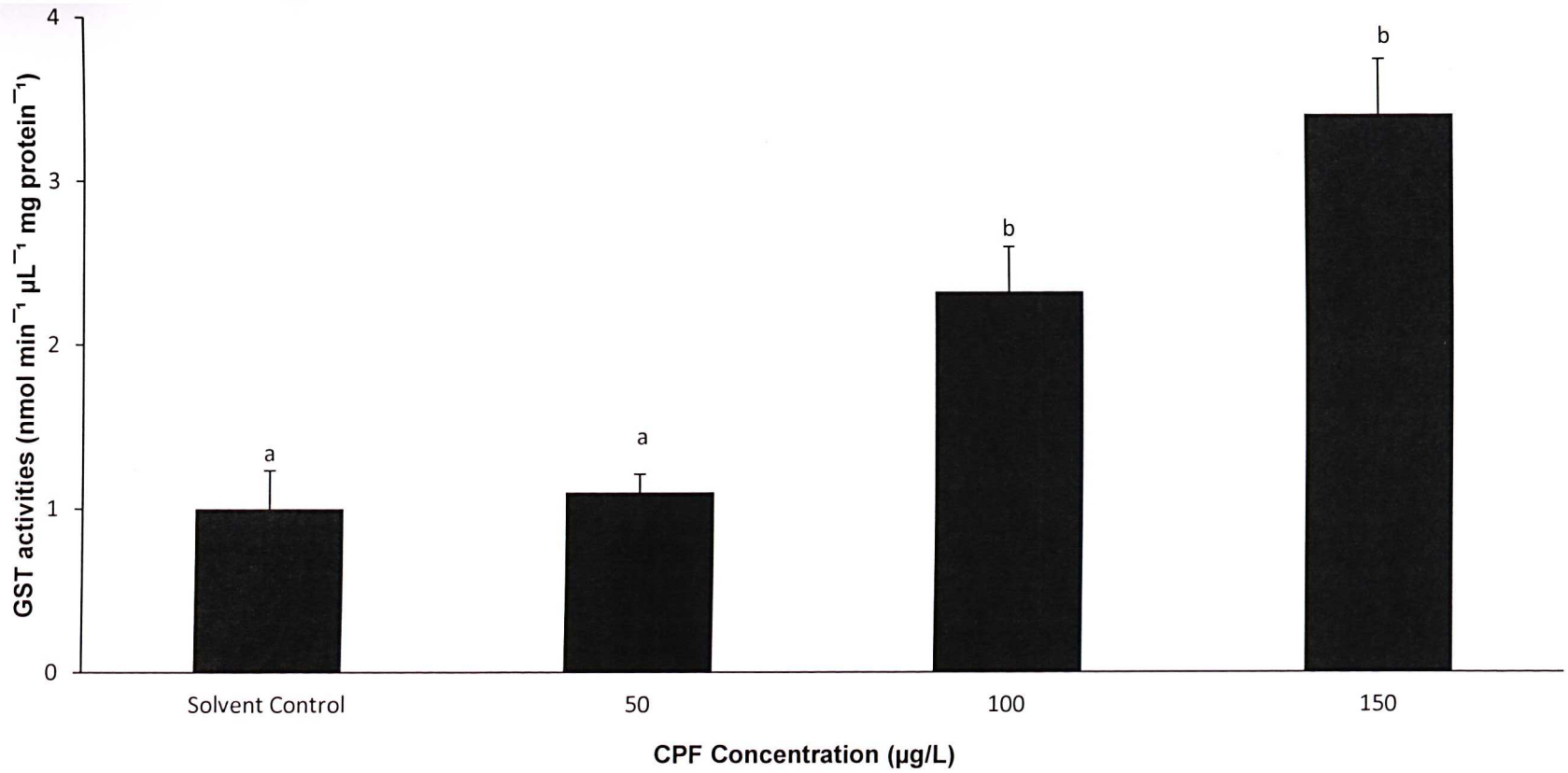

Figure 4.2: GST activity across CPF treatments. Treatments labelled with different letters are significantly different (P<0.05 Tukey's multiple-range test)

The widespread use of CPF in the environment is causing increasing concern about the effects on the health of humans, wildlife and ecosystems. In contrast to in mammals, studies examining toxicity of these chemicals in aquatic vertebrates have been limited, particularly on their toxicity in combination. GSTs have important functions in protection against xenobiotic (Blanchette et al., 2007). Thus, the activation, detoxification, and antioxidant mechanisms of xenobiotic have been investigated in many species of fish.

Induction of CYP1A and glutathione S-transferase activities with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was studied in human hepatocytes in primary culture to investigate the variability of inducibility and the potency of TCDD. Dioxin is a group of chemically related compound that is persistent environmental pollutant. Approximately half-maximal induction of CYP1A was observed (Schrenk et al., 1995). Besides, study by Singha et al., (1999) observed induction of hGST 5.8, which is involved early response to oxidative stress in RPE cells, it suggested the detoxification of the lipid peroxidation products 4-HNE and hydroperoxides, may be an early adaptive response of RPE cells exposed to low levels of transient oxidative stress (Singhal et al., 1999).

4.3 The effects of CPF to human health

The exposure of CPF to human can caused adverse health effect. It caused AChE activity inhibition and GST activity elevation. The finding from this study has shown the AChE activity and GST activity of the fish was disturbed after exposed to CPF and it may give the same responses if it was exposed to the human. The usage of CPF by the user will goes through inhalation, skin absorption or accidentally ingested and will distributed by the blood to the brain, liver and other organ of the human. It is reported in many study exposures to pesticides will cause adverse health effect such as Alzheimer disorder, Parkinson disease and many more can be explained by this finding. The finding in this study has shown that the exposures of CPF cause alteration in enzymatic activities due to its toxicity which can accumulate in the body for long period of time and may cause adverse health effects to human health. Although, other factors must be consider because the alterations of the enzymes are not the only one that can caused adverse health effects to the human health.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In conclusion, CPF is OP very toxic to fish, aquatic invertebrates and human. It may build up in the tissues of fish and other animals which are known as bioaccumulation. Juvenile *C. gariepinus* exposed to three grade concentration of CPF for 21 days caused inhibition of AChE activity in the fish brain due to CPF toxicity. The increasing levels of antioxidant enzyme GST activities in the liver of exposed *C. gariepinus* indicated the responses of the fish to xenobiotic compound present in their body. Although, GST and AChE enzymes are available in human, the effect of exposures to CPF may be different due to different species. But they have same functions and the finding data obtained can be used as a bioindicator to evaluate the health effect of CPF to human by using fish as a model.

5.2 RECOMMENDATIONS

The finding results have shown CPF was toxic to aquatic animal and maybe toxic to the human health. CPF should not be used near to the water and water source. Furthermore, protection must be applied to protect the consequences of health effect from exposure to CPF is by avoiding eye and skin contact with CPF as well as inhalation of its vapors, dusts or sprays.

Due to timeline factor, this study missed behavioral change and other important biomarkers parameter which can be used to evaluate the effect of CPF. Therefore, further study should be conducted as the following aspects:

- Do the behavioral study by observing behavior of the fish after exposures to CPF.
- Add more biomarkers such as catalase activities and lipid peroxidation level.
- To test longer exposure duration order to evaluate the effect of CPF in chronic effects.

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