HISTOPATHOLOGICAL ALTERATION AND *METALLOTHIONEIN* GENE EXPRESSION IN THE ORGANS OF SWAMP EEL (*Monopterus albus*) COLLECTED FROM THE MAE SOT DISTRICT, TAK PROVINCE, THAILAND

MASYITHA WAHID

A Thesis Submitted to the Graduate School of Naresuan University

in Partial Fulfillment of the Requirements

for the Master of Science Degree in Biological Science

July 2017

Copyright 2017 by Naresuan University

Thesis entitled "Histopathological Alteration and *Metallothionein* Gene Expression in the Organs of Swamp Eel (*Monopterus albus*) Collected from the Mae Sot District, Tak Province, Thailand"

By Miss Masyitha Wahid

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Biological Science of Naresuan University

Oral Defense Committee

..... Chair

(Pantip Hinhumpatch, Ph.D.)

..... Advisor

(Assistant Professor Surisak Prasarnpun, Ph.D.)

..... Co – Advisor

(Nonglak Yimtragool, Ph.D.)

..... External Examiner

(Sittichai Urtgam, Ph.D.)

Approved

.....

(Panu Putthawong, Ph.D.)

Associate Dean for Administration and Planning

for Dean of the Graduate School

27 JUL 2017

ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah for the strengths and His blessings in completing this thesis. The completion of this thesis would not have been possible without the support and guidance to whom I would like to deliver my sincere thanks. First and foremost, I would like to express my deepest thanks and appreciation to my advisor, Assistant Professor Dr. Surisak Prasanrnpun, for his supervision and constant support. His useful comments, remarks, guidance and the valuable insight have contributed to deliver the best version of my thesis work. I would also express my sincere thanks to my co-advisor, Dr. Nonglak Yimtragool, for providing the guidance, caring, patience throughout the experimental and thesis works. Her invaluable helps and support have contributed to the success of this research.

I also deliver my sincere thanks to the committee member, Dr. Pantip Hinhumpatch and Dr. Sittichai Urtgam, for providing suggestions for the improvement of my thesis work. I am also indebted to Asisstant Professor La-Or Chompuk, M.D. in Naresuan University Hospital Pathology Unit, Dr. Supat Ponza in Fisheries Department and Assistant Professor Dr. Katechan Jampachaisri in Mathematic Department for all the useful helps and suggestions for this work. I am thankful to all the lectures in Biology Department who had been so generous in sharing their knowledge and their wisdom during my study in Naresuan University. My thank also goes to all the technicians and office staffs of Biology Department and Science Faculty for their cooperation.

My special thanks also goes to Miss Sopita Piangpraichom, Miss Sirinoot Khemkong, Miss Kamonchanok Bunmee, Miss Naruemon Nimlob, Mr. Thanaban and Mr. Jatuphol Pholtaisong who helped me in numerous ways during various stages of my study, for their friendship and sincere companion throughout my study in Thailand. Never will I forget the moments I shared with them, particularly their kind help.

My sincere thanks also goes to my fellow international students, Miss Andi Citra Pratiwi, Miss Nguyen Ho Nhat Thuan, Miss. Chorn Sokuntheary, Miss Norma Pawestri, Miss Amaraa Amarjargal and all members of Indonesian Students Association in Phitsanulok, who made me found a family away from home.

I gratefully acknowledge Naresuan University for providing scholarship through ASEAN Scholarship during my study in Thailand. The opportunity of studying in Naresuan University really has broadened my perspective and introduced many new cultures and customs and also provided me with a huge amount of useful knowledge.

My sincere thankful goes to my parents, Drs. Abdul Wahid and Masni Madeali and also my sisters and brother for their endless love, prayers and encouragements. To those who indirectly contributed in this research, your kindness means a lot to me. Thank you very much.

Masyitha Wahid

Title	HISTOPATHOLOGICAL ALTERATION AND *METALLOTHIONEIN* GENE EXPRESSION IN THE ORGANS OF SWAMP EEL (*Monopterus albus*) COLLECTED FROM THE MAE SOT DISTRICT, TAK PROVINCE, THAILAND
Author	Masyitha Wahid
Advisor	Assistant Professor Surisak Prasarnpun, Ph.D.
Co Advisor	Nonglak Yimtragool, Ph.D.
Academic Paper	Thesis M.S. in Biological Science Naresuan University, 2016
Keywords	Cadmium, Histopathological Alteration, *Metallothionein* Gene Expression, Swamp Eel, Mae Sot.

ABSTRACT

Cadmium (Cd) is produced mainly as a by-product of zinc mine activities. In Thailand, the largest zinc mine is located in Mae Sot district, Tak Province. Samples of *Monopterus albus* were collected from paddy fields in 4 sites, three downstream and one upstream from the zinc mine. The upstream site was considered to be uncontaminated, while the three downstream sites were considered to be contaminated with Cd. Studies on the accumulation level of cadmium was conducted on the organs (liver, kidney and gills) of the fish as well as the sediment from respective sites using Atomic Absorption Spectrophotometer (AAS). The histopathological alterations of these organs were examined using H&E staining technique. The *metallothionein* (*MT*) gene expression level in the liver, as a potential biomarker for long-term Cd exposure in their natural habitat, was also assessed. The level of hepatic *MT* gene expression was performed by quantitative Real-Time PCR. The result showed that Cd accumulation of the sediment and the three subject organs were much higher in swamp eels collected from the downstream sites, when compared to those collected from the upstream site. Histopathological alteration in the liver and the kidney showed similar lesions in all study sites. However, the severity of damage fish liver collected from downstream sites seemed to be more prominent compared to those from the upstream site. Meanwhile,

the histopathological determination of the gills was difficult due to the lack of documentation about the histological architecture of gills in this species. Study on *MT* gene expression showed that the hepatic *MT* level in the upstream site was 0.75 fold, while the other three downstream sites were 0.36, 4.44 and 0.94 fold. There is no parallel correlation between the hepatic cadmium level and the hepatic *MT* gene expression neither between the Cd level in the sediment and the *MT* gene expression level. This study then suggests that *MT* gene expression biomarkers might be not suitable for swamp eels with prolonged exposure to Cd.

LIST OF CONTENTS

Chapter		Page
I	**INTRODUCTION**	1
	Background	1
	Statement of the Problems	4
	Objectives of the Study	4
	Scopes of the Study	4
	Hypotheses	5
	Significances of the study	5
II	**REVIEW OF LITERATURE**	6
	Cadmium (Cd)	6
	Gills	21
	Kidney	22
	Liver	22
	Metallothionein (MT)	23
	Zinc Mine in Mae Sot District, Tak Province, Thailand	26
	Swamp Eel (*Monopterus albus*)	28
III	**RESEARCH METHODOLOGY**	30
	Study Design	30
	Materials and Instruments	30
	Methods	31
	Statistical Analysis	38

LIST OF CONTENTS (CONT.)

Chapter		Page
IV	**RESULTS**	39
	Cadmium Level in the Sediment and the Organs of Swamp Eel (*M. albus*)	39
	Histopathological Alteration in the Organs of Swamp Eel (*M. albus*)	41
	Metallothionein (*MT*) Gene Expression Level in the Liver of Swamp Eel (*M. albus*)	46
V	**DISCUSSION**	52
VI	**CONCLUSION AND SUGGESTION**	63
REFERENCES		65
APPENDIX		84
BIOGRAPHY		102

LIST OF TABLES

Table		Page
1	GPS coordinates and codes of four study sites in Mae Sot district	33
2	Primers sequence of *18 S* and *Metallothionein* gene for Real-Time PCR	38
3	Cadmium level in sediment (mg/kg dry weight) from four study sites	40
4	Cadmium level in swamp eel organs (mg/kg dry weight) from four study sites	41

LIST OF FIGURES

Figures		Page
1	Model of source pathway for cadmium in the environment	7
2	Cd uptake and handling in three major target organs in fish	10
3	Scheme illustrating the mechanism responsible for the selective accumulation of cadmium in proximal tubular cells	14
4	Zinc mine operated by Padaeng Industry PLC in the Tak Province	27
5	Map of the four study sites according to their respective GPS coordinates in Mae Sot District.....	32
6	Multiple sequence alignment (Clustal Omega) of 32 sequence of metallothionein of teleost.....	35
7	Photomicrograph of liver section of *M. albus* in four study sites.....	43
8	Photomicrograph of renal section of *M. albus* in four study sites	45
9	Photomicrograph of gills section of *M. albus* in four study sites	46
10	Amplification of 3'-RACE of *M. albus* MT gene using UPM-1 and MT gene specific primer.....	47
11	Electrophoresed gel from colony cracking method showing the predicted size of MT swamp eel.....	48
12	Electrophoresed gel from colony PCR method showing the size of pJET 1.2 combining with the size of predicted MT gene.....	48
13	A typical plasmid reparation.....	49
14	Sequence of MT cDNA of *M. albus* including the coding sequence and the deduced amino acids.....	49
15	A typical relative quantitative analysis output (melting curve).....	50
16	Expression profile of MT and 18S rRNA	50
17	Quantitative expression of Metallothionein of fish liver which was normalized using 18S rRNA and site S1 (uncontaminated site) was used as the calibrator	51

LIST OF FIGURES (CONT.)

Figures		Page
18	pJET 1.2/blunt vector map.....	82
19	Standard curve compromising six standard calibration of 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/L of Cd.....	83

ABBREVIATIONS

µM	Micro molar
µl	Micro liter
2X YT	2x Yeast Extract Tryptone
3'RACE	3' rapid amplifications of cDNA ends
3'UTR	3' untranslated region
AREs	Antioxidant responsive element
BLAST	Basic local alignment search tool
Ca	Calcium
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
Cds	coding region/coding sequence
cm	Centimeter
Ct	Threshold cycle
Cu	Copper
Cys	cysteine
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
GIT	gastrointestinal tract
GSH	Glutathione
H&E	Hematoxylin and Eosin
Hg	Mercury
Kg	kilogram
M. albus	*Monopterus albus*
Min	Minutes
mM	Millie molar
MREs	Metal-responsive elements

ABBREVIATIONS (CONT.)

mRNA	Messenger ribonucleic acid
MT	Metallothionein
MTF-1	Metal-regulatory transcription factor-1
PCR	Polymerase chain reaction
PVC	Pavement cells
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SE	Standard error
Sec	Seconds
SOB	Super optimal broth
SOC	Super optimal broth with catabolite repression
TBE buffer	Tris borate EDTA buffer
Zn	Zinc

CHAPTER I

INTRODUCTION

Background

One of the major problem that the world is facing today is environmental pollution. The effects of pollution are varied and wide-ranging. Pollution, can be pollution in the air, water, or land, is causing a lot of distress not only to humans but also animals and plants, driving many animals and plants species to endangerment and even extinction. Pollutants are introduced into the environment by wide diversity of human activities. Environment can be exposed continuously by pollutants often as a consequence of industrial activities, for instance heavy metal cadmium (Cd) which is generated during zinc ore processing.

Cadmium (Cd) is a heavy metal with no known role in biological system, no evidence that it is either biologically necessary or beneficial (Eisler, 1985; Canli and Atli, 2002). It is distributed in the earth's crust, generally in association with zinc ores. Over time, it has increasingly caused concern as an environmental toxin (Thévenod, 2009). Cd, a rare but widely distributed element, is emitted by various natural and anthropogenic activities to the atmosphere, aquatic system and terrestrial environment (UNEP, 2010). Anthropogenic activities release 3–10 times more Cd into the environment than natural activities (Joseph, 2009). Cd is naturally discharged into the environment from volcanic eruptions, forest fires, production of sea salt aerosol or other natural phenomena (Ashizawa et al., 2012). It is frequently used in wide range of technological applications, such as Cd-coating (electroplating), plastic and paint pigments, cadmium-nickel batteries and as a component of various alloys (Martelli et al., 2006). It is generated mostly as a by-product of zinc mining activities and, to a lesser degree, as a by-product of lead and copper production (UNEP, 2010).

Cd which enters to the aquatic system from industrial source is quickly adsorbed by particulate matter, where it may settle out or remain suspended, depending on local condition (IPCS, 1992). It is considered as highly toxic element and is readily absorbed by living things directly from the water in its free ionic form. In freshwater

fish, the possible areas of cadmium uptake mainly through three routes namely, gills as respiratory tract, gastrointestinal (GI) tract through ingestion, and skin (Kumar and Singh, 2010; Jalaludeen, et al., 2012). In general, the accumulation of Cd depends on several factors such as concentration, exposure time, way of uptake (waterborne or dietary pathway), the condition of environment and intrinsic factors (Perera, et al., 2015). Prior to being transported to other tissues through circulatory system, the Cd levels increase at the site of exposure, for instance, at gills in a waterborne exposure or at gastrointestinal tract in a diet borne exposure (McGeer, et al., 2012). Fish living in heavy metal contaminated waters tend to accumulate heavy metals in their tissues (Perera, et al., 2015). Cd in fish body rarely accumulated evenly but is accumulated by particular target organs (Wangsongsak, et al., 2007), where it may reach maximal level in the kidney, liver, gills, and GI tract, to a lesser extent in the blood and not significant amount could be found in the brain or muscle. However, the trend of accumulation also varies depending on the exposure route (Szebedinszky, et al., 2001; Chowdhury, et al., 2005). During waterborne exposure, trend of Cd accumulation follows the order kidney>gills>liver>intestine, while for diet borne exposure the Cd accumulations are in order intestine>kidney>live>gill (McGeer, et al., 2012).

The effects of Cd could be directly or indirectly lethal to aquatic living things and could affect individuals, populations and ecosystems. As a toxic element, Cd might behave as stress inducing agent for fish (Perera, et al., 2015). Cd has a high affinity for the sulfhydryl groups of cysteine, and are able to competes against essential metals such as zinc (Zn) and copper (Cu) for the structural and active sites of various enzymes, resulting the impairment of their catalytic activities. Cd toxic also impacts on the calcium (Ca) metabolism in cells (Santovito, et al., 2012). Cd is a carcinogenic that can interfere with cellular signaling and the gene expression regulation (Duncan, et al., 2008). Cd Chronic exposure leads to accumulation of Cd majorly in liver and kidney as well as other organs (Ognjanović, et al., 2008).

To evaluate the heavy metal pollution in aquatic ecosystem, fish are frequently used as a bio-monitor in many studies (Yin, et al., 2012). The accumulation of Cd in living things is major ecological concerns due to its ability to accumulate very quickly yet the Cd excretion from living organisms is a slow process. In fish, Cd can cause structural and phatomorphological alteration in various organs (Raskovic, et al., 2011).

Both in laboratory and field studies, histopathological alterations have been frequently applied as biomarkers to evaluate the health of fish exposed to contaminants. Using this biomarker in environmental monitoring allows examining specific target organs, such as gills, kidney and liver that are known to have responsibility for important functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish (Hadi and Alwan, 2012).

Once Cd inside the cell, it will induce various types of signal transduction mechanisms and activate many genes. A disruptive process of cell homeostasis is one of the immediate effects caused by cadmium. Mechanism of cell homeostasis was recovered by the existence of metal binding protein called metallothionein (Rumahlatu, et al., 2012). Metallothineins (MTs) are present in a range of aquatic organism and is responsible for binding heavy metal and reducing its toxicity (Abdel-Tawwab and Wafeek, 2014). MTs are low molecular weight peptides that have a high cysteine content and give rise to metal-thiolate clusters. Most MTs consist of two metal clusters containing three and four bivalent metal ions, respectively (Carpene, et al., 2007). The MTs function in transport, detoxification and storage of essential metal ions (Zn, Fe, and Cu) and also non-essential metal ions (Cd, Pb and Hg). When the Cd concentration is high, the MT detoxification system can become overwhelmed and the excess Cd (Cd that cannot be bound to MTs) will be available to produce toxic effects (Perera, et al., 2015) by interacting with high molecular mass protein (Kovarova, et al., 2009). In fish, tissue expression and accumulation of MTs primarily occur in the liver, kidney and gills (Santovito, et al., 2012), yet the most intense MTs production is usually observed in the liver (Kovarova, et al., 2009).

The largest zinc mineral source of Thailand and also high contamination of Cd area is located in Mae Sot district, Tak Province (Thamjedsada, et al., 2012). Several findings have reported regarding the contamination of Cd in this area. Simmons, et al. (2005) found that the rice grain in Mae Sot district contain cadmium in range 0.05 to 7.7 mg/kg, it was already exceed permisible level of Cd in rice namely 0.2 mg/kg. In Mae Tao also found that sediment of river contain 5.67 to 112.40 mg/kg with the maximum permissible level is 37 mg/kg (Thamjedsada, et al., 2012). The previous research also reported that swamp eel in mae tao river contain Cd which exceed the maximum level allowance on the fish (Krissanakriangkrai, et al., 2009). The

bioaccumulation of Cd in trophic food chain is cause of concern since they can have deleterious effects on human health (Annabi, et al., 2013). When fish are consumed by human with high levels of accumulated Cd, 3–7% of the ingested Cd is absorbed (Liu, et al., 2015). Therefore, the level of cadmium in aquatic organism such as fish must be given attention, and the level of Cd in different tissues must be taken into account.

In the current study, we used histopathological and molecular approaches to examine the effect of cadmium on swamp eel (*Monopterus albus*). It is one of fish consumed by people in Mae Sot District. It is predator and scavenger in fresh water ecosystem, also its position is on top level of food chain where it can accumulate more contaminants (Krissanakriangkrai, et al., 2009).

Statement of the Problems

Based on the background that has been explained above, the proposed statement of the problem are as follows:

1. How is the level of cadmium (Cd) of the gills, kidney and liver of the swamp eel and also sediment collected from downstream and upstream from zinc mine?
2. How are the histopathological changes of the gills, kidney and liver of the swamp eel collected from the downstream and upstream from zinc mine?
3. How is the alteration of metallothionein gene expression in the liver of swamp eel collected from downstream and upstream from zinc mine?

Objectives of the Study

Based on the statement of the problem that has been explained above, the objectives of the research are as follows:

1. To compare the level of cadmium in swamp eel gills, kidney and liver of the swamp eel as well as the sediment collected from downstream and upstream from zinc mine.
2. To compare the histopathological changes of the gills, kidney and liver of the swamp collected from downstream and upstream from zinc mine.
3. To compare the alteration of metallothionein gene expression in the liver of swamp eel collected from downstream and upstream from zinc mine.

Scopes of the Study

There are three experiment in this study. The first is to determine the cadmium level of swamp eel gills, kidney and liver as well as the sediment collected from downstream and upstream from zinc mines areas using Atomic Absorption Spectrophotometer. The second is to examine the histopathological changes of the gills, kidney and liver of swamp eel collected from downstream and upstream from zinc mines areas using hematoxylin and eosin (H&E) staining. The third is to examine the alteration of metallothionein gene expression of liver of swamp eels collected from downstream and upstream from zinc mines areas using Real-Time Polymerase Chain Reaction (qPCR) techniques.

Hypotheses

1. The swamp eels that live in the contaminated area may uptake cadmium into their bodies and result in accumulation and adverse effect to the target organs such as gills, kidney and liver.

2. Continuously accumulation of cadmium in the swamp eel causes the histopathological changes of gill, kidney, and liver.

3. Continuously accumulation of cadmium in the swamp eel causes the alteration of metallothionein gene expression in liver.

Significances of the Study

1. This study will provide data indicating that the swamp eels living on cadmium contaminated areas can accumulate in several organs.

2. As an indicator that shows the level of cadmium pollution around Mae Tao River.

3. The accumulation of heavy metals such as Cd in fish organs might reflects the health status of fish which could be considered as important warning for human consumption.

CHAPTER II

REVIEW OF LITERATURE

Cadmium (Cd)

General Introduction

Cadmium (Cd) belongs to group of metallic element, together with zinc and mercury to group IIb of the periodic table (IPCS, 1992). This element present naturally in the earth's crust. Cd in the environment usually not occurs as a pure metal, but as a mineral in association with other elements such as chlorine (cadmium chloride), oxygen (cadmium oxide), sulfur (cadmium sulfate, cadmium sulfide) and carbonates in zinc, lead, and copper ores (Scoullos, et al., 2001; ATSDR, 1999). Cd rarely exist in large quantities as the chlorides and sulfates. These different forms of cadmium compounds are solids (ATSDR, 1999). The pure Cd is a soft, silver white metal with a distorted hexagonal structure. Its atomic weight is 112.41 g/mol, its relative density, melting point and boiling are 8.642 g/ml, at 321°C and at 767 °C respectively, a relatively 'volatile' metal (Scoullos, et al., 2001).

Cd clearly was the most toxic metal in comparative acute toxicity testing of all 63 atomically stable heavy metals in the periodic table (Perera, et al., 2014). In the environment, heavy metal Cd is present as a pollutant which is resulted from industrial and agricultural activities, however, it also can occur naturally (IPCS, 1992). Approximately 30.000 tons are emitted into the atmosphere annually, with an estimated in the range of 4.000 to 13.000 tons are from anthropogenic activities (Institute, 2013). In the geosphere, the levels of Cd are usually low except for enrichment in association with Zn, Pb, and Cu sulfidic ore deposits, and also the formation of some phosphate rock. Cd is naturally released to the environment from volcanic eruptions, forest fires, sea salt aerosols generation, as well as other natural phenomena (Scoullos, et al., 2001). Cd is one of chemical element that is neither created nor destroyed but is redistributed in the environment through the human activities also is a persistent contaminant (Erickson, et al., 2008). The source pathway model for cadmium in the environment can be seen in Figure 1.

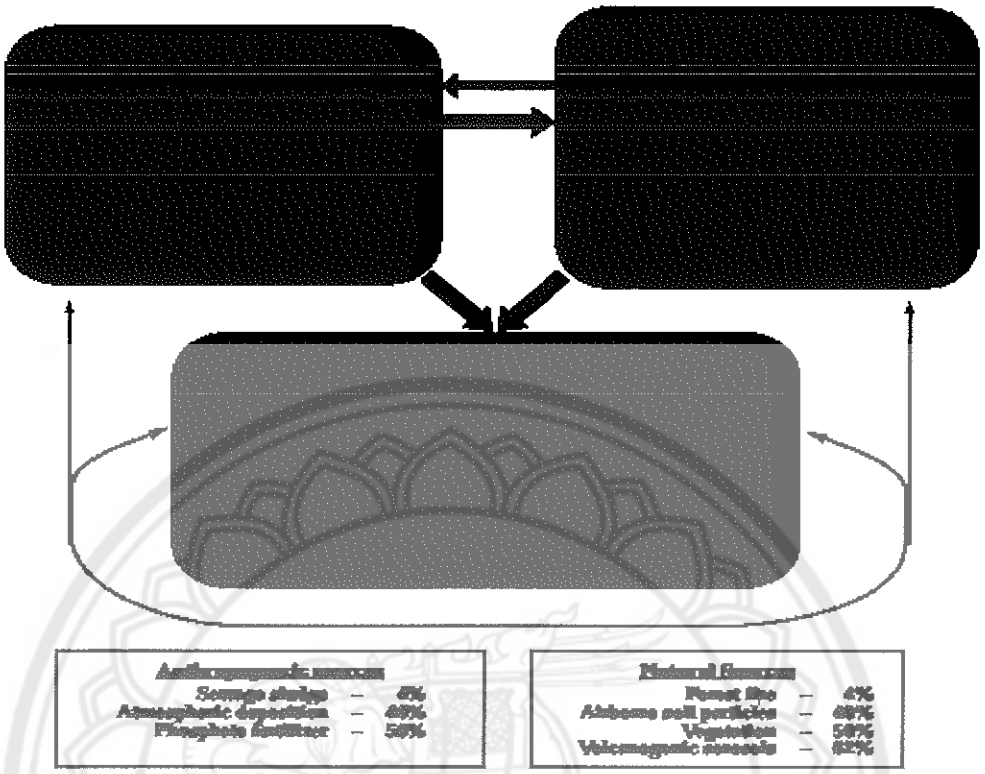

Figure 1 Model of source pathway for cadmium in the environment

Source: McGeer, et al., 2012

Cd is generated majorly as a by-product of mining, smelting and refining of zinc and, to a lesser degree, as a by-product of production of lead and copper. It is therefore primarily a function of zinc production rather than cadmium demand (UNEP, 2010). Cd is typically the 20th century's metal, even though vast amounts of this heavy metal have been released by non-ferrous smelters during the 19th century (Bernard, 2008). The Cd content of the zinc concentrate is usually around 0.3% to 0.5% (Llewellyn, 1994).

Cd is mostly used in the industrial application, for instance, as a colour pigment, an anticorrosive agent, as a colour pigment, as a stabilizer in PVC products, a neutron absorber in nuclear power plants, and in the production of nickel-cadmium batteries. In the agricultural field, phosphate fertilizers also seem to contribute a large amount of Cd. Even though, some cadmium-containing products can be recycled, a large

share of the general pollution of Cd is caused by dumping and incinerating cadmium-polluted waste (Godt, et al., 2006). Cd possess particular properties such as low melting temperature, excellent corrosion resistance, high ductility, high thermal and electrical conductivity, making it suitable for a wide variety of industrial applications (National Resources Canada, 2007).

Cadmium in aquatic ecosystem

According to Darmono (2010), living things in the water are affected by the presence of dissolved metals in the water, especially at concentrations in excess of normal. There are several factors affecting the toxicity of metals in aquatic organism i.e 1) chemical bonds form of dissolved metal; 2) effect of interaction between metals and other toxic species; 3) environmental factors such as temperature, salinity , pH and dissolved oxygen levels in the water; 4) animal condition, the phase of the life cycle (egg, larva, adult), the size of organisms, sex, and nutritional adequacy requirements; 5) the ability of the animal to escape from the effects of pollution; 6) the ability of the organism to acclimatization against toxic metals.

Cd is classified to the group of highly toxic heavy metals. It occurs in water naturally only in trace amounts, but recently the levels have elevated due to human activities (Kovarova, et al., 2009; ATSDR, 2004). A represent of main source of Cd emit into the aquatic environment is non-ferrous metal mines. The contamination can arise from overflow of mine drainage, overflow from tailings reservoirs and the run-off of rainwater from the area surrounding the mine. Thus, effluent flowing into local creeks and other water sources, lead to extensive contamination downstream of the mining operation (IPCS, 1992). Fish generally have the ability to avoid being influenced by pollution. However, the fish that live in a habitat that is limited (such as rivers, lakes, and bays) will be difficult to avoid contamination or pollution. As one result is the accumulation of pollutant elements including heavy metals into the body of the fish (Murtini and Rachmawati, 2007). The process of bioaccumulation of metals in fish tissue is quite varied, depending on the type of metal and fish species. Some types of metal that accumulates through the food chain as predatory fish have large concentrations of metals (Darmono, 2010).

The concentrations of cadmium in fish are considerable interest because consuming fish is one of intake source of cadmium for the general population. Most of

the Cd content in fish or other seafood is highly absorbable in the form of $CdCl_2$ (Squadrone, et al., 2013). The typical accumulation trends of contaminants in fish and other aquatic organisms depend both on uptake and elimination rates (Kumar and Singh, 2010). There are several factor which may affect the accumulation of some metal in fish such as presence of the others metals and the interactions among various metals may be related to their different affinities to various organs (Perera, et al., 2015).

Cadmium uptake into fish body

In freshwater fish, there are three possible areas of cadmium uptake namely, gills as respiratory tract, intestine through ingestion, and body surface (skin) uptake (Kumar and Singh, 2010; Yousafzai, et al., 2010; Jalaludeen, et al., 2012). Fish are able to accumulate heavy metal in their tissues through those areas gastrointestinal tract to higher concentrations than the heavy metal concentration in their environment (Annabi, et al., 2013).

Cd can be taken by fish directly from cadmium contaminated medium (water or sediment) or by consuming the food containing Cd (USEPA, 2002). The essential metals (e.g. calcium (Ca), iron (Fe), and zinc (Zn) which are required by the fish for the metabolism, must be taken up from water, food or sediment. However, the non-essential ones (e.g. mercury (Hg), cadmium (Cd) and lead (Pb)) that have no known function in biological system are also found to be taken by fish via similar route to the essential metals leading to the accumulation in their tissue (Canli and Atli, 2012). One hypothesis which has earnt concern by numbers of researcher regarding the uptake of non-essential metals, states that selective uptake of Cd involves, at least in part, mechanisms whereby Cd interacts with, and competes for, binding site(s) on membrane proteins involved in the transport of essential elements into target epithelial cells, possibly through some form of ionic mimicry (Zalups and Ahmad, 2003). The cadmium uptake and handling in three major target organs in fish i.e gills, gastrointestinal tract and liver is shown in Figure 2.

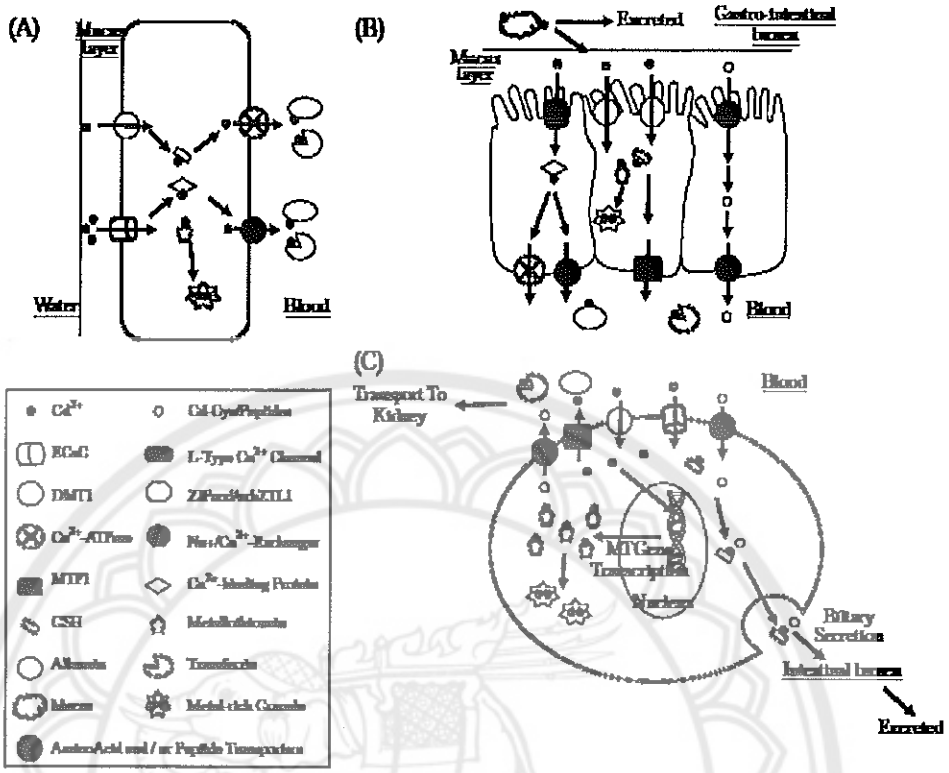

Figure 2 Cd uptake and handling in three major target organs in fish

Note: (A) gill (mitochondria-rich chloride cell), (B) GI tract, and (C) liver (hepatocytes).

Source: McGeer, et al., 2012

1. Cadmium uptake via gills

Due to the direct exposure to the external environment, the gills are often found to be very sensitive to contaminants (Arini, et al., 2015). Metal ions can be taken up through passive diffusion or carrier mediated transport through the gills while metals associated with organic materials are ingested and absorbed by endocytosis over intestine (Perera, et al., 2015). Cd enters the fish from water over the gills and are able to reach the circulatory system can be explained in three steps as follows: 1) apical membrane uptake into the epithelial cells, 2) intracellular Cd buffering system by metal-binding ligands, and 3) basolateral transfer from the cells to the circulatory system (influx). Several studies have demonstrated that the influx of Cd is usually considerably

lower than the apical uptake into the cells, probably due to the intracellular buffering of Cd^{2+} after the uptake from the water (Glynn, 1996).

Apical uptake of Cd at the fish gill occurs via a lanthanum-sensitive voltage-independent epithelial Ca^{2+} channel situated in the mitochondria-rich chloride cells and basolateral transfer via Ca-ATPase and Na/Ca exchanger (McGeer, et al., 2012). Like Ca^{2+}, Cd^{2+} enters the fish predominantly via a transcellular route because of the tight character of the branchial epithelium. Interference of Cd^{2+} with the Ca^{2+} influx route may occur at at least three sites i.e 1) the apical membrane, where Ca^{2+} enters the cell via Ca^{2+} channels; 2) the intracellular Ca^{2+} buffering systems and; 3) the basolateral membrane, where Ca^{2+} is translocated to the blood by a high-affinity Ca^{2+} pump (Verbost, 1989). Dutta and Kaviraj (2001) found that pre-exposure of fish to Ca^{2+} (CaO) leads to the calcium accumulation on the surface of the gill rendering these surfaces most impermeable. It shows that the Ca^{2+} ions can effectively compete with Cd^{2+} ions and prevent the later from entering through gill. Baldisserotto, et al. (2005) also noted that increased dietary Ca^{2+} protected against both dietary and waterborne Cd uptakes.

2. Cadmium uptake via intestine

If chemicals present in the water are strongly into solid forms or present as dissolved forms which are poorly absorbed at gill and skin surfaces, the main route of exposure to fish can be through ingestion of contaminated food or sediment for some species and following the absorption within the gastrointestinal tract (GIT) (Erickson, et al., 2008). The xenobiotics absorption through GIT involves series of steps of interaction as follows: 1) within the intestinal lumen, 2) at the absorptive surface, 3) within the enterocytes, and 4) at the enterocyte–vascular interface. On the apical surface of the enterocytes, physical and chemical properties of the membrane and associated unstirred layer, as well as the transporter complement within the membrane, significantly influence xenobiotic uptake. Within the enterocytes, biotransformation enzymes, carrier molecules, and cellular components further impact xenobiotic concentration and movement. Transport across the enterocyte basolateral membrane into blood is dependent on factors similar to those operating at the apical membrane (Kleinow, et al., 2008).

The uptake of Cd^{2+} through gastrointestinal happens in all sections of the intestine which include anterior, mid, and posterior section as well as in the stomach

(McGeer, et al., 2012). Klinck and Wood (2013) noted that stomach provides the major role in the internalization of Cd in comparison with the other GIT segments. The posterior intestine also appears to plays an important route of Cd entry into the fish body. Bucking and Wood (2006) conducted an *in vivo* experiment on rainbow trout and has suggested that the stomach is the most important segment of the GIT for Ca^{2+}, Na^{+}, and K^{+} transport.

GIT plays a significant barrier against Cd uptake (Klinck and Wood, 2013). From dietary exposure, Cd has several routes of entry (Klinck and Wood, 2011). Cd^{2+} uptake seems to occur via a calcium-sensitive mechanism in the stomach that has different from that in the gills in terms of pharmacological and kinetic properties (McGeer, et al., 2012). Klinck and Wood (2011) provided evidence that Cd is in part transported via channels intended for Ca along the entire GIT. In stomach, Ca and Cd metals are transported via a mechanosensitive L-type Ca channel as well as via a transporter that carries Zn. Evidence shows that Ca and Zn transporter also found in anterior intestine. In mid intestine, in addition to L type Ca channels and Zn transporter(s) which transport Cd, there is evidence for a lanthanum sensitive Ca channel as Cd transporter. In the posterior intestine, Cd is likely transported via an L-type Ca channel, Zn transporters, as well as through divalent metal transporter 1 (DMT1). Cadmium transported by DMT1 thus allows the toxic metal to enter the intestinal epithelium, particularly under iron deficient condition (Martelli, et al., 2006).

3. Cadmium uptake via skin (body surface)

Cadmium uptake through body surface is relatively small in amount compared to that through the gills or intestine, and seems to occur through different mechanisms than that in the gills (McGeer, et al., 2012). Generally, the uptake of substances from water through the skin would be much less significant than uptake over gills because the fish skin typically provides less surface area, a thicker and less permeable diffusion barrier, slower transport of water to the exchange surface, less blood flow, and no countercurrent flow of water and blood. For smaller fish, however, uptake through skin could be significant due to, as size decreases, generally, skin is more permeable and has an increasing surface area relative to the gills. (Erickson, et al., 2008).

Internal handling of cadmium in fish body

Upon uptake, the metals then be delivered to internal organs through blood. In the blood plasma, various proteins participate in metal binding following transport to internal organs for utilization, storage, and excretion (Chowdhury, et al., 2003). After enter within the epithelial cells, diverse of low molecular weight thiols (such as Metallothionein (MTs), glutathione (GSH)), cysteine (Cys) and Ca^{2+} binding proteins (such as calbindin) sequester Cd. A number of Cd is then transported across the basolateral membrane into the circulatory system, either in the form of Ca^{2+} or in conjugated forms (e.g. MT–Cd, GSH–Cd–GSH, Cys–Cd–Cys). In the bloodstream, Cd is delivered in association with a various of free proteins in plasma, albumin, and transferrin, as well as by MTs, albeit in small proportion because of their low concentration in plasma relative to other tissues (McGeer, et al., 2012). In brown trout and human, most of Cd in the plasma is bound to albumin or an "albumin-like" protein (Kleinow, et al., 2008). Cadmium from the plasma is rapidly absorbed by internal organs of fish, liver, however, absorbs the most followed by the kidney. The absorption of Cd from plasma into various internal organs likely occurs via any of the same transporters/mechanisms as occur in the gills and GIT (McGeer, et al., 2012).

The intracellular detoxification of Cd is primarily mediated by glutathione GSH and MTs (Figure 3). MTs serve protection against the toxicity of metal through sequestration of free metal ions as well as by scavenging intracellular reactive oxygen species (ROS) (McGeer, et al., 2012). The Cd-MT complexes is mainly formed in the liver as detoxification organ (Klaassen, et al., 2009; Kovarova, et al., 2009). In mammals, the kidney responds to Cd by both reabsorbing circulatory Cd-MT complex that has been released from the liver and gut, and filtered into the renal tubules, as well as by synthesizing renal MT for Cd storage. It is likely that fish also respond in a similar way and that in the dietary Cd-exposed trout, a relatively greater amount of Cd-MT complex released from extrarenal tissues was reabsorbed in the kidney and more renal MT was synthesized (Chowdhury, et al., 2005).

Once in the liver, Cd binds to already exist MT by displacing zinc. Then, Cd induces the synthesis of MT and the newly synthesized MT is sequestering Cd ion from other binding sites, thus protecting liver cells from Cd toxicity (Nordberg and Nordberg, 2009). When liver reaches the threshold of Cd (Cd saturation), Cd-MT complexes are

then released into the blood stream and are delivered to kidney (Wangsongsak, et al., 2007; Klaassen, et al., 2009), where it is filtered through the glomerulus and taken up by adsorptive endocytosis. MT is catabolized in the lysosomes of the tubules and the free cadmium ions then induce the new synthesis of MT in the cell while free Cd may also interact with other binding sites and Cd toxicity may follow (Nordberg and Nordberg, 2009). Wangsongsak, et al. (2007) noted that MT in the liver seems to show time-dependent expression but MT in the kidney is likely to show dose-dependent expression. GSH, which represents the major non-protein thiols of cells, is able to modify the toxicity of Cd by changing the metal uptake and elimination rates and by chelation of metal ions as soon as they enter the cell. GSH also acts as an antioxidant by scavenging intracellular ROS (McGeer, et al., 2012). A highly acute dose of waterborne Cd can produce a depression in GSH (Health, 1999). In mammals, some proof is supporting excretion of GSH-Cd complexes into the bile and presentation of Cd to renal tubules (Martelli, et al., 2006).

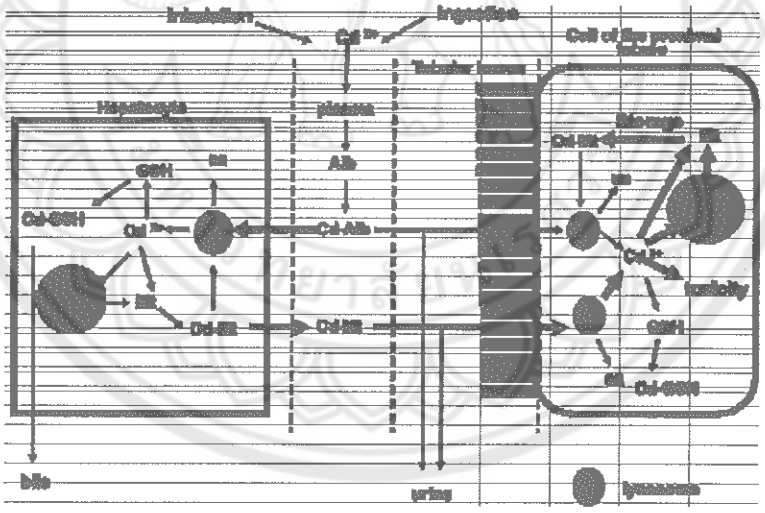

Figure 3 Scheme illustrating the mechanism responsible for the selective accumulation of cadmium in proximal tubular cells

Note: Alb, albumin; Mt, metallothionein; GSH, glutathione; aa, amino acid.

Source: Bernard, 2008

The heavy metals eliminations in the body depend on the biological half-time of the metals and difference between various species of water animals. Biological half time of liver and kidney cadmium was estimated to be more than one year (Kargin and Cogun, 1999). Fish have routes for possible excretion of harmful chemicals which is different from mammals; these include the bile (via feces), kidney (urine), gills and skin. However, gills and skin may tend to take it up rather than excrete it depending on the concentration of metal in the water (Health, 1995). The absorption of Cd through dietary exposure and a large proportion of ingested Cd is excreted from the body via feces by mucosal sloughing (McGeer, et al., 2012). Chowdhury, et al. (2004) demonstrated that only a small fraction of the infused dose (non-acclimated: 2.4%; Cd-acclimated: 6.6%) was internalized across the gut wall, while most was bound in the gut tissues (10–24%) or remained in the lumen (16–33%) or lost from the fish (~50%) over 24 h. A little amount might be excreted through the bile and gills; In mammals many metals are excreted via the bile which is formed by the liver. The bile does not necessarily rid the body of the metal in question. The bile flows from the gallbladder through the bile duct into the anterior end of intestine where it then mixes with the foodstuffs that are being digested (Health, 1995). Following the Cd-MT cleavage by lysosomal action, Cd^{2+} might be re-excreted into the tubular fluid and then the Cd is eliminated in the urine. The ability of Cd to induce hepatic and renal lesions exacerbates its toxic effects, and compounds its propensity to accumulate over years (Annabi, et al., 2013). Acclimation to dietary Cd exposure dramatically increases the urinary excretion rate of Cd (McGeer, et al., 2012). Loss of Cd via the skin and gills probably involve the mucus. This proteinaceous material is constantly secreted and sloughed off by these organs (Health, 1995).

Bioconcentration and bioaccumulation of cadmium in fish

Cadmium are able to accumulate and concentrate in aquatic living things. The increased levels of Cd in gills and intestine probably reflected the source of uptake of metals from water and food, respectively, since gills primarily accumulate higher level of metals via waterborne exposure, while intestine accumulate metals through dietary exposure (Chowdhury, et al., 2005). In laboratories under control conditions accumulated levels of Cd among organs varied following the different treatment of Cd, amount of dose and exposure time. In natural environment, describing the reasons that

influence bioaccumulation of Cd may be complicated because the complex and unidentified time related changes (Perera, et al., 2015). Cd in fish body rarely accumulated evenly but is accumulated by particular target organs (Wangsongsak, et al., 2007), maximally in the kidney, gills, liver, and GIT, to a lesser extent in the blood, but not significantly in the brain or muscle, although the pattern of accumulation differs depending on the exposure route (Szebedinszky, et al., 2001; Chowdhury, et al., 2005). During waterborne exposure, the accumulation of Cd occurs in the order kidney> gills>liver>intestine, whereas in dietary exposure the tissue Cd accumulation follows the order intestine>kidney>live>gill (McGeer, et al., 2012). Regardless of the route, once absorbed, cadmium is rapidly cleared from the blood and concentrates in several tissues. Hepatic and renal cadmium usually make up the bulk of the total body burden (Waalkes, 2003).

Many studies have been carried out regarding the tissue-specific Cd accumulation (Reviewed in Perera, et al., 2015). The greatest Cd concentrations were observed in the kidney followed by the gills and liver of the fish exposed to Cd via water, but in the gut tissues followed by the kidney, liver, and gills for dietary-exposed fish (Chowdhury, et al., 2005). Wu, et al. (2006) found that after the first day exposure of *A. paradoxus* and tilapia to Cd, gills contain the highest accumulation of Cd then decreased after 3 days of exposure. They suggest that gills are able to exchange the Cd very quickly in order to maintain their normal physiological function. In line with Arini, et al. (2015), they suggested that the fast Cd depuration in the gills likely resulted from a metal transfer to the liver.

The are several factors which influence the bioaccumulation of heavy metal in hish such as feeding behaviour, ambient temperature, water hardness, pH, salinity, age, sex and metal interactions etc (Yousafzai, et al., 2010). Various species of fish from the same water body may accumulate different amounts of metals. Interspecies differences in metal accumulation may be related to living and feeding habits (Jezierska and Witeska, 2006).

Toxicity of Cadmium in fish

The toxicity of Cd to aquatic species is generally dependent on concentrations of its bioavailable forms (species), as defined by the total dissolved concentration in combination with the underlying water chemistry. Among others geochemical

speciation of Cd, it is particularly the concentration of free Cd^{2+} ions that is generally associated with toxicity. Therefore, as with many other transition metals, complexation reactions that reduce the concentration of Cd^{2+} tend to reduce uptake and decrease toxicity (McGeer, et al., 2012). It is very rare that only one toxic element, at a time, is released into the aquatic ecosystem. Most of the heavy metals interact with each other and also influenced by other ions (e.g. Ca^{2+}, Mg^{2+}, Na^{2+}, Mn^{2+}, Fe^{2+}, Pb^{2+}, S^{2+}, Se^{2+} and Ni^{2+}) (Kumar and Singhs, 2010). Gill concentration of cadmium are used to monitor acute exposure to fish whereas, hepatic, renal and gastrointestinal Cd levels are useful in evaluating chronic exposures. Renal and hepatic tissues are critical in detoxification, as evidence by marked cadmium accumulation in these organs (Health, 1995). In general, Cd is toxicant with extremely long biological half-life that can cause hepatic and renal injuries in mammals and fish (Liu, et al., 2015).

High concentrations of Cd in a body of water can be very hazardous to the organisms in that ecosystem. When Cd inside the cell, it will induce various types of signal transduction mechanisms and activate many genes (Rumahlatu, et al., 2012). Cadmium, as a non-essential element, can disrupt cellular homeostasis, resulting adverse effect such as DNA damage, membrane depolarization and cytoplasm acidification. Moreover, it may stimulate production of ROS, leading to alterations in antioxidant enzyme systems and oxidative stress in affected organisms (Qu, et al., 2014). Chowdhury, et al. (2004) reported increased levels of plasma cortisol, glucose, lactate, and total ammonia, all classical stress parameters, in rainbow trout exposed to waterborne Cd.

Even though cadmium is only weakly mutagenic (Rousselet, et al., 2016), it is carcinogenic and teratogenic in several animal species (Rumahlatu, et al., 2012). Liver and kidney plays an important role in Cd metabolism, but the liver is the most critical organ for detoxification in acute exposure, whereas the kidney is found to be the storage site in chronic exposure (Cirillo, et al., 2012). Cd is a highly toxic metal, in particular, it was reported to produce cardiotoxicity, hepatotoxicity, as well as neuropathological and neurochemical alterations in the central nervous system resulting in irritability and hyperactivity (Wang, et al., 2004).

1. Acute toxicity

Free ionic form (Cd^{2+}) acute toxicity involves disturbance of ion homeostasis, particularly Ca, but also Na and Mg. Disturbance of Ca balance has been showed in various fish studies. Homeostasis of Ca in freshwater fish relies on gill uptake processes involving basolateral Ca-ATPase and an Na/Ca exchanger and the apical epithelial Ca channel (ECaC) (McGeer et al., 2012). Cd is a Ca^{2+} antagonist and is proved to cause toxicity in fish majorly by disrupting Ca^{2+} homeostasis during acute exposures (Driessnack, et al., 2016). Acclimation of fish to Ca are able to reduces Cd uptake from water and transfer of Cd from gill to blood. Cd is absorbed through the gill through high affinity calcium channel and thus it is readily encountered by Ca^{2+}. The Ca^{2+}ions can effectively compete with Cd^{2+} and prevent the later from entering through gill (Dutta and Kavirajk, 2001). Banaee, et al. (2015) demonstrated during the acute toxicity tests, fish exposed to cadmium often showed abnormal swimming and lethargy. Mucus secretion increased, the fish swam in a vertical posture and became motionless and some died when the toxic effects became dominant. These behavioral dysfunctions may be attributed to the decreased acetylcholinesterase (AChE) activity and simultaneously increased brain lipid peroxidation. Acute mortality to Cd typically occurs as a result of ionregulatory dysfunction. After the initial shock phase of metal exposure, the fish physiologically adapts to compensate for ion losses by secreting mucus and altering gill structure at the cellular and subcellular level (Hansen, et al., 2002).

2. Chronic toxicity

Chronic toxicity values for fish in freshwater range from approximately 0.5 to 160 μg Cd/ L (McGeet., et al., 2012; USEPA, 2001) and much less is known on the mechanisms of chronic impact of Cd compared to acute toxicity (McGeet, et al., 2012).

2.1 Ionregulatory effect

Chronic sublethal Cd exposure is unequivocally linked to ionoregulatory disturbance (McGeer, et al., 2012). Larsson, et al. (1981) found disturbed ion balance in flounder, *Platichtys flesus* L., exposed to sublethal levels (5–500 μg Cd/l) of cadmium, potassium and calcium ion concentrations decreased in blood plasma, whereas inorganic phosphate and magnesium ion increased.

2.2 Oxidative damage

Cadmium accumulation is known to result in the production of ROS and this is presumed to be one of the key mechanisms of toxic action. ROS include hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), singlet oxygen, hydroperoxyl radical (HO_2•), superoxide anion radical (O2•-) and other forms of oxygen-derived species (McGeer, et al., 2012).). Cd is a redox-stable metal, so ROS production after acute Cd intoxication must be mediated by indirect mechanisms (Liu, et al., 2009). Wang, et al. (2004) suggested that Cd appears to bind to a site on complex III (between semiubiquinone and cytochrome b_{566} of the Q_0 site of cytochrome *b*) of the electron transfer chain (ETC), resulting in an accumulation of semiubiquinones. The semiubiquinones, being unstable, are prone to transfer one electron to molecular oxygen to form superoxide, providing a possible mechanism for Cd-induced generation of ROS in mitochondria;

Lipid peroxidation is a major consequence of Cd-induced oxidative stress (Liu, et al., 2009). Due to high affinity of Cd for thiols, GSH and the sulphydryl rich MTs are the primary targets for free Cd ions. Cd-induced depletion of the decreased GSH pool can enhance oxidative stress (Gobe and Crane, 2010). Alterations in ROS-related gene expression during chronic exposures are less significant compared to acute Cd poisoning. This is probably due to induced adaptation mechanisms (e.g., metallothionein and glutathione) following chronic Cd exposures, which in turn diminish Cd-induced oxidative stress (Liu, et al., 2009). SOD plays an important role in protecting the cell against the potentially toxic effects of environmental pollutants. Liu, et al. (2015) noted that hepatic SOD activity decreased with the increasing dietary Cd levels.

2.3 Growth and survival

Szczerbik, et al. (2006) reported a significant decrease of appetite and growth rate *Rattus norvegicusas* well as the decrease of the locomotion activity in tilapia after exposure to dietary Cd. Lizardo-Daudt and Kennedy (2008) found Cd-induced hatching and developmental abnormities in rainbow trouts (*Oncorhynchus Mykiss*) exposed for 28 days. Chow and Cheng (2003) noted that zebrafish embryos exposed to Cd during the gastrulation period had defects in somite structure, which probably

contributed to altered axial curvature, and also abnormal notochordal morphology, with the notochord failing to extend into the tail region.

2.4 Reproduction

In General, the reproductive performance, particularly fecundity, has been suggested to be the most sensitive endpoint (more sensitive than any effects on early life stages) in fish during chronic exposure to toxicants (Wang, et al., 2014). The mechanisms underlying the reproductive toxicity of Cd remains to be fully understood, the reproductive effects are likely to be linked to impaired gametogenesis and/or steriodogenesis in fish. Cd has been found to impair gametogenesis in both male (decreased spermatids and spermatozoa) and female (decreased mature oocytes) fish during chronic waterborne exposure (Driessnack, et al., 2016). Lizardo-Daudt and Kennedy (2008) exposed rainbow trout eggs to Cd and followed responses from shortly after fertilization through to juvenile stages. Premature hatching at concentrations of 0.05 and 0.25 mg Cd/L and delayed hatching at 2.5 mg Cd/L. Growth of juveniles was slightly reduced at the highest concentration of 2.5 mg Cd/L.

2.5 Histopathology

Chronic sublethal exposure to Cd clearly degrades tissue ultrastructure in gills, liver, and kidney. Wangsongsak, et al. (2007) described that gills of silver barb which were exposed to 0.006 mg/L Cd for 60 days showed thickening of the primary lamellar epithelium and clubbing of secondary lamellae. Similar result with Annabi, et al. (2011), they investigated the histopathology of exposure of *Gambusia affinis* to Cd and found that greater changes in gills, kidney and liver tissues after chronic exposure than those recorded during acute Cd exposure the changes in gills were characterized by epithelial lifting, total and partial lamellar fusion, epithelial necrosis as well as telangiectasis.

2.6 Behavioral effects

Blechinger, et al. (2007) conducted experiment where they exposed juvenile zebrafish to cadmium during early larval development and found that zebrafish display deficits in olfactory-dependent predator avoidance behaviors 4-6 weeks after a return to clean water.

Gills

Gross anatomy of gills

In all fishes, the gills are bilaterally situated on either side of the pharynx and are composed of a series of pouch-like or arch-like structures that provide the physical support for the delicate gill filaments also termed primary lamellae. The gill filament is considered the basic functional unit or subdivision of the gill. Its epithelium contains ionoregulatory cells while it also supports the lamellae, which are the basic respiratory unit (Wilson and Laurent, 2002).

Histology of gills

Gills consist of two sets of four branches, with numerous rows of filaments; each filament has two rows of lamellae, which represent the respiratory surface. The afferent arteries to the gills open into blood sinuses in the lamellae-flat spaces lined by cytoplasmic extensions of the pillar cells. The spaces are drained by efferent arterioles, which anastomose with a central venous sinus in the filament, drained by venolymphatic vessels. Whereas the lamellae are covered by a uniform one- to two-layer flat epithelium of respiratory cells, the filament epithelium is multilayered and consists mainly of filament cells, with an apical layer of flattened pavement cells with mucus cells and chloride cells. (Bonga and Lock, 2008). The gill arch is a curved osseous, or bony, structure from which radiate double rows of paired primary lamellae or filaments. Each of these primary lamellae has a series of secondary lamellae located perpendicular to the primary lamellae. The gill arch is covered by typical teleost epidermal tissue but at the origin of the primary lamellae the epidermis is much thicker and usually contains numerous mucous cells. Below this epidermis there is usually an array of lymphoid tissue (Mumford, et al., 2007). The gill epithelium is composed of several distinct cell types but primarily consists of pavement cells (PVCs) and mitochondrion-rich cells (MRCs), which comprise >90% and <10% of the epithelial surface area, respectively (Evans, et al., 2005). Secondary gill lamellae were composed cells, which were contractile and separated the capillary channels. One to two erythrocytes were usually observed within each capillary lumen. Chloride cells were identified as large epithelial cells with light cytoplasm, usually present at the base of lamellae. Mucous cells were present in the epithelium of the filament at the base of lamellae, but they lacked the light cytoplasm and were smaller than chloride cells (Hadi and Alwan, 2012).

Kidney

Gross anatomy of kidney

The kidney of fish is usually located in a retroperitoneal position up against the ventral aspect of the vertebral column. It is a light or dark brown or black organ normally extending the length of the body cavity. It is usually divided into anterior or head kidney, which is largely composed of hematopoietic elements, and posterior or excretory kidney. The ureters, which conduct urine from the collecting ducts to the urinary papilla, may fuse at any level and may be dilated, after fusion, to form a bladder. The urinary ducts open to the outside posterior to the anus (Mumford, et al., 2007).

Histology of kidney

The kidney of the teleost is a mixed organ comprising hematopoietic, reticuloendothelial, endocrine and excretory elements (Mumford, et al., 2007). It consists of anterior kidney (also known as head kidney) and posterior kidney (also known as body or trunk kidney) (Silva and Martinez, 2007). The kidney was composed of numerous renal corpuscles with well-developed glomeruli and a system of tubules. The proximal segment was covered by tall columnar epithelial cells with basal nuclei and brush border located along the cell apices. The distal segment was lined with large, relatively clear columnar epithelial cells with central nuclei and the brush border was reduced or absent. The collecting duct or glomerulus was larger in diameter than the distal segment, containing columnar epithelial cells with basal nuclei and no brush border (Hadi and Alwan, 2012). In freshwater, the kidney must conserve salt and eliminate excess water. This is accomplished by a high glomerular filtration rate, reabsorption of salts in the proximal tubules, and dilution of urine in the distal convoluted tubule (Mumford, et al., 2007).

Liver

According to Hinton and Lauren (1990), fish liver comprises two compartments: parenchyma and stroma. Parenchyma consists of the epithelial cells that perform the organ's major function whereas stroma consist of blood vessel and connective tissue, it also includes the various cells located within the liver as well as the respective extra-cellular space.

Gross anatomy of liver

The fish liver is a relatively large organ. In wild fish, carnivores usually possess reddish brown in color and lighter brown in herbivores, but at particular times of year it may be yellow or even off white. In farmed fish, it can be lighter in color than in an equivalent wild specimen but this is diet dependent. The liver may be a localized organ in the anterior abdomen or may, in some species, have processes which extend the length of the abdomen or are closely applied to the other viscera (Mumford, et al., 2007).

Histology of liver

The histology of fish liver is different from that in mammalian (Mumford, et al., 2007). Fish liver histology is characterized by the absence of liver lobules and portal triads which are the basic morphological unit of liver structure in mammals (Rašković, et al., 2011). The fish liver features the same general circulatory components as the mammalian liver; that is, blood is supplied by hepatic arterioles and portal veins, and is drained by hepatic veins. The biliary apparatus of fish is also comparable to that of mammals (Wolf and Wolfe, 2005). Arterial blood reaches the liver by way of the hepatic artery, a branch of the celiacomesenteric artery from the dorsal aorta. An afferent venous supply, the hepatic portal vein, is the major source of blood to the liver. Whether the source is arterial or venous, blood from both sources enters the capillary bed, hepatic sinusoids, or microvasculature of the liver (Hinton, et al., 2008).

Metallothionein (MT)

General characteristic

The first publication Metallothioneins (MTs) was in 1957 on a cadmium-binding protein in horse tissue. The first detailed report on metallothionein was published in 1960. The cadmium-containing protein, isolated from kidney of horse, was described and named "metallothionein" because of its extremely high content of sulfur of 4.1%/g dw and 2.9% of Cd and 0.6% of Zn (Nordberg and Nordberg, 2009). MTs are abundant throughout the whole animal kingdom (Kovarova, et al., 2012).

Metallothioneins (MTs) belong to superfamily of low-molecular-weight cysteine- (characteristically with 6 to 7 KDa) and metal-rich polypeptides conserved through evolution and does exist in all eukaryotes and certain prokaryotes (Vašák and Meloni, 2008). MTs are soluble, cytosolic proteins and due to their high cysteine

content, they are able to selectively bind metals such as Cd, Zn and Cu (Knapen, et al., 2007). The typical MT protein contain 20 cysteines (30%), methionine (N-terminal), alanine (C-terminal), comprise 60–68 residues, no histidine, no aromatics, it has a unique amino acid sequence with a tertiary structure forming two domains of metal clusters, which are the α- and β- clusters, (Nordberg and Nordberg, 2009), and have a characteristic pattern of cysteine residues in Cys–X–Cys or Cys–X–X–Cys motifs (Capasso, et al., 2003).

The high content of cysteine residues, which consist of 11 residues in the C-terminal α domain and 9 residues in the N-terminal β domain, are responsible for a high capacity of MTs to coordinate covalent binding of divalent metal cations in metal/thiolate clusters. For instance, MT molecules can bind up to a total of seven zinc or cadmium ions, or up to 12 copper ions, in aggregate between the two domains (Koropatnick, 2010).

MTs are able to bind various metals, however, in normal condition or in the absence of unusually high levels exposure to other metal ions in the environment or diet, they are bound primarily to zinc and secondarily to copper (Koropatnick, 2010). Under experimental conditions, exposure of fish to heavy metals, such as zinc (Zn), copper (Cu), and cadmium (Cd) causes induction of MT (Linde, et al., 2001). The liver is one of the major sites of MT expression in fish, especially during reproduction and development (Hidalgo, et al., 2009).

The Role of MT

Metallothionein in the physiological system has not only one but several roles, especially in the metabolism and kinetics of metals, such as transport of metal ions, detoxification and protection from metal toxicity, scavenging of free radical, storage and metabolism of essential metal ions, immune response, and genotoxicity and carcinogenicity (Nordberg and Nordberg, 2009).

MTs display the unique ability to coordinate up to 7 divalent metal ions (e.g., Zn^{2+}) via 20 cysteine residues. These residues allow MT to bind, transport, and store variety of transition metals via thiolate bonding (Abumourad, et al., 2013). Seven zinc atoms, shared between a 3- and a 4-metal cluster, are bound to metallothionein monomers, yet cadmium can substitute zinc in both 3- and 4-metal clusters of these proteins. MT's functions remain elusive; they possibly distribute and deliver zinc to

other protein in a chaperon-like activity. They might also serve in protecting cells against excess zinc-induced toxicity (Martelli, et al., 2006). The binding of toxic metals to MT molecules shows a sequestration role that renders them unable to react with other proteins, such as enzymes, and thus generates protection against metal toxicity at the cellular level (Chowdhury, et al., 2005).

MT mRNA and or protein is dose and time dependent in fish upon being exposed to certain ranges of waterborne heavy metals, suggesting the possible role of MT in heavy metal detoxification (Wu, et al., 2006). For example, MT concentration in increased in Nile tilapia organs as Cd concentration increased (Abdel-Tawwab and Wafeek, 2014). Another important role of MT is the cellular defense mechanism against free radicals where methionine may act as free radical scavenger (Liu, et al., 2015). Due to the high number of cysteine content, MTs are able to sequester metal ions and scavenge free radicals (Oliva, et al., 2009).

As mammals, fish are able to synthesize MT in response to endogenous and environmental stimuli, and metals are strong inducers of MT expression in different fish cell lines (Vergani, 2009). Bourdineaud, et al. (2006) suggested that under low contamination pressure the physiological function of MT gene induction is to compensate for MT degradation but not to increase the metal sequestration capacities as it was generally thought. This is suitable with a function of MT gene induction related to zinc homeostasis rather than to metal detoxification.

MT-Cd complex in plasma is filtered through the renal glomeruli and reabsorbed in the tubuli, where cadmium is released. Upon a long-term exposure, the unbound cadmium induces new MT synthesis which binds cadmium and protects the renal tubular cells. When this process is insufficient, toxic effects may occur, probably due to cadmium interference with zinc-dependent enzymes and membrane functions (Balamurugan and Schaffner, 2009).

MT gene and isoform

Fish MTs have been less extensively studied than those of other taxonomic groups. Only recently, studies have been carried out on cDNA or genomic DNA genes from many bony fish species including carp, goldfish, rainbow trout, and plaice. By contrast, few reports on MT genes in cartilaginous fish are available, and, only recently, a MT gene from the tiger shark has been cloned. In mammals, a characteristic of the MT

promoters is the presence of multiple metal-responsive elements (MREs) mediating the response to metal ions. MREs have been found in the MT promoters of several teleosts, including rainbow trout, pike, common carp, zebrafish, icefish, and tilapia (Vergani, 2009). MTF-1 requires elevated zinc concentrations for optimal DNA binding (Balamurungan and Schaffner, 2009).

Induction of MTs in fish is mediated by multiple copies of metal-responsive elements (MREs) present in the 5'-regulatory regions of the MT. Metal Transcription Factors (MTFs) are zinc finger proteins that are activated (either directly or indirectly) by heavy metals such as Zn^{2+} and Cd^{2+}, and they regulate transcription through binding to MREs in the 5'-regulatory region of genes. (McGeer, et al., 2012). MT expression is also regulated by oxidative stress by antioxidant responsive elements (AREs) or by MREs which are also responsive to oxidants (Nordberg and Nordberg, 2009).

In fish, the two MT isoforms are differentially regulated by heavy metals. In rainbow trout, MT-B is expressed constitutively, whereas MT-A preferentially accumulates in response to cadmium exposure. In the icefish, a similar behavior is observed for MT-2 and MT-1, respectively (Vergani, 2009). In addition, Kwong, et al. (2011) found that MT-A was more sensitive to Cd in the intestine and liver, meanwhile MT-B was more sensitive in the kidney.

Regulation of MT protein degradation

Degradation of MT proteins occurs through several proteolytic pathways. A sub-maximal number of bound metal atoms stimulates the degradation of MT and concurrent titrations indicate that most of the zinc is released from MT whereas, at lysosomal pH, most of the cadmium is not. This could be the reason why Cd-MT has a higher half-life in vivo than Zn-MT (Martelli, et al., 2007).

Zinc Mine in Mae Sot District, Tak Province, Thailand.

In Thailand, the biggest reported zinc deposit is located in Mae Sot district, Tak province. The mine production capacity up to 214,023 metric tons or costs around 5,550 million baht (Unhalekhaka and Kositanont, 2008). After Mae Sot district in Tak Province being classified as the richest source of zinc mineral in Thailand by Department of Mineral Resources, Ministry of Industry, in 1977, zinc mining activities of 3 companies were started after this area (Padungtod, et al., 2007).

Drilling, material transfer, and removal of mine tailings and drainage are the example of mining activities which may influence the Cd contamination throughout the environment (Netpae, et al., 2015). The Mae Tao creek is known as the most worrisome site of Cd contamination in Thailand (Netpae, et al., 2015). Mae Tao stream passes through a zinc rich area where the zinc mining has been actively operated for more than 30 years (Weeraprapan, et al., 2015). A 25 kilometers long stream, Mae Tao Creek, flows from the mountainous area in the east to the low land areas in the west, passes through zinc deposits and finally discharges into the Mae Moei River, supplying irrigation water for the over 3,000 hectares of agricultural land (Karoonmakphol and Chaiwiwatworakul, 2010).

The first mine, currently owned by Padaeng Industry (Figure 4), is the biggest Zn mine in Southeast Asia. The second mine is no longer active. The sites of these two mines are located uphill of the Mae Tao creek, along the river path. It has been then claimed that at least one of these industrial sites is responsible for the Cd contamination at Mae Sot (Khaokaew and Landrot, 2015).

Figure 4 Zinc mine operated by Padaeng Industry PLC in the Tak Province

Source: Katz, et al., 2013

Several studies have been carried out regarding the contamination of Cd around Zinc Mine area in Mae Sot District, Tak Province. For instance, Simmons, et al. (2005)

found that the Cd concentrations in these impacted paddy soils can be up to 284 mg/kg. At this rate, it is much higher than the Cd background concentrations in Thai soils, which is 0.15 mg/kg (Khaokaew and Landrot, 2015). Simmons, et al. (2005) also reported that the rice grain in Mae Sot district contain cadmium in range 0.05 to 7.7 mg/kg, it already exceeds permisible level of Cd in rice namely 0.2 mg/kg. Another study which was conducted by Krissanakriangkrai, et al. (2009).that swamp eel in mae tao river contain Cd which exceed the maximum level allowance on the fish (0.2 mg/kg ww).

Weeraprapan, et al. (2015) investigated Cd concentrations in the sediments of Mae Tao Stream in four different sites. The first two sites located at the upstream of the zinc mine have the lowest concentration of 0.84 and 2.47 mg/kg, respectively. Meanwhile, the last two sites which are located at the downstream of the zinc mine, had much higher Cd concentration than the first two sites, i.e 5.32 and 7.86, respectively. This level is higher than the Permissible Levels of Cd in sediment (>3.0 mg/kg). It is clear that the source of Cd is the zinc mine, as Mae Tao Stream accepts the water from the zinc mine.

Swamp Eel (*Monopterus albus*)

Monopterus albus (Zuiew)is also known as Asian eel or swamp eel is one of the common fish found mainly in several Asian countries such as India, Southern China to Malaysia and Indonesia (Ponsen, et al., 2009). The Asian swamp eel does not belong to the family of eel (Anguillidae) but is a bony fish that belongs to the Synbranchidae family. *M. albus* has an eel-shaped body, absence of scales, the pectoral and pelvic fins are also absent while the dorsal, caudal and anal fins are confluent and reduced to a skin fold (Hii, et al., 2007). Most (possibly all) mature as females, after which some change to males. In their native range, swamp eels are rarely observed incidentally because most are cryptically colored, they are mostly active at night (nocturnal), often bury themselves in soft sediments, occupy burrows, and/or hide in crevices (Shafland, et al., 2010). It can be found in muddy ponds, canals, medium to large rivers, rice fields and swamps. It is a categorized as predator that consumes animals such as crayfish, tadpoles, small fish and worms. They can eat some larger pray as well, by grabbing them with their mouths, and spinning until they are torn in half (Bricking, 2002).

According to Ponsen, et al. (2009) the classification of swamp eel are as follows:

Kingdom Animalia

Phylum Chrodata

Class Actinopterygii

Order Synbranchiformes

Family Synbranchidae

Genus Monopterus

Species *Monopterus albus* and binomial name *Monopterus albus* (Zuiew).

Most members of the Synbranchidae family are air breathers and are able to survive weeks or months out of water (if kept moist) and without food (Nico, et al., 2011). It is also characterized by reduced gills, and was originally categorized as an obligate air-breather, like its close relative *M. cuchia*, but because *M. albus* maintains blood O_2 concentrations during forced submersion in normoxic water, it was argued to be a facultative rather than obligate air-breather. Unlike most air-breathers, *M. albus* lacks a distinct air-breathing organ (ABO) and relies on extrabranchial gas exchange using a highly vascularised epithelium in the buccopharyngeal cavity as well as a vascularized esophagus and integument (Damsgaard, 2014).

Nowadays, the habitats of *M. albus* have been decreasing, however, *M. albus* consumption has increased. Aquaculture of *M. albus* is adversely affected by production related disorders and infectious diseases. In the rainy season, or summer, people would catch them for consumption. This fish is an important protein source for people in the northeastern part of Thailand (Ponsen, et al., 2009).

CHAPTER III

RESEARCH METHODOLOGY

Study Design

This study investigated the level of cadmium in sediment and in organs (gills, kidney and liver) as well as their histopathological changes, and also the metallothionein (MT) gene expression in the liver of swamp eel collected from Mae Sot District, Tak Province, Thailand. The fish and sediment samples were obtained from the upstream and downstream of zinc mine. The Cd level, histopathological changes, and MT gene expression were then compared between these two representative sites.

Materials and Instruments

1. Instruments

1.1 Atomic Absorption Spectrophotometer (Perkin Elmer)
1.2 Centrifuge
1.3 Dissection set
1.4 Electrophoresis set
1.5 Gel documentation
1.6 Light microscope (Olympus)
1.7 Microtome
1.8 Microplate reader
1.9 Oven
1.10 PCR T100 Thermo Cycler (Bio Rad)
1.11 Real Time PCR machine (ESCO)
1.12 Shaking incubator
1.13 Tissue embedding machine
1.14 Tissue processing machine
1.15 UV box
1.16 Water Bath

2. Materials

2.1 0.5X TBE buffer

2.2 100 bp DNA ladder

2.3 10% Neutral buffer formalin (NBF)

2.4 Agarose

2.5 Chloroform

2.6 *E. coli* JM 109

2.7 Eosin solution

2.8 Ethidium bromide (EtBr)

2.9 GeneJET Plasmid Miniprep Kit (Fermentas, USA)

2.10 Hi-Yield Gel/PCR DNA fragment extraction kit (RBC Real-Genomic)

2.11 Loading dye

2.12 Haematoxylin solution

2.13 Nitric Acid (65%)

2.14 Paraffin

2.15 pJET 1.2/blunt cloning vector (Thermo Scienctific, USA)

2.16 Perchloric acid (70%)

2.17 RNAlater (Thermo scienctific, USA)

2.18 SensiFAST SYBR (Bioline, USA)

2.19 Trizol (Ambion, USA))

2.20 Tetro cDNA synthesis kit (Bioline, USA)

2.21 Xylene

Methods

1. Study area

The study sites were four different paddy fields around Mae Tao Creek, Mae Sot District, Tak Province, Thailand. The zinc mines are located uphill of the Mae Tao Creek, along with the river path (Khaokaew and Landrot, 2015). The paddy fields have been receiving water irrigation from Mae Tao Creek, which passes through zinc mine area (Simmons, et al., 2005). Mae Tao Creek is regarded as the most worrying site for Cd contamination in Thailand (Netpae, et al., 2015). Site S1

(Table 1) was considered as an uncontaminated or less polluted site as it is located in upstream and far from the zinc mine and the river. Meanwhile sites S2, S3 and S4 were considered as Cd contaminated sites as they are located downstream from zinc mine. The study site is approximately 4–7 km downstream and is around 2–3 km upstream from the Zn mineralization zone (Figure 5).

2. Sample collection and fish tissue preparation

Thirty-two *Monopterus albus* specimens, were collected in July-August 2016 from the four study sites. The size of the individual samples ranges between 70 -110 grams were collected from each location and brought to the laboratory for analysis. The sediment of respective site was also collected. The fish samples were then anesthetized using clove oil and then carefully cut open using sharp scissors. The livers, kidneys, and gills were removed for metal analysis using Atomic Absorption Spectophotometer (AAS) Perkin Elmer and submerged into 10% neutral formalin buffer prior to histopathological analysis using H&E staining technique. Slices of the livers were also taken for metallothionein gene expression analysis. After removing the livers, they were submerged into RNAlater (Ambion, USA) immediately to stabilize the RNA and kept at -20 °C until used.

Figure 5 Map of the four study sites according to their respective GPS coordinates in Mae Sot District (★= study site)

Table 1 GPS coordinates and codes of four study sites in Mae Sot district

Location (sub district)	Code	Latitude and Longitude
Phratat Padaeng	S1	N 16°.40' 36. 55" E 098°.41'18. 54"
Phratat Padaeng	S2	N 16°.39' 59. 14" E 098°.36'56. 19"
Phratat Padaeng	S3	N 16°.39' 32. 04" E 098°.36'38. 31"
Mae Tao	S4	N 16°.40' 25. 29" E 098°.36'01. 37"

3. Measuring the level of Cadmium

The liver, kidney and gills tissues of 2 swamp eels from each site were pooled to make a subsample (replicate). Four replicates were taken from each site. Liver, kidney and gills organs were dried and put in an oven at 150° C until reaching a constant weight. Tissues were homogenized and ground to a powder, 0.5 grams of each dried tissue were weighed out, transferred into polyethylene tubes, 5 ml of freshly prepared nitric acid-perchloric acid (10:4) were added to the sample, and left overnight at room temperature for digestion. Final digestion was done by putting the digestion tubes in a water bath and set to boiling water temperature, 100°C and the contents boiled for about 2 hours until all the tissues were dissolved. The digests were allowed to cool, filtered through Whatman, transferred to 25 ml volumetric flasks and made up to mark with deionized water (FAO, 1983; Al-Weher, 2008). The digests were kept in plastic bottles prior to be analyzed by using an atomic absorption Atomic absorption spectrophotometer.

The sediment samples were analyzed according to the modified method of (de Astudillo, et al., 2005), sediment samples were collected from the same sites of collected swamp eels. Sediments were brought to laboratory and were oven-dried at 60°C to constant weight and subsequently ground with an agate pestle and mortar. The sediment sample were then sieved through 2 mm sieve. The samples were made into 3 replications (each of 0.5 g) per locations. Sediment samples were placed in volumetric flask and 10 ml of HNO_3 per sample was added for overnight pre-digestion followed by a 24 hr-digestion time at 130°C. Similar with the tissues preparation, the digests were allowed to cool, filtered through Whatman filter paper, transferred to 50 ml volumetric flasks and made up to mark with deionized water. The digests were kept in

plastic bottles prior to be analyzed by using an atomic absorption Atomic absorption spectrophotometer.

According to Al-Weher (2008), determination of any heavy metals including cadmium in the sample can be calculated by using the formula:

Real concentration of the metal in the sample (mg / kg) = ppmR x dilution factor

Where:

ppmR = reading solution by AAS (ppm)

Dilution factor

= volume of solution (mL)/weight of the dissolved sample (gram)

4. Hematoxylin 7 Eosin (H&E) staining

Fish gills, kidney and liver were fixed in 10% neutral buffered formalin, and the samples were then processed for routine wax histological evaluation (dehydrated and embedded in paraffin). Sections of 5 µm were prepared and stained with hematoxylin and eosin stains (see APPENDIX C part II-2).

5. Cloning and sequencing of metallothionein

5.1 Total RNA isolation

Liver slices (50–100 mg) were immediately submerged into RNA*later* reagent (Thermo Fisher Scientific) to stabilize RNA and kept at -20 °C until they were used. Tissues were homogenized in a microcentrifuge tube and the total RNA was extracted by using the TRIzol reagent (Ambion) according to manufacturer's protocol (see APPENDIX C part II-3.1). The RNA concentration was determined by the absorption at 260 and 280 nm using microplate reader.

5.2 Synthesis of the first-strand cDNA

Five microgram RNA was reverse transcribed with Tetro cDNA synthesis kit™ (Bioline) using dT-UPM primer (5'-AAGCAGTGGTATCAACGCAG AGTAACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3') according to the manufacturer's protocol (see APPENDIX C part II-3.2).

5.3 -3'-RACE amplification from cDNA

To obtain the MT sequence, 3'-rapid amplification of cDNA ends (3'-RACE) was performed. The cDNA synthesis was initiated from a dT-UPM primer containing a 3'adapter sequence. A Gene specific of MT swamp eel (forward primer to performed 3'RACE) was obtained from aligned sequence of 32 sequence of

metallothionein gene of fish in Genbank (NCBI). Those sequences are aligned by using Clustal Omega program (Figure 6). The first 22 bases were chosen as primer for forward primer. Choosing base for non-conserved region will follow the IUPAC code. The gene-specific primers were used in conjunction with provided adapter primers to amplify 5′ and 3′ cDNA ends. The gene specific primers of MT swamp eel (5'-ATGGAYCCYTGYGANTGCKCCAA-3') served as forward primer and UPM-1 served as reverse primer (5'-CTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT-3'). The amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, (EU) Lithuania) with reaction mixture consisted of 4 µl of 5X phusion buffer, 0.2 µl of Phusion DNA polymerase, 0.4 µl of 10 mM dNTPs, 2 µl of each 10 µM UPM-1 and gene specific primer, 2 µl of cDNA template and nuclease free water to adjust to 20 µl. The PCR condition were as follows: an initial denaturation for 2 min and 30 sec at 98°C, followed by 35 cycles of 15 sec at 98°C for denaturation, 15 sec at 60°C for annealing, 15 sec at 72°C for extension and 5 min at 72°C for final extension.

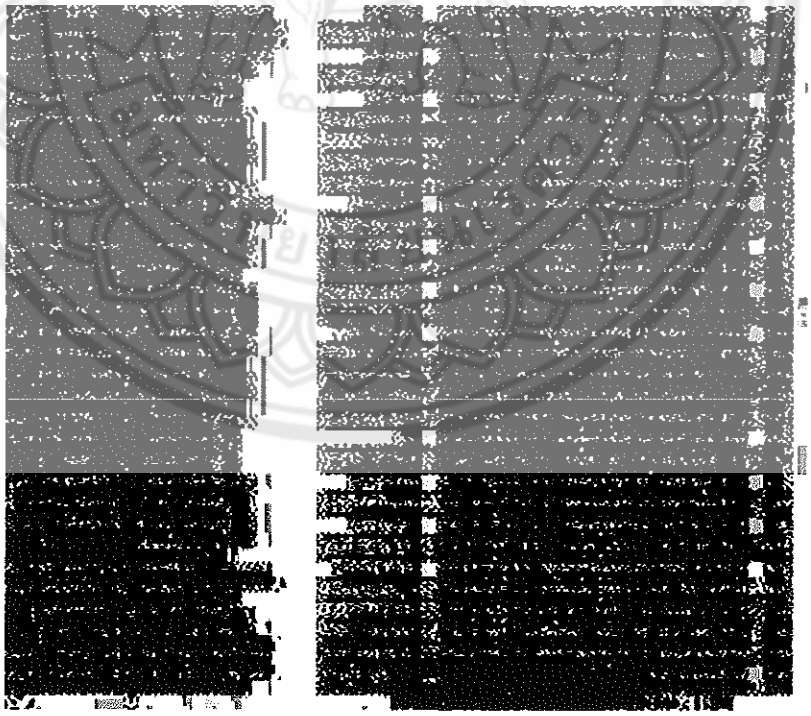

Figure 6 Multiple sequence alignment (Clustal Omega) of 32 sequence of metallothionein of teleost

5.4 Gel purification

PCR products were excised from a 1% agarose gel and purified with the RBC Real Genomics Hi Yield Gel/PCR DNA fragment extraction kit followed the manufacturer's protocol (see APPENDIX C part II-3.4).

5.5 Ligate DNA fragment to vector and transform plasmid to competent cell

Purified gel fragment then ligated into the pJET 1.2/blunt cloning vector (see APPENDIX A) according to the manufacturer's protocol (see APPENDIX C part II-3.5). It is a positive selection cloning vector. The vector contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. Following transformation into *E. coli* JM109 by using heat shock method (see APPENDIX C part II-3.6; 3.7).

5.6 Colony cracking and colony PCR

The screening and transformed colonies were conducted using both colony cracking and colony PCR technique (see APPENDIX C part II-3.8; 3.9). Cracking buffered was used in colony cracking technique to lyse the *E. coli* resulting the release of its DNA (both genomic and plasmid), which was then electrophoresed on agarose gel. The positive clones can be identified by observing the movement of band on agarose gel. The genomic DNA move very slowly whiles the plasmid DNA move faster. Plasmid DNA containing insert move slower than those without insert. In colony PCR technique, the blue solution of colony cracking mixture was used as template. UPM-1 as reverse primer and gene specific primer of MT swamp eels as reverse primer with PCR condition: an initial denaturation for 2 min and 30 sec at 98°C, followed by 35 cycles of 15 sec at 98°C for denaturation, 15 sec at 60°C for annealing, 15 sec at 72°C for extension and 5 min at 72°C for final extension

5.7 Plasmid extraction

E. coli containing the plasmid of interest were first cultured, then the samples were centrifuged in order to concentrate cellular material (including DNA) at the bottom of centrifuge tubes, the supernatants were then discarded. The plasmid extraction was performed using GeneJET Plasmid Miniprep Kit (Fermentas, USA) according to manufacturer's protocol (see APPENDIX C part II-3.10).

5.8 DNA sequencing and analysis

After measuring the quality and concentration, the chosen plasmid DNA were sent to Macrogen (Korea), to be sequenced by using universal primer T7 promoter as a primer. The sequences were then aligned using Clustal Omega program (EMBL-EBI, UK) to obtain the MT gene sequence of *M. albus*. Protein prediction was performed using ExPASy program (Swiss Institute of Bioinformatics, Switzerland) and compared to other reported sequences using BLAST (NCBI) to identify similarity.

6. Real time Polymerase Chain Reaction (Real-time PCR)

6.1 Total RNA isolation and cDNA synthesis

Total RNA was isolated from liver using TRIzol reagent (Ambion, USA) according to manufacturer's protocol. Absorbances at 260 and 280 nm were measured on a microplate reader to check the concentration and the quality of RNA. Two microgram of RNA was reverse transcribed with Tetro cDNA synthesis kit™ (Bioline, USA). The cDNA mixture was stored at -20°C until used in real-time PCR reaction.

6.2 Quantitation of MT by Real-time PCR

Amplification and quantitation of cDNA were performed in the Swift SpectrumTM 48 Real Time Thermal Cycler (ESCO Micro Pte. Ltd, Thailand). The primers of MT gene were designed from the *M. albus* MT sequence (Figure 14) based on the conserved region of other fish MT sequences. The primers of 18S rRNA gene were selected according to Hu, et al. (2014). The 18S rRNA gene of swamp eel was served as an internal control for normalization and sample from uncontaminated site (site S1) was served as calibrator. The relative gene expression data were calculated using comparative Ct method ($2^{-\Delta\Delta Ct}$ method) as described by Livak, & Schmittgen (2001). Amplification were carried out at final volume of 20 µl containing 10 µl 2x SensiFAST SYBR No-ROX kit (Bioline, USA), 0.3 µl of 10 µM each forward and reverse primer, and 8.4 µl of nuclease free water. The primers of MT and 18S rRNA sequence for Real-time PCR are listed in Table 2. The real time PCR condition were as follows: 95°C for 3 min, followed by 40 cycles of 15 sec at 95°C, 15 sec at 58°C, 15 sec at 72°C. Each sample was run in triplicate and mean values were reported.

Table 2 Primers sequence of *18 S* and *Metallothionein* gene for Real-Time PCR

Primer	Sequence
18 S	F 5'-GTGGAGCGATTTGTCTGGTTA-3'
	R 5'-CGGACATCTAAGGGCATCAC-3'
MT	F 5'-CTGCTCATGTTGTCCTCCCG-3'
	R 5'-GTCGCACTTCTTCCCTTTGC-3'

Statistical Analysis

All data were expressed as mean ± standard error (mean ± SE) value. One Way ANOVA was used for determining the significant difference in Cd and MT gene expression level followed by Duncan's Multiple Range Test for statistical comparison in each experiment. Spearman's Correlation Test was applied to study the correlation between Cd level in three organs and Cd level in sediment as well as correlation between hepatic Cd content and hepatic MT level. Significance is accepted for $P<0.05$. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER IV

RESULT

Cadmium Level in the Sediment and the Organs of Swamp Eel (*M. albus*)

The level of Cd in the sediment and organs of fish were analyzed by Perkin Elmer Atomic Absorption Spectrophometer (AAS) at wavelength (λ) 213.9 nm. Table 3 shows the level of Cd (mg/kg dry weight) in sediment from four different sites.

Table 3 Cadmium level in sediment (mg/kg dry weight) from four study sites

Sites	Cadmium level
S1	
Mean	ND
Min	ND
Max	ND
S2	
Mean	2.33 ± 0.64b
Min	1.06
Max	3.04
S3	
Mean	3.12 ± 1.63b
Min	1.43
Max	6.40
S4	
Mean	7.82 ± 0.86a
Min	6.32
Max	9.29

Note: Mean Cd level in the same row having a different letter are significantly different, $P<0.05$. ND: not detected.

The highest mean of Cd level in sediment was found in site S4 of 7.82 mg/kg dw, followed by site S3 and S2 of 3.12 and 2.33 mg/kg dw, respectively, whereas non-detectable level was found in site S1. Cd level in sediment differed significantly between site S4 and the other two sites. All sediment samples which were collected from three sites in the downstream area appeared to have much higher Cd level compared to the upstream site.

Table 4 Cadmium level in swamp eel organs (mg/kg dry weight) from four study sites

Tissue	Study site			
	S1	S2	S3	S4
Liver				
Mean	1.10 ± 0.10c	10.26 ± 3.04b	6.70 ±1.26bc	22.88 ± 2.60a
Min	0.86	3.01	3.48	17.70
Max	1.27	20.61	9.18	25.75
Kidney				
Mean	0.48 ± 0.18b	3.49 ± 0.59b	3.92 ± 0.71b	9.00 ± 2.84a
Min	ND	2.34	2.85	3.83
Max	0.86	5.63	6.00	16.96
Gills				
Mean	0.12 ± 0.12b	8.48 ± 1.71a	7.07 ±1.99a	4.43 ± 0.62ab
Min	ND	4.46	4.40	3.18
Max	0.48	13.06	12.87	5.83

Note: Mean Cd level of particular of tissue in the same row having a different letter are significantly different, $P<0.05$. ND: not detected.

The result of Cd level in liver, kidney and gills is summarized in Table 4. A significant difference of hepatic Cd level was found between site S4 and the other two sites i.e. site S1 and site S3. In kidney, Cd level was found to be significantly different between site S1 and the other three sites. On the other hand, site S2 and site S3 were

observed to be significantly different between site S1. Over all, site S1 (upstream site) appears to has lower Cd accumulation compared to the other three upstream sites (site S2, S3 and S4) in the three subject organs.

The trend of accumulation of Cd in tissue varied according to the locations. In site S1, the highest Cd of level was found in liver followed by kidney and gills respectively. The similar trend was found in site S4 where the liver had the highest Cd level followed by kidney and gills, respectively. In site S2, the highest Cd accumulation was detected in liver followed by gills and kidney, respectively. In site S3, gills were observed to have the highest Cd accumulation of Cd followed by liver and the lowest Cd level was found in kidney.

In order to see whether the accumulated Cd in the liver, kidney and gills were correlated with the Cd accumulation in sediment, Spearman Correlation Test was applied. The result showed that correlation between tissue cadmium content and sediment cadmium level was not statistically significant in any but kidney ($R=0.80$, $N=4$, $P>0.05$ for liver; $R=1.00$, $N=4$, $P<0.01$ for kidney; $R=0.2$, $N=4$, $P>0.05$ for gills). The correlation was positive meaning that the higher level of Cd in sediment, the higher content of Cd in the kidney.

Histopathological Alteration in the Organs of Swamp Eel (*M. albus*)

1. Histopathology of liver

Histopathological analysis of swamp eel liver showing injured liver in all site (Figure 7). Generally, all liver showed several lesions such as nuclear degeneration, cytoplasmic degeneration, intracellular bile stagnation, hepatocyte swelling (hydrophic change), dilation of sinusoid and congestion in sinusoid and main vessel. However, bile stagnation was found less in number in site S1 compared to the other three sites. In addition, presence of melano-macrophage centers (MMCs) were found in liver from site S2, S3, and S4.

2. Histopathology of kidney

The swamp eel kidney consists of hemopotoetic cells which is typically the characteristic of head kidney (anterior kidney). Numerous melanomacrophage centers (MMCs) were observed scattered through the organ in all study sites (contaminated and

uncontaminated). Furthermore, clustered of protein cast-like vesicle in different size were also observed dispersed the organ in all study sites (Figure 8).

3. Histopathology of gills

M. albus gills is known to have reduced gills. The histological structure also seemed to be different with the gills of common fish. Figure 9.A and 9.2 from site S1 showed the filament of gills without any extension of it. It is similar with gills from site S3 (9 C and 9 D) and S4 (9 E and 9 F). However, distinct difference was found in the gills from site 2 (9 B and 9 C). It somewhat showed the structure of filament and lamellae but in random pattern.

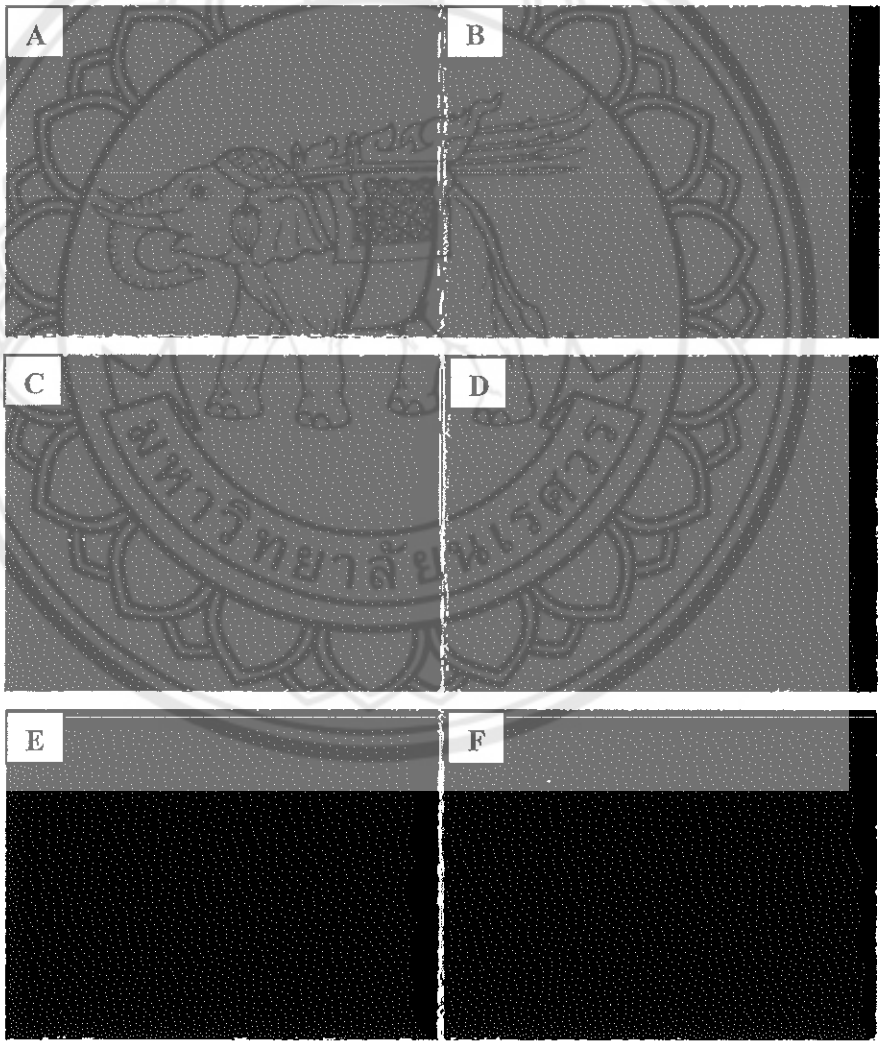

Figure 7 Photomicrograph of liver section of *M. albus* in four study sites

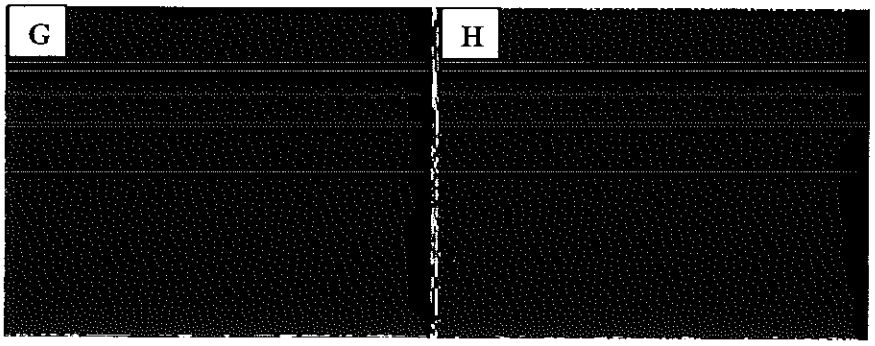

Figure 7 (Cont.)

Note: A, B (site S1); C, D (site S2); E, F (site S3); G, F (site S4). A) dilation and congestion of sinusoid (black arrow); B) nuclear degeneration (blue arrow), intracellular bile stagnation (black arrow), dilation and congestion of sinusoid (yellow arrow). C) presence of melanomacrophage center (MMC), close to blood vessel (blue arrow), congestion in sinusoid and main vessel (black arrow); D) nuclear degeneration (blue arrow), intracellular bile stagnation (black arrow), cytoplasmic degeneration (blue asterisk), and hepatocyte swelling (black asterisk); E) presence of MMCs (blue arrow), dilation and congestion of sinusoid (black arrow); F) nuclear degeneration (blue arrow), intracellular bile stagnation (black arrow), dilation of sinusoid (yellow arrow); G) presence of MMC (blue arrow), congestion in sinusoid (black arrow); H) nuclear degeneration (blue arrow), intracellular bile stagnation (black arrow), dilation and congestion of sinusoid (yellow arrow); CV, central vein, Bar = 100 μm (A, C, E G); 20 μm (B, D, F, H).

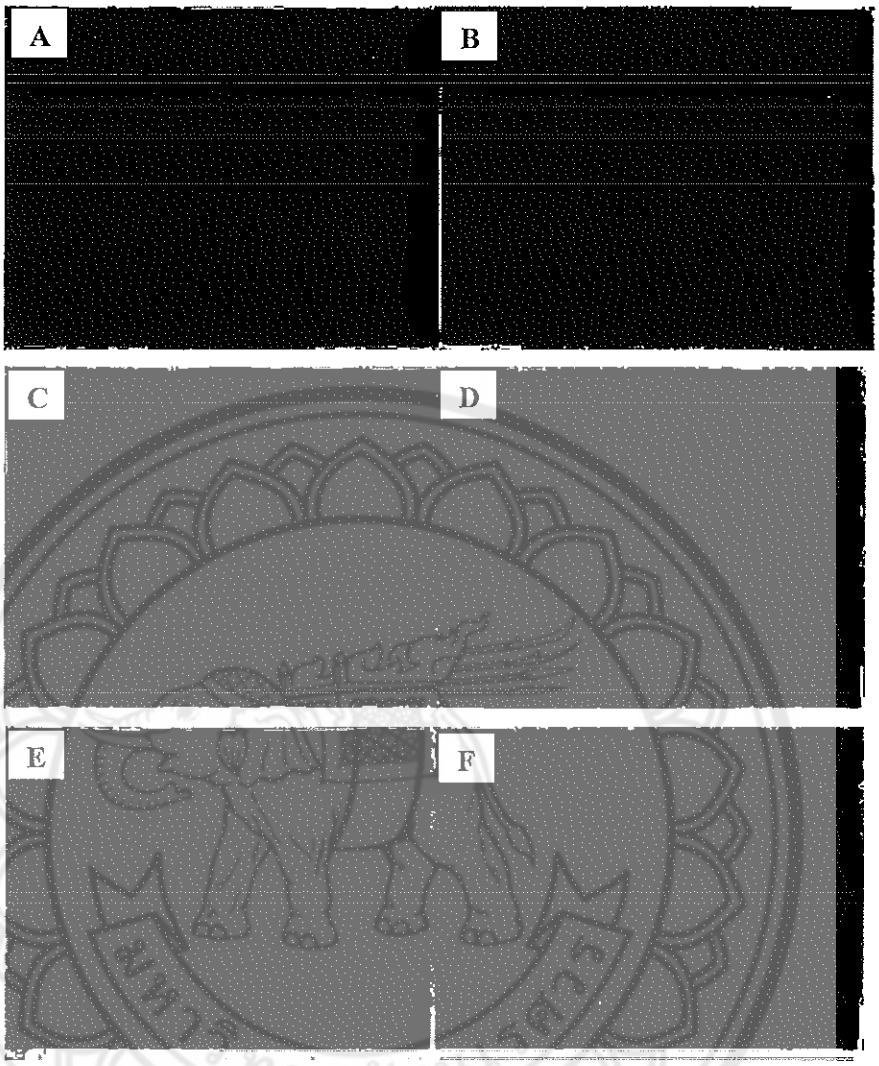

Figure 8 Photomicrograph of renal section of *M. albus* four study sites

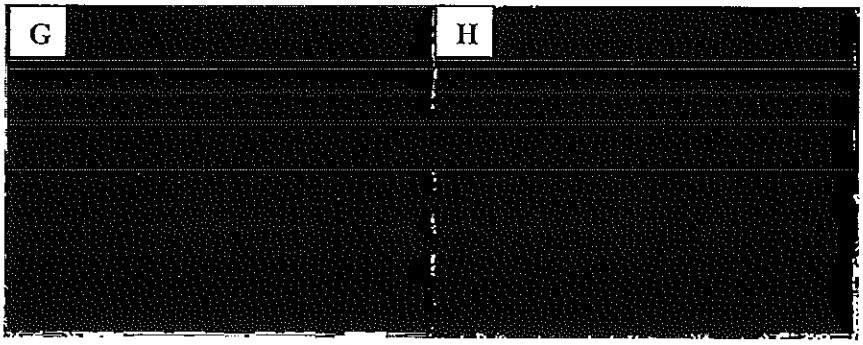

Figure 8 (Cont.)

Note: A, B (site S1); C, D (site S2); E, F (site S3); G,F (site S4). Presence of casts or cast like-protein (short arrow); melano machrophage center (long arrow); hematopoietic tissue (HT). Bar = 500 µm (A, C, E G); 200 µm (B, D, F, H).

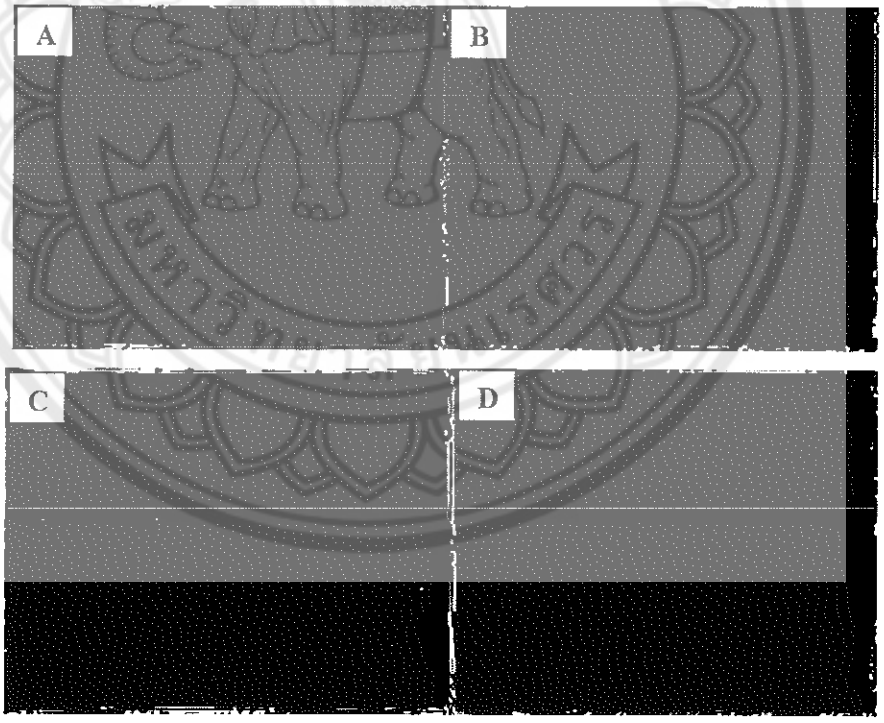

Figure 9 Photomicrograph of gills section of *M. albus* four study sites

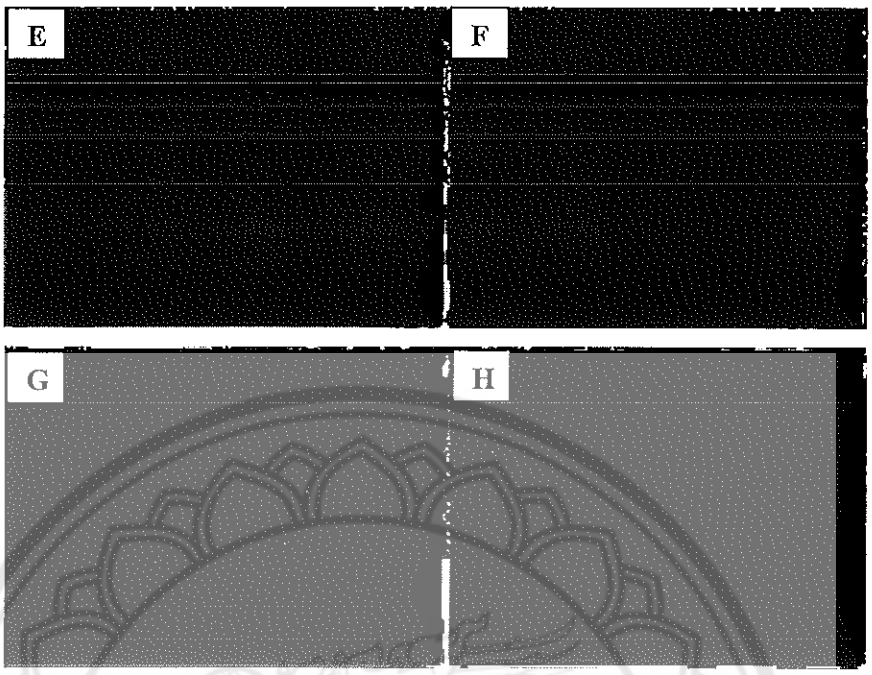

Figure 9 (Cont.)

Note: A, B (site S1); C, D (site S2); E, F (site S3); G,F (site S4). Gills filament/lamellae (black arrow); gill filament/primary lamellae (blue arrow); gills lamellae/secondary lamellae (yellow arrow).

Metallothionein* (*MT*) Gene Expression Level in the Liver of Swamp Eel *(M. albus)

1. Cloning and sequencing of *MT* gene

To obtain the MT sequence (not including 5'UTR), the 3'RACE was performed. The length of MT sequences according to aligned of 32 MT fish sequences were ranged from 300-500 bp (Figure 7). Figure 10 shows that a band in lane 2 was in the range of 300-400 (arrow) and it was chosen as predicted MT band. After the ligation and transformation, 10 transformed colonies were chosen for screening using colony cracking and colony PCR method, where the successfully transformed colonies are shown with arrow (Figure 11 and 12). Those colonies were then picked for plasmid extraction and were screened (Figure 13) to be sequenced.

The sequence of MT is shown in Figure 14. The MT mRNA sequence of 333 bp which consists of a complete coding sequence (cds) and 3' untranslated region

(3'UTR), was identified from swamp eel (*Monopterus albus*). The MT cDNA sequence obtained from *M. albus* consists of coding region in length of 180 bp encoding 60 amino acids with 20 cysteine residues. The cystenil residuses of *M. albus* MT protein were distributed in Cys-X-Cys and or Cys-X-Y-Cys pattern (X and Y are amino acid other than cysteine) which is the typical distribution of cystenil residues in this protein.

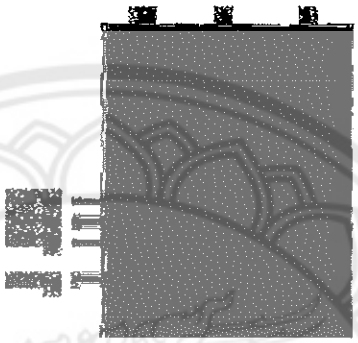

Figure 10 Amplification of 3'-RACE of *M. albus* MT gene using UPM-1 and MT gene specific primer

Note: The black arrows shows the predicted band of MT (300-400 bp). M, Marker; 1 and 2, PCR products.

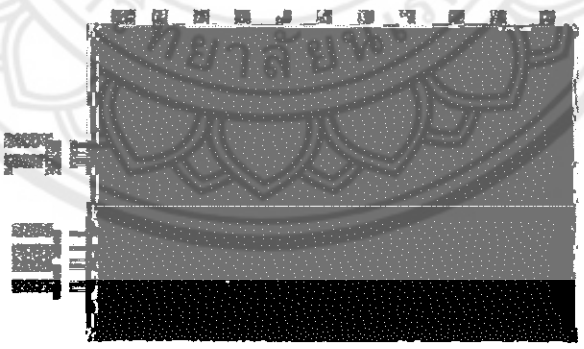

Figure 11 Electrophoresed gel from colony cracking method showing the predicted size of MT swamp eel

Note: The black arrows shows the predicted band of MT (300-400 bp). M, Marker; 1-10, transformed colonies.

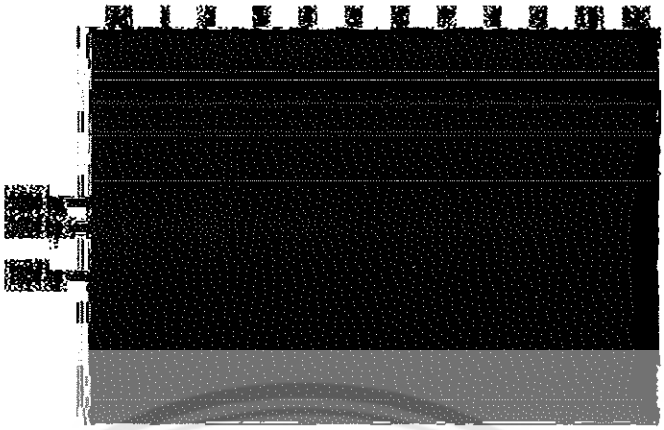

Figure 12 Electrophoresed gel from colony PCR method showing the size of pJET 1.2 combining with the size of predicted MT gene

Note: The black arrows shows the predicted band of MT (300-400 bp). M, Marker; 1-10, transformed colonies.

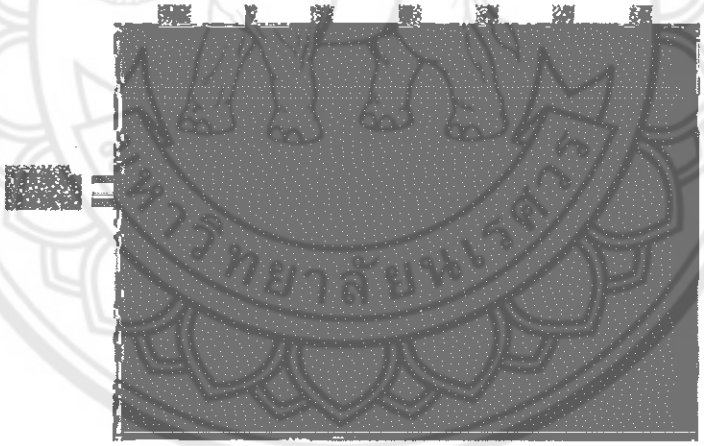

Figure 13 A typical plasmid preparation

Note: A typical plasmid preparation showing: first band is nicked DNA, second band is linear DNA, and third band is supercoiled DNA.

```
ATG GAC CCT TGC GAG TGC TCC AAA ACC GGA ACC TGC AAC TGC GGA GCA AAC TGC AGC TGC   60
  M   D   P   C   E   C   S   K   T   G   T   C   N   C   G   A   N   C   S   C

AAA GAC TGC TCC TGC ACA ACC TGC AAA AAG AGT TGC TGC TCA TGT TGT CCT TCC GGC TGC  120
  K   D   C   S   C   T   T   C   K   K   S   C   C   S   C   C   P   S   G   C

AGC AAG TGC GCC GCT GGG TGC GTG TGC AAA GGG AAG AAG TGC GAC ACC AGC TGC TGT CAG  180
  S   K   C   A   A   G   C   V   C   K   G   K   K   C   D   T   S   C   C   Q

TGA GGA GTC TGC ACC CTT CAC TCT TGT GAT GGA GCC TGT GCA AAC GAA ACA GGA TTG TAG  240
TGT AAA TGT CTA GAA GAC GTG TTT TCT ACC GTT TCA ATG TTG AAA TAA TAA AAA ATC CTT  300
AAT GTA AAA AAA AAA AAA AAA AAA AAA AAA AAA                                     333
```

Figure 14 Sequence of MT cDNA of *M. albus* including the coding sequence and the deduced amino acids

2. Expression level of MT gene

Specificity of the primers used in the Real Time PCR technique were validated via melting curve analysis (Figure 15) and gel electrophoresis (Figure 16), where single banded amplicons were observed.

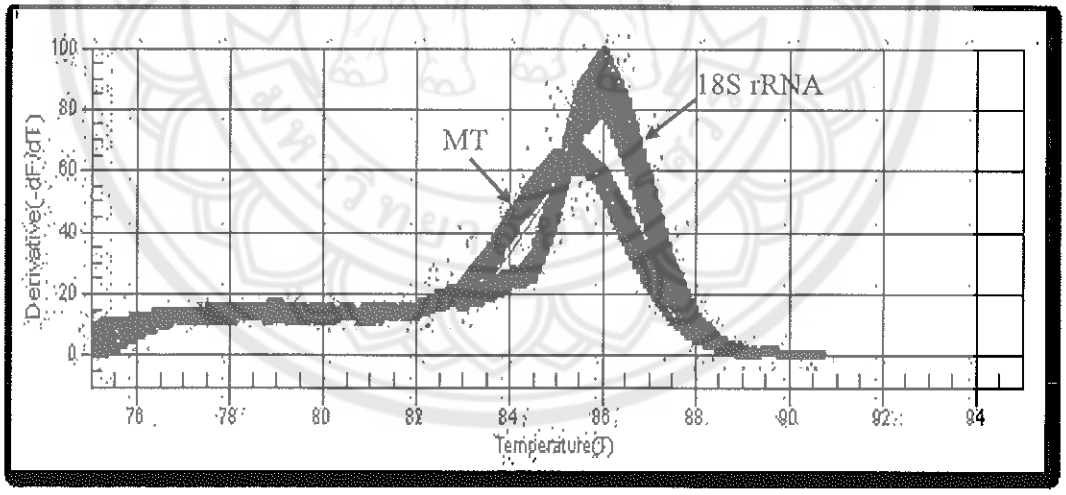

Figure 15 A typical relative quantitative analysis output (melting curve)

Note: Each peak reflects the specificity of the primer pairs to form single amplicon which is showed with black arrow.

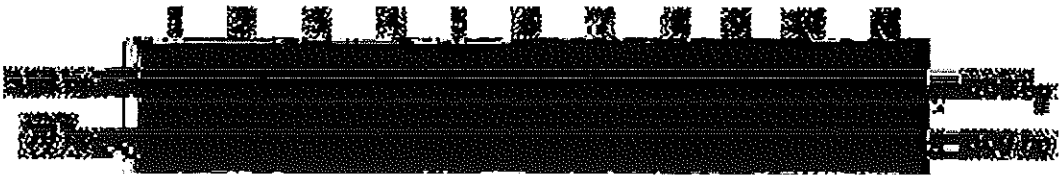

Figure 16 Expression profile of MT and 18 S rRNA

Note: Lane 1-5 were MT's (70 bp) amplicons whereas 6-10 were 18S's (162 bp).

Expression levels of MT in the liver of swamp eels collected from four study sites are shown in Figure 17. Statistically significant differences in MT gene expression level were found between site S3 and the other three sites (S1, S2, and S4). The highest level MT mRNA was found in site S3 while the lowest level was found in site S4.

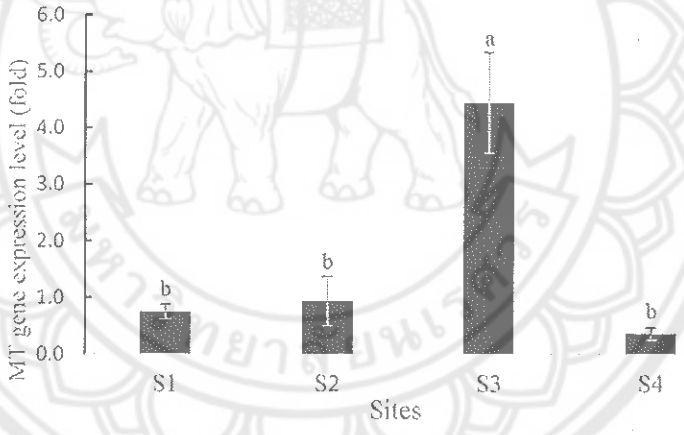

Figure 17 Quantitative expression of Metallothionein of fish liver which was normalized using 18S rRNA and site S1 (uncontaminated site) was used as the calibrator

Spearman's correlation coefficient showed moderate negative correlation and not significant between hepatic Cd level and MT gene expression (R=-0.400, N=4, $P>0.05$) and a low negative correlation between Cd level in sediment and MT gene expression (R=-0.200, N=4, $P>0.05$). The MT levels didn't reflect the trend of Cd level in liver and the MTs distribution also did not correlate with the Cd burden in the

sediment. For instance, the sediment samples collected from site S3 and site S2 showed no significant difference of Cd level ($P > 0.05$, 3.12 ± 1.63 and 2.33 ± 0.64 mg/kg dw, respectively). In addition, the hepatic Cd content from these two sites also showed no significant difference ($P > 0.05$, 6.70 ± 1.26 vs 10.26 ± 3.04 mg/kg dw, respectively), whereas the MT mRNA levels on these two sites were significantly different ($P < 0.05$, 4.44 ± 0.89 vs 0.94 ± 0.43 fold of MT mRNA). Conversely, it was found that no significant differences in MT expression ($P > 0.05$, 0.75 ± 0.13 vs 0.36 ± 0.11 fold) between site S1 and site S4 whereas the hepatic Cd levels were found to be the lowest and the highest, respectively and were significantly different (1.10 ± 0.10 vs 17.62 ± 5.57 mg/kg dw, respectively). Moreover, the Cd level in the sediment sample collected from site S1 and site S4 also showed the significance difference of Cd level ($P>0.05$, ND vs 7.82 ± 0.86 mg/kg dw, respectively).

CHAPTER V

DISCUSSION

The current study was carried out around Mae Tao Creek in four different locations. *M. albus* is one of the most common fish that can be found in the paddy fields, therefore it has been used as subject study for monitoring of heavy metal pollution in the paddy fields (Yin, et al., 2012). From the Cd analysis of sediment samples, the Cd was non-detectable in sediment from site S1 (upstream site). Meanwhile, the Cd level in sediment collected from site S2, S3 and S4 (downstream site) were, 2.236, 3.155 and 7.821 mg/kg dry weight, respectively. The Cd level in site S3 and site S4 were exceeded the permissible level of Cadmium in sediment of 3.0 mg/kg dry weight according to European Union (EU) and Food and Drug Administration (FDA) Guidance/Action/Tolerance level for fish tissue concentration (Gale, et al., 2003). In addition, the Cd level in sediment collected from these three contaminated areas were much higher compared to Thai 'background' total soil Cd level which range from 0.002 - 0.141 and Thai Investigation Level for Cd in soil of 0.15 mg/kg (Simmons, et al., 2005). This result is similar with the previous study by Weeraprapan, et al., (2015) who reported that the Cd level in the sediment of Mae Tao Creek which is located downstream of the zinc mine was much higher than in the sediment obtained from upstream of the zinc mine areas. As the Mae Tao River accepts water from the zinc mine, it is apparent that the source of Cd is the zinc mine (Weeraprapan, et al., 2015). This is also in line with Thamjedsada, et al., (2012) who reported that Cd in the sediment of Mae Tao Creek tends to be higher in sites downstream sites from the zinc mine both in the dry and the wet seasons. Since site S1 is located far above the zinc mine, it receives irrigation water which has not passed the zinc mine area, thus minimizing Cd contamination. The downstream sites, on the other hand, receive water from the river which passes through the zinc mine which is highly contaminated with Cd. A number of mining activities such as drilling, material transfer, the removal of mine tailings and drainage may influence the contamination of the environment with Cd (Netpae, et al., 2015). When Cd enters to aquatic system, a vast amount of metal precipitates and resides

in the bottom of sediments. Thus, sediment can be a major source for Cd emitted to the aquatic environment (reviewed in Perera, et al., 2009). The most available of form to aquatic species is the free metal ion (Cd^{2+}) (IPCS, 1994). Cd can be obtained directly by fish either through cadmium contaminated medium (water or sediment) or by ingested Cd-contaminated food (USEPA, 2002). Freshwater fish are able to uptake the Cd in three possible ways i.e. via gills as respiratory tract, intestines through ingestion and body surface (skin) (Kumar and Singh, 2010; Yousafzai, et al., 2010; Jalaluddeen, et al., 2012).

The current study reveals that the average of Cd accumulation in swamp eels' differed depending on location and organs. The concentration of Cd in freshwater fish tissues such as muscles, intestine, gill, kidney, spleen, skin, brain and liver usually does not exceed 2 mg/kg (Kondera, et al., 2014). The result showed that the fish from three downstream sites accumulated much higher cadmium in three subject organs i.e. liver, kidney and gills, compared to those from the upstream site. The Cd levels in swamp eel tissues were considerably higher than their concentration in the sediment which indicates bioaccumulation of this element in fish organism. This result is in agreement with Kodera, et al. (2014) who found that the level of Cd and Cu in *Cyprinus carpio* L blood, gill, liver, trunk kidney and head kidney were higher than in the medium in both short and long term waterborne exposure. As Cd is classified as a heavy metal which is non-biodegradable and non-thermo degradable and hence it readily accumulates to toxic levels (Taghipour, et al., 2012).

Cd rarely accumulate uniformly in organs (Wangsongsak, et al., 2007). This study shows that the pattern of cadmium accumulation in organs differed following the different locations. Liver accumulated the highest Cd in almost all location (except site S3), following by either gills or kidney. These variations of Cd distribution in various organ of fish were primarily addressed to the difference in the physiological function of particular organ (Jelodar, et al., 2011) and also the exposure route (Chowdury, et al., 2005). In fish the gill is considered as the most vulnerable organs to acute exposure while liver and kidney are vulnerable during prolonged exposure (Olsik, et al., 2000). Liver and kidney is known as metabolically active organ. Many studies have been conducted regarding the accumulation of Cd in particular organs (Reviewed in Perera, et al., 2015). In laboratory studies the pattern of Cd distribution differs depending upon

the exposure route, during waterborne exposure, the trend of Cd accumulation in fish from the highest to lowest level were kidney, gills, liver and gut, respectively, while during dietary borne exposure the pattern was gut, kidney, liver and gills, respectively (Chowdury, et al., 2005). Yin, et al. (2012) reported that the pattern of Cd accumulation in *M. albus* which was collected from paddy field in Klangtan for gills, kidney, and liver were 4.45, 2.83, 1.78 μg/g respectively. This Cd contamination was then addressed to the used of fertilizers by the farmers in the rice fields. The current study also shows high accumulation of Cd in gills (in site S3, gills was the highest), as the gill is the first organ to be exposed to re-suspended sediment particles (Wei, et al., 2014) leading to significant effect in the total Cd levels of the gills (Eneji, et al., 2011). Another study suggested that the higher level of metal in gills might be due to the presence of mucus in the gills that is impossible to be removed completely from the gills prior to tissue analysis preparation (Jelodar, et al., 2011). Kidney is primary target following the chronic exposure of Cd. (Klaassen, et al., 2009). A significant strong positive correlation was found between Cd accumulation in sediment and Cd content in kidney suggesting that renal Cd levels could be a good indicator of environmental exposure to Cd.

Cadmium accumulation varies from species to species and depends upon exposure duration (Yeşilbudak and Erdem, 2014). Cadmium levels in *Oreochromis* niloticus after exposure to 0.5 ppm Cd for 30 days were higher in gill and kidney, whereas in the *Cyprinus carpio*, the higher accumulation was found in liver and muscle. This, perhaps due to the differences in osmoregulation and detoxification processes of the two species studied (Yeşilbudak and Erdem, 2014). Wangsongsak, et al. (2007) investigated that Cd accumulation in *Puntius gonionotus* tissue during the sub-chronic exposure to Cd increased with the increasing exposure duration with kidney accumulated the highest level followed by liver and gills. Furthermore, a number of environmental factors, such as temperature, salinity, hardness, pH of water (Abdel-Tawwab and Wafeek, 2014), and also seasonal variation (Tekin-Özan and Kir, 2008) also could affect the accumulation of heavy metal. In this study, the fish were collected during rainy season in July-August 2016. It is therefore unlikely that the Cd accumulation observed in the fish tissues would be related to seasonal variations.

It has been suggested that high concentration of heavy metal might have associated to the increase of pathologic abnormality frequencies (Dane and Şişman,

2015). In this study the fish liver, kidney and gills were subjected to histopathological analysis. This type of biomarker is known to be highly sensitive and is suitable to study the ecosystem impairment due to the contaminants, yet the result present low specificities to toxicants, since particular injuries can be derived from different source of contaminations (Stori, et al., 2014).

The liver is an organ most associated with the detoxification and biotransformation process due to its function, position, and blood supply making it one of the most affected by contaminant. The histology of fish liver consists of hepatocytes that not oriented into distinct lobules but arranged in branched laminae two cells thick, separated by sinusoids. Hepatocytes are polygonal cells with a central spherical nucleus and a densely stained nucleolus (Hadi and Alwan, 2012). Wangsongsak, et al. (2007) observed the alteration in the livers of *Puntius gonionotus* exposed to sub-chronic cadmium including hepatocytes swelling, vacuolation in hepatocytes cytoplasm, and focal necrosis. Younis, et al. (2013) reported that the severity of pathological symptoms in the liver of Nile tilapia exposed to $CdCl_2$ (10%, 20% and 30% of 96-d LC_{50}) varied according to the concentration exposure. The increase of hepatocyte vacuolation, coarse granulation of hepatocytes cytoplasm and large amount of erythrocyte infiltrations were the general lesions found in this study. In other observations about fish exposure to $CdSO_4$, sign of hepatic injuries such as necrosis, proliferation of hepatocyte, formation of sponge mass and the pancreatic tissue impairment were observed after 20 days of exposure (Jalaludeen, et al., 2012). Earlier field study by Camargo and Martinez (2007) investigated *Prochilodus lineatus* that were subjected to *in situ* tests (caged) for 7 days in a disturbed urban stream, generally the liver displayed lesions such as irregular-shaped nuclei, nuclear hypertrophy, nuclear vacuolation and the presence of eosinophilic, granules in the cytoplasm, bile stagnation, cytoplasmic and nuclear degeneration and presence of melano-macrophage aggregates.

In the present study, histopathological observation of organ using light microscopy showed several lesions in the liver such as hepatocyte swelling (hypertrophy), sinusoid dilation, blood congestion in sinusoid and central vein, degeneration of cytoplasm, nuclear degeneration, bile stagnation, and presence of melano-macrophage center. The liver tissue in all sites exhibit similar lesion but with different degree of severity, which probably due to the different hepatic Cd

accumulation in each site. For instance, the blood congestions in sinusoid were found in all study sites, however, the most severe ones were found in the site S2 and site S4. The frequency of presence of bile stagnation in the liver from the three downstream sites were also observed to be higher compared to the liver collected from upstream site. Furthermore, melano-macrophage aggregates (melano-machropage centers) were only found in fish liver collected from downstream sites.

MMCs are group of pigment-containing cells generally found in a number of organs in fish majorly in hematopoetic organ such spleen and kidney. These structures usually contain a variety of pigments including melanin, lipofuscin, ceroid and hemosiderin (Agius and Robert, 2003). The presence of MMCs in the tissue has been associated with chronic inflammatory injuries and cell degeneration (Stori et al., 2014). A number of pathological state of organism such as starvation, inflammatory and immunological processes, viral infections, parasitic infestations, as well as changing environmental condition could alter the amount and the morphology of MMCs (Pronina, et al., 2014).

The current study shows that the hepatic histopathological alterations especially in downstream sites could lead to metabolic disturbance. It can be seen by the presence of bile stagnation in the liver, which characterized by yellow- brown granules in the hepatocyte cytoplasm indicating that the remaining bile is not being released from the liver. This accumulation of bile could lead to metabolic disturbance of liver (Camargo and Martinez, 2007). The presence of melano-machrophage and more frequency of bile stagnation in *M. albus* livers collected from downstream sites confirms that the liver exhibit structural and metabolic impairment due to exposure to Cd, strengthening the idea that these environments is really damaged.

It is worth noting that fish can easily be exposed to variety stressors such viruses, parasites, bacteria, fungi, pollutants, nutritional insufficiency, oxygen deficiency, temperature fluctuation and salinity variation. These stressors and the interaction among them might affect the fish health at different degree (Dang, et al., 2017). In addition to Cd contamination, the swamp eels living in the paddy field might have exposed to the other pollutants such fertilizers and pesticides where farmers might have applied them in paddy field in order to increase the crop production (Yin, et al., 2012). Tabassum, et al. (2016) reported several histopathological alterations such as

degeneration of cytoplasm of hepatocyte, necrosis, atrophy, vacuole formation, blood vessels rupture, and cell wall disappearance in hepatic of *Channna striata* after exposed to sub-lethal concentration of pendhimethalin (herbicide). In another study, Velmurugan, et al. (2007) found several lesions such as karyohexis, karyolysis, congestion and dilatation of sinusoids, cloudy degeneration, hepatocytes hypertrophy and focal necrosis have been reported in the *Cirrhinus mrigala* after exposed to lambda-cyhalothri (pesticide).

Likewise, kidney also one of the major target organ of Cd toxicity. In the present study the kidney tissue section showed the hematopoietic tissue and lack of tubules which is the characteristic of head kidney (anterior kidney). The head kidney is composed of a great variety of cells, the most abundant being leucocytes and red blood cells in different stages of maturation. The head kidney (phenoperos) is a basic organ which responsible in the blood elements formation, and it is also the reservoir of blood cells. The head kidney is also an important endocrine organ, equivalent to adrenal glands in mammals, releasing hormones such as corticosteroids, catecholamines, and others (Kondera, et al., 2014).

In the current study, all study sites exhibit similar pathology i.e. the presence of MMCs and also formation of protein casts. The cast formation itself in kidney were found in the tubules which is the sign obstructive nephropathy (Ito, et al., 2009). The casts derived from sloughed cells, tubular debris, and protein (Basile, et al., 2012). However, there was no nephron found in these kidney section. Possibly, all tubules might have already changed to cast forms, indicating that these tissue were impaired.

Kondera and Witeska (2013) observed that *Cyprinus carpio* L. head kidney exposed to Cu and Cd exhibit the reduction of the hematopoietic potential measured as the ratio of proliferating to apoptotic precursor cell frequency. Mishra, et al. (2008) investigated the head kidney of *Channa punctatus,* in the endocrine component, which consisted of interrenal and chromaffin cells, exhibited the hypertrophy and degranulation after exposed to 20 and 40 mg/L of chromium. Salazar-Lugo, et al. (2013) noted that after chronic Cd exposure in the freshwater fish *C. macropomum* produced an anomalous head kidney structure, and induced an inflammatory process in this organ, affecting hematopoietic cell differentiation, especially with regard to granulocytes and perhaps affecting its function.

In fish, gills are important organs that function in respiration, osmoregulation and excretion process. A high rate of contaminants absorption through gills also makes fish a susceptible target of their toxicity (Cengiz, 2006). To date, there have been no studies investigating either the normal histological architecture or histopathological pathologies in regards to the anthropogenic contaminant exposure in swamp eel gills. Asian swamp eel has a reduced gill area on branchial arches I-III and lack of lamella on arch IV. Wu and Liu (1940) found that preventing branchial gas exchange by destroying the gills of *M. albus* leads no effect on the rate of aquatic oxygen consumption suggesting that the vasculature of the bucco-pharynx was responsible for the vast majority of gas exchange. Given the reduced gills, *M. albus* which is classified to facultative air breathing fish (Damsgaard, 2014), appears to possess different architecture of gills compared to common water breathing fish. If normally, the gills histology shows distinct different between primary lamellae (filament) and secondary lamellae, however, the swamp eel only possess filament without any extension of it. This possibly, at least in this case, due to the fact that swamp eels have the reduced gills and/or the size of gills were too small (probably due to it was derived from small sized fish). In view of this last possibility, the gills might be still in developing processes so that in some samples, which were derived from small sized fish, gills histological structure only shows filament/lamellae (Figure 10 A, B, E, F, G, H). However, in sample which had larger sized gills, it somewhat shows a structure of filament and lamellae, even though, there is no fixed pattern of the development (Figure 10 C-D).

In the current study, it is difficult to assess the histopathological changes occurrences in the swamp eel gills due to the lack of knowledge and documentation regarding the histological structure of swamp eel gills. Nile tilapia exposed to copper exhibit several histological changes, such as epithelium proliferation, lamellar epithelium lifting, lamellar axis vasodilation, edema in the filament, fusion of lamellae and lamellar aneurisms (Figueiredo-Fernandes, et al., 2007).

In the current study, molecular approach to study the adverse effects of Cd in the fish was also performed through metallothionein gene expression determination. Cloning of MT sequence in *M. albus* was conducted prior to MT expression examination. The *M. albus* MT protein sequence shows the typical cystenil residues pattern of MT protein i.e Cys-X-Cys and or Cys-X-Y-Cys pattern. This protein also

shows other typical MT characteristics such as the absence of aromatic amino acid and the presence of 5 glycine residues (Ceratto, et al., 2002).

The MT gene expression of swamp eel liver was determined quantitatively using real time-PCR. The most intense MT synthesis was usually observed in liver (Kovarova, et al., 2009) as a major detoxification organ in fish (Espinoza, et al., 2012). Yang, et al. (2014) also reported that the MT gene were detected in all eleven tissues (gills, heart, skin, spleen, head kidney, blood, kidney, stomach, intestine, brain and liver) of healthy black porgy, indicating that MT mRNA was ubiquitous in various tissues, however, liver has the highest MT gene expression. The liver was thus chosen as the subject organ in this study.

In the present study, the MT gene expression level was used in an attempt to understand the suitability as a biomarker of metal exposure (especially Cd) as it is frequently used in pollution monitoring programs (Laurie, 2004; Quirós, et al., 2007). MT has been suggested as an ideal biomarker to monitor metal contamination in fish, due to its ability and specificity to bind metal ions (Chan, 1995). Hepatic MT gene expression was observed in all study sites including in the non-detectable sediment Cd level site and/or in the lowest hepatic Cd level samples, implying that Cd detoxification is not the only role of MT and/or that the constitutively expression of MT is as a basal level of protection from Cd toxicity (Timmermans, et al., 2005). It has previously been suggested by Bourdineaud, et al. (2006) in which under low contamination pressure, the purpose of the MT gene induction is to compensate MT degradation rather than to increase the metal sequestration capacities as it was generally expected. This is suitable with the role of MT gene induction in regard to zinc homeostasis rather than to metal detoxification.

Generally, metallothionein is expected to be induced under conditions of elevated metal concentrations, allowing more binding sites to sequester the metal ions and preventing the potential adverse effect (Langston, et al., 2002). A number of studies in a variety of fish such as killifish (Van Cleef-Toedt, et al., 2001), silver barb (Wangsongsak, et al., 2007), mandarin fish (Gao, et al., 2009), catfish *Clarias gariepinus* (Rose, et al., 2014), and grass carp (Tan, et al., 2016) showed the elevation of MT expression level after being exposed to Cd. In the current study, the highest MT gene expression of liver was found in site S3 of 5.92 fold followed by site S2 of 1.25

fold compared to site S1 (reference site). Earlier field studies have shown that hepatic MT gene expression of *Oreochromis niloticus* was found to be higher in heavy metal polluted fish farms (Abumourad, et al., 2013). Interestingly, the present study shows no parallel correlation between MT gene expression levels and hepatic cadmium content. Moreover, the current study also reveals that the lowest expression of 0.48 fold (compared to S1) was found in S4 which turned out to be the highest hepatic Cd level site. These accumulations of Cd in liver might exceed its regulation capacity, leading to disruption in the induction of MT synthesis and eventually loss of the correlation between MT and Cd levels as generally found at lower exposed concentrations (Shariati, et al., 2011). When the metal accumulations have surpassed the capability of animal to produce MT, metal can bind to high molecular mass proteins but with a weaker affinity and that Cd toxicity may follow (De Boeck, et al., 2003). For this reason, MT gene expression biomarker was probably more sensitive and suitable to acute (short-term) exposure but not to chronic (long-term) exposure to heavy metals (Quirós, et al., 2007; Rhee, et. 2009). However, in another species such as springtail, the animal might develop Cd tolerance after chronic exposure by increasing the basal level of MT expression. It was proved by the higher expression of MT basal level of this animals' population collected from metal contaminated environment compared to those obtained from the clean environment, even after the animals have been cultured in laboratory with the clean food for several generations (Timmermans, et al., 2005).

No parallel correlation also was found between MT mRNA level and level of Cd in the sediment as the exposure site. The areas that had lower levels of Cd showed higher hepatic MT gene expression. Moreover, the most heavily polluted Cd area (site S4) showed lowest hepatic MT gene expression. A similar result was reported in a laboratory study which was conducted by Kovarova, et al. (2009), in which hepatic MT in carp reached its maximum level (>130 ng/g) in lower exposure of Cd (2.5, 5, 7.5 mg/L of CdCl) yet hit the low MTs levels (<50 ng/g) in highest exposure of Cd (12.5 mg/L of CdCl) and control group (Cd-free treatment) in all time period of exposure (24, 48,72 and 96 hr). Wangsongsak, et al. (2007) also demonstrated that during 56 days of exposure of silver barb to three series amounts of Cd i.e. 0.012, 0.06 and 0.12 mg/L, respectively, the hepatic MT mRNA expression increases significantly upon the exposure and reached highest expression at 28 days, however the MTs expression were

gradually dropped after 35 days. Combining these together thus than suggested that synthesis of MT and its binding capacity is restricted (Kovarova, et al., 2009). A study of human monocyte exposed to CdCl showed that 10 μM of Cd^{2+} which induced significant toxicity, induced significantly less MT mRNA than 1 μM of Cd^{2+}. It was than believed that MT levels perhaps have to be in relatively narrow range to mediate the induction of activation, either increasing or decreasing those levels would cause negative effect. Another possibility hypothesis that this study proposed was that metal exposure might affect the other expression of genes as in addition to MT's induction. The non-MT factors induced by metal might act on their own or in concert with MT to inhibit the activation potential (Koropatnick and Zalups, 1997).

MT expression was found to be strongly induced by Cd in the laboratory (Wangsongsak, et al., 2007) and field condition (Fernandes, et al., 2008). However, not every species of fish is suitable for bio-monitoring (Kovarova, et al., 2009). It was reported that MT was suitable as a bio-indicator for monitoring heavy metals pollution in brown trout, but not in European eel (Linde, et al., 2001). In animal, it is a well-known that the increase of susceptibility to Cd toxicity is usually associated with the incapability of animal to synthesize MT (Włostowski, et al., 2008). Wu, et al. (2006) observed that three species of fish i.e. *Acrossocheilus paradoxus*, *Oreochromis mossamibicus* and *Chanos chanos* appear to have different tolerance to Cd which might be associated with difference in Cd accumulation rate and the ability to synthesize the additional MT of each species of fish.

Furthermore, several stressors – other than metal – have been reported to predispose the MT mRNA synthesis. Yang, et al. (2014) reported a significant up-regulation of MT gene expression in black porgy after 48 hours of bacterial infection, suggesting that MT gene in this species involved in the immune-associated response. Abdel-Tawwab and Wafeek (2014) observed that the temperature significantly increases the MT levels in respect to the increase of Cd accumulation. Rhee, et al. (2009) indicated that some intrinsic factors, such as age and gender were found to affect the MT gene expression level in *Kryptolebias marmoratus* (Mangrove killifish). It is worth noting that, with swamp eels, it was not always possible to distinguish the gender or estimate the age precisely. In addition, the variation of swamp eel size in this study may be related to the variation of accumulated Cd thus affecting the MT gene expression

level. Even though, MT mRNA expression can be stimulated by various stressors, hepatic MT in eel (*Anguilla Anguilla*) was found to be strongly correlated with hepatic heavy metal, even after taking other variables (sizes and seasonal variations) into consideration. This study indicates that the MT is a good biomarker for heavy metal contamination (Bird, et al., 2007).

CHAPTER VI

CONCLUSION AND SUGGESTION

From this study it is concluded that sediment and swamp eels tissue collected from downstream site tend to accumulated higher Cd level compare to those obtained from the upstream site. These high accumulations in downstream sites were attributed to the Cd accumulation in paddy fields irrigated with Cd-contaminated water from the upstream zinc mine. Hence, the swamp eels collected from downstream sites would most likely accumulate higher Cd concentration compared to upstream site. Taking findings from the current and from previous studies together, there is some evidence that the downstream from zinc mine areas tend to contaminate with Cd in higher level than upstream from zinc mine. The trend of cadmium accumulation in organs differed following the different locations. The Cd concentration order in various organs were: site S1, liver>kidney>gills; site S2, liver>gills>kidney; site S3, gills>liver>kidney and S4, liver>kidney>gills.

Generally, liver and kidney showed similar lesions in all study site, although to some extent, fish liver collected from downstream sites seemed to display more tissue impairments compared to those from the upstream site. Perhaps, fish have been affected by other stressors other than Cd in the environment, resulting the injured of tissues even in the lowest Cd accumulated tissue. In gills, the histopathological alteration assessment was difficult due to the lack of documentation about the histological architecture of swamp eel gills. Further study, it needs to consider the size and the age swamp eel fish prior to histological study of the gills. In addition, using more samples in variety of size range would help in result validation.

No parallel correlation was found between MT gene expression level and hepatic Cd burden neither between MT gene expression level and Cd concentration in sediment. This study suggests that Cd may induced MT gene expression within certain range levels only. Due to the prolonged duration of exposure and/or high concentration exposure to Cd, the Cd accumulation might have exceeded the limit of its regulatory capacity resulting the restriction of MT mRNA synthesis. In addition, some intrinsic and

extrinsic factors need to be taken into consideration. Therefore, prior to application of MT gene expression biomarker in the natural environment, it is important to develop laboratory studies to provide data regarding the basal and maximum induced levels of swamp eel MT mRNA and also environmental variables which may regulates the MT gene expression in this species.

REFERENCES

REFERENCES

Abdel-Baki, A. S., Dkhil, M. A., & Al-Quraishy, S. (2011). Bioaccumulation of some heavy metals in tilapia fish relevant to their concentration in water and sediment of Wadi Hanifah, Saudi Arabia. *African Journal of Biotechnology*, *10*(13), 2541-2547. doi: 10.5897/AJB10.1772

Abdel-Tawwab, M., & Wafeek, M. (2014). Influence of water temperature and waterborne cadmium toxicity on growth performance and metallothionein–cadmium distribution in different organs of Nile tilapia, *Oreochromis niloticus* (L.). *Journal of Thermal Biology, 45*, 157–162. doi:10.1016/j.jtherbio.2014.09.002

Abumourad, I. M. K., Authman, M. M. N., & Abbas, W. T. (2013). Heavy metal pollution and metallothionein expression: A survey on Egyptian tilapia farms. *Journal of Applied Sciences Research*, *9*(1), 612-619.

Agency for Toxic Substances and Disease Registry (ATSDR). (1999). *Toxicological profile for cadmium*. Georgia: Agency for Toxic Substances and Disease Registry.

Agius, C., & Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology. *Journal of Fish Diseases, 26*(9), 499-509. doi:10.1046/j.1365-2761.2003.00485.x

Al-Weher, S.M. (2008). Levels of heavy metal Cd, Cu and Zn in three fish species collected from the Northern Jordan Valley, Jordan. *Jordan Journal of Biological Sciences, 1*, 41-46.

Annabi, A., Said, K., & Messaoudi, I. (2013). Cadmium: bioaccumulation, histopathology and detoxifying mechanisms in fish. *American Journal of Research Communication, 1*(4), 60-79.

Arini, A., Gourves, P., Gonzalez, P., & Baudrimont, M. (2015). Metal detoxification and gene expression regulation after a Cd and Zn contamination: An experimental study on *Danio rerio*. *Chemosphere, 128*, 125-133. doi:10.1016/j.chemosphere.2015.01.022

Authman, M. M. N. (2008). *Oreochromis niloticus* as a biomonitor of heavy metal pollution with emphasis on potential risk and relation to some biological aspect. *Global Veterenaria, 2*, 104-109.

Baldisserotto, B., Chowdhury, M., & Wood, C. M. (2005). Effects of dietary calcium and cadmium on cadmium accumulation, calcium and cadmium uptake from the water, and their interactions in juvenile rainbow trout. *Aquatic Toxicology, 72*(1-2), 99-117. doi:10.1016/j.aquatox.2004.11.019

Banaee, M., Mohammadipour, S., & Madhani, S. (2015). Effects of sublethal concentrations of permethrin on bioaccumulation of cadmium in zebra cichlid (*Cichlasoma nigrofasciatum*). *Toxicological & Environmental Chemistry, 97*(2), 200-207. doi:10.1080/02772248.2015.1031668

Basile, D. P., Anderson, M. D., & Sutton, T. A. (2012). Pathophysiology of acute kidney injury. *Comprehensive Physiology*. doi:10.1002/cphy.c110041

Bernard, A. (2008). Cadmium & its adverse effects on human health. *Indian Journal of Medical Research, 128*, 557-564.

Beširović, H., Alić, A., Prašović, S., & Drommer, W. (2010). Histopathological effects of chronic exposure to cadmium and zinc on kidneys and gills of brown trout (*Salmo trutta m. fario*). *Turkish Journal of Fisheries and Aquatic Sciences, 10*, 255-262.

Bird, D., Rotchell, J., Hesp, S., Newton, L., Hall, N., & Potter, I. (2008). To what extent are hepatic concentrations of heavy metals in *Anguilla anguilla* at a site in a contaminated estuary related to body size and age and reflected in the metallothionein concentrations? *Environmental Pollution, 151*(3), 641-651. doi:10.1016/j.envpol.2007.03.018

Blechinger, S., Kusch, R., Haugo, K., Matz, C., Chivers, D., & Krone, P. (2007). Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. *Toxicology and Applied Pharmacology, 224*(1), 72-80. doi:10.1016/j.taap.2007.06.025

Bonga, S. E. W., & Loack, R. A. C. (2008). The osmoregulatory system. In Giulio, R. T. D., & Hinton, D.E, *The toxicology of fishes* (pp. 401-415). New York, NY: CRC Press.

Bourdineaud, J., Baudrimont, M., Gonzalez, P., & Moreau, J. (2006). Challenging the model for induction of metallothionein gene expression. *Biochimie, 88*(11), 1787-1792. doi:10.1016/j.biochi.2006.07.021

Bricking, E. M. (2002). *Introduced species summary project Asian swamp eel (Monopterus albus).* Retrieved January 14, 2016, from http://www.columbia.edu/itc/cerc/danoffburg/invasion_bio/inv_spp_summ/Monopterus_albus.html.

Bucking, C., & Wood, C. M. (2006). Gastrointestinal processing of Na, Cl-, and K during digestion: implications for homeostatic balance in freshwater rainbow trout. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 291*(6). doi:10.1152/ajpregu.00224.2006

Camargo, M. M., & Martinez, C. B. (2007). Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. *Neotropical Ichthyology, 5*(3), 327-336. doi:10.1590/s1679-62252007000300013

Canli, M., & Atli, G. (2003). The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. *Environmental Pollution, 121*(1), 129-136. doi:10.1016/s0269-7491(02)00194-x

Capasso, C., Carginale, V., Crescenzi, O., Di Maro, D., Parisi, E., Spadaccini, R., & Temussi, P. A. (2003). Solution structure of MT_nc, a novel metallothionein from the Antarctic fish *Notothenia coriiceps*. *Structure, 11*, 435–443

Carpene, E., Andreani, G., & Isani, G. (2007). Metallothionein functions and structural characteristcs. *Journal of Trace Elements in Medicine and Biology, 21*, 35-39.

Cengiz, E. I. (2006). Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. *Environmental Toxicology and Pharmacology, 22*(2), 200-204. doi:10.1016/j.etap.2006.03.006

Ceratto, N., Dondero, F., Loo, J. V., Burlando, B., & Viarengo, A. (2002). Cloning and sequencing of a novel metallothionein gene in *Mytilus galloprovincialis* Lam. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 131*(3), 217-222. doi:10.1016/s1532-0456(02)00008-x

Chan, K. M. (1995). Metallothionein: Potential biomarker for monitoring heavy metal pollution in fish around Hong Kong. *Marine Pollution Bulletin, 31,* 411–415.

Cheung, A. P., Lam, T. H., & Chan, K. M. (2004). Regulation of Tilapia metallothionein gene expression by heavy metal ions. *Marine Environmental Research, 58*(2-5), 389-394. doi:10.1016/j.marenvres.2004.03.084

Chow, E. S. (2003). Cadmium affects muscle type development and axon growth in zebrafish embryonic somitogenesis. *Toxicological Sciences, 73*(1), 149-159. doi:10.1093/toxsci/kfg046

Chowdhury, M. J., Baldisserotto, B., & Wood, C. M. (2005). Tissue-specific cadmium and metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. *Archives of Environmental Contamination and Toxicology, 48*(3), 381-390. doi:10.1007/s00244-004-0068-2

Chowdhury, M., Grosell, M., Mcdonald, D., & Wood, C. (2003). Plasma clearance of cadmium and zinc in non-acclimated and metal-acclimated trout. *Aquatic Toxicology, 64*(3), 259-275. doi:10.1016/s0166-445x(03)00074-2

Chowdhury, M., Mcdonald, D., & Wood, C. (2004). Gastrointestinal uptake and fate of cadmium in rainbow trout acclimated to sublethal dietary cadmium. *Aquatic Toxicology, 69*(2), 149-163. doi:10.1016/j.aquatox.2004.05.002

Cirillo, T., Cocchieri, R. A., Fasano, E., Lucisano, A., Tafuri, S., Ferrante, M. C. Carpene`, E., Andreani, G., & Isani, G. (2011). Cadmium accumulation and antioxidant responses in *Sparus aurata* exposed to waterborne Cadmium. *Archives of Environmental Contamination and Toxicology, 62*(1), 118-126. doi:10.1007/s00244-011-9676-9

Cleef-Toedt, K. A., Kaplan, L. A., & Crivello, J. F. (2001). Killifish metallothionein messenger RNA expression following temperature perturbation and cadmium exposure. *Cell Stress & Chaperones, 6*(4), 351. doi:10.1379/1466-1268(2001)006<0351:kmmref>2.0.co;2

Damsgaard, C., Findorf, I., Helbo, S., Kocagoz, Y., Buchanan, R., Huong, D. T. T., Weber, R. E., Fago, A., Bayley, M., & Wang, T. (2014). High blood oxygen affinity in the air-breathing swamp eel *Monopterus albus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 178,* 102-108. doi:10.1016/j.cbpa.2014.08.001

Dane, H., & Şişman, T. (2015). Histopathological changes in gill and liver of *Capoeta capoeta* living in the Karasu River, Erzurum. *Environmental Toxicology, 30*, 904-917. doi:10.1002/tox.21965

Dang, M., Nørregaard, R., Bach, L., Sonne, C., Søndergaard, J., Gustavson, K., . . . Nowak, B. (2017). Metal residues, histopathology and presence of parasites in the liver and gills of fourhorn sculpin (*Myoxocephalus quadricornis*) and shorthorn sculpin (*Myoxocephalus scorpius*) near a former lead-zinc mine in East Greenland. *Environmental Research, 153*, 171-180. doi:10.1016/j.envres.2016.12.007

Darmono. (2010). *Lingkungan Hidup dan Pencemaran*. Jakarta: Penerbit Universitas Indonesia.

de Astudillo L.R., Yen I.C., Berkele I. (2005). Heavy metals in sediments, mussels and oysters from Trinidad and Venezuela. *Revista de Biologia Tropical, 53*, 41–53.

De Boeck, G., Ngo, T. T., Campenhout, K. V., & Blust, R. (2003). Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquatic Toxicology, 65*(4), 413-424. doi:10.1016/s0166-445x(03)00178-4

Driessnack, M. K., Matthews, A. L., Raine, J. C., & Niyogi, S. (2016). Interactive effects of chronic waterborne copper and cadmium exposure on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 179*, 165-173. doi:10.1016/j.cbpc.2015.10.009

Duncan, K. E., Kirby, C. W., & Stillman, M. J. (2008). Metal exchange in metallothioneins - a novel structurally significant Cd5 species in the alpha domain of human metallothionein 1a. *FEBS Journal, 275*(9), 2227-2239. doi:10.1111/j.1742-4658.2008.06375.x

Dutta, T. K., & Kaviraj, A. (2001). Acute toxicity of cadmium to fish *Labeo rohita* and copepod *Diaptomus forbesi* pre-exposed to CaO and $KMnO_4$. *Chemosphere, 42*(8), 955-958. doi:10.1016/s0045-6535(00)00166-1

Eisler, R. (1985). *Cadmium Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review*. U.S. Fish and Wildlife Service, Washington, DC. Biological Report No 85 (1.2) NY, 143-166.

Eneji, I. S., Sha'Ato, R., & Annune, P. A. (2011): Bioaccumulation of heavy metals in fish (*Tillapia zilli* and *Clarias gariepinus*) organs from River Benue, North Central. *Pakistan Journal of Analytical & Environmental Chemistry, 12*(1-2), 25-31.

Erickson, R., Nichols, J., Cook, P., & Ankley, G. (2008). Bioavailability of chemical contaminants in aquatic Systems. In Giulio, R. T. D & Hinton, D.E. (Eds.), *The toxicology of fishes* (pp. 9-54). New York, NY: CRC Press.

Espinoza, H. M., Williams, S. R., & Gallagher, E. P. (2012). Effect of cadmium on Glutathione S-Transferase and metallothinein gene expression in coho salmon liver, gill, and olfactory tissue. *Aquatic Toxicology, 110-111*, 37-44. doi: 10.1016/j.aquatox.2011.12.012

Fernandes, D., Bebianno, M. J., & Porte, C. (2008). Hepatic levels of metal and metallothioneins in two commercial fish species of the Northern Iberian shelf. *Science of the Total Environment, 391*(1), 159-167. doi:10.1016/j.scitotenv.2007.10.057

Figueiredo-Fernandes, A., Ferreira-Cardoso, J. V., Garcia-Santos, S., Monteiro, S. M., Carrola, J., Matos, P., & Fontaínhas-Fernandes, A. (2007). Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. *Pesquisa Veterinária Brasileira, 27*(3), 103-109. doi:10.1590/s0100-736x2007000300004

Gao, D., Wang, G. T., Chen, X. T., & Nie, P. (2009). Metallothionein-2 gene from the mandarin fish *Siniperca chuatsi*: cDNA cloning, tissue expression, and immunohistochemical localization. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 149*(1), 18-25. doi:10.1016/j.cbpc.2008.05.014

Glynn, A. W. (1996). The concentration dependency of branchial intracellular cadmium distribution and influx in the zebrafish (*Brachydanio rerio*). *Aquatic Toxicology, 35*(1), 47-58. doi:10.1016/0166-445x(96)00006-9

Gobe, G., & Crane, D. (2010). Mitochondria, reactive oxygen species and cadmium toxicity in the kidney. *Toxicology Letters, 198*(1), 49-55. doi:10.1016/j.toxlet.2010.04.013

Godt, J., Scheidig, F., Grosse-Siestrup, C., Esche, V., Brandenburg, P., Reich, A., & Groneberg, D. A. (2006). The toxicity of cadmium and resulting hazards for human health. *Journal of Occupational Medicine and Toxicology*, *1*, 22. Doi:10.1186/1745-6673-1-22

Hadi, A. A., & Alwan, S.F. (2012). Histopathological changes in gills, liver and kidney of freshwater fish, *Tilapia zillii*, exposed to aluminium. *International Journal of Pharmacy & Life Sciences, 3*(11), 2071-2081.

Hansen, J., Welsh, P., Lipton, J., & Suedkamp, M. (2002). The effects of long-term cadmium exposure on the growth and survival of juvenile bull trout (*Salvelinus confluentus*). *Aquatic Toxicology, 58*(3-4), 165-174. doi:10.1016/s0166-445x(01)00233-8

Health, A.G. (1995). *Water Pollution and Fish Physiology* (*2nd Ed.*). USA: CRC Press.

Hidalgo, J., Chung, R., Penkowa, M. & Vašák, M. (2009). Structure and Function of Vertebrate Metallothioneins. In Sigel, A., Sigel, H., & Sigel, R. K. O (Eds.), *Met. Ions Life Sci: Metallothioneins and Related Chelators Vol. 5* (pp. 279–317). Chichester, UK: John Wiley & Sons, Ltd.

Hii, Y. S., Lee, M. Y., & Chuah, T. S. (2007). Acute toxicity of organochlorine insecticide endosulfan and its effect on behaviour and some hematological parameters of Asian swamp eel (*Monopterus albus* Zuiew). *Pesticide Biochemistry and Physiology, 89*(1), 46-53. doi:10.1016/j.pestbp.2007.02.009

Hinton, D.E., & Lauren, D.J. (1990). Integrative histopathological approaches to detecting effects of environmental stressors on fishes. *Am. Fish Soc. Symp. 8*, 51–66.

Institute, M. (2013). *Cadmium*. Retrieved January 25, 2016, from http://www.eoearth.org/view/ article/150844.

International Programme on Chemical Safety (IPCS). (1992). C*admium-environmental aspects*: *Environmental health criteria 135*. Geneva: WHO.

Ivanina, A. V., Cherkasov, A. S., & Sokolova, I. M. (2008). Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. *Journal of Experimental Biology, 211*(4), 577-586. doi:10.1242/jeb.011262

Jalaludeen, M. D., Arunachalam, M. Raja, M., Nandagopal, S., Bhat, S. A., & Sundar, S. (2012). Histopatological of the gill, liver and kidney tissue of the freshwater fish *Tilapia Mossambica* exposed to cadmium sulphate. *International Journal of Advanced Biological Research*, *2*(4), 572-578.

Jelodar, H. T., Baei, M. S., Najafpour, Sh., & Fazli, H. (2011). The comparison of heavy metals concentrations in different organs of *liza aurata* inhabiting in southern part of Caspian Sea. *World Applied Sciences Journal, 14*, 96-100.

Jezierska, B., & Witeska, M. (2006). The Metal Uptake And Accumulation In Fish Living In Polluted Waters. In Twardowska I., Allen H. E., Häggblom M. M., Stefaniak S. (Eds.), *Soil and Water Pollution Monitoring, Protection and Remediation. NATO Science Series, Vol 69*. Dordrecht: Springer. doi:10.1007/978-1-4020-4728-2_6

Joseph, P. (2009). Mechanisms of cadmium carcinogenesis. *Toxicology and Applied Pharmacology, 238*(3), 272-279. doi:10.1016/j.taap.2009.01.011

Kargin, F., & Çogun, H.Y. (1999). Metal interactions during accumulation and elimination of zinc and cadmium in tissues of the freshwater fish *Tilapia nilotica*. *Bulletin of Environmental Contamination and Toxicology, 63*(4), 511-519

Karoonmakphol, P., & Chaiwiwatworakul, P. (2010). Evaluation of cadmium contamination due to bed load sediment transport in Mae Tao Creek, Mae Sot District, Tak Province, Thailand. *2010 International Conference on Chemistry and Chemical Engineering,* 114-118. doi:10.1109/iccceng.2010.5560375

Katz, T., Martens, P. N., & Sakamornsnguan, K. (2013). The minerals industry of Thailand current status and mineral regulatory framework. *World of Mining – Surface & Underground, 65*, 300-303.

Khaokaew, S., & Landrot, G. (2015). A field-scale study of cadmium phytoremediation in a contaminated agricultural soil at Mae Sot District, Tak Province, Thailand: (1) Determination of Cd-hyperaccumulating plants. *Chemosphere, 138*, 883-887. doi:10.1016/j.chemosphere.2014.09.108

Klaassen, C. D., Liu, J., & Diwan, B. A. (2009). Metallothionein protection of cadmium toxicity. *Toxicology and Applied Pharmacology, 238*(3), 215-220. doi:10.1016/j.taap.2009.03.026

Kleinow, K.M., Nichols, J. W., Hayton, W. L., McKim, J. M. & Barron, M. G. (2008). Toxicokinetics in Fishes. In Giulio, R. T. D., & Hinton, D.E. (Eds.), *The toxicology of fishes* (pp. 55-152). New York, NY: CRC Press.

Klinck, J. S., & Wood, C. M. (2011). In vitro characterization of cadmium transport along the gastro-intestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology, 102*(1-2), 58-72. doi:10.1016/j.aquatox.2010.12.009

Klinck, J. S., & Wood, C. M. (2013). In situ analysis of cadmium uptake in four sections of the gastro-intestinal tract of rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicology and Environmental Safety, 88*, 95-102. doi:10.1016/j.ecoenv.2012.10.028

Knapen, D., Reynders, H., Bervoets, L., Verheyen, E., & Blust, R. (2007). Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquatic Toxicology, 82*(3), 163-172. doi:10.1016/j.aquatox.2007.02.008

Kondera, E., Ługowska, K., & Sarnowski, P. (2014). High affinity of cadmium and copper to head kidney of common carp (*Cyprinus carpio* L.). *Fish Physiology and Biochemistry*, *40*(1), 9–22. http://doi.org/10.1007/s10695-013-9819-1

Koropatnick, J., & Zalups, R. K. (1997). Effect of non-toxic mercury, zinc or cadmium pretreatment on the capacity of human monocytes to undergo lipopolysaccharide-induced activation. *British Journal of Pharmacology, 120*(5), 797-806. doi:10.1038/sj.bjp.0700975

Kovarova, J., Kizek, R., Adam, V., Harustiakova, D., Celechovska, O., & Svobodova, Z. (2009). Effect of cadmium chloride on metallothionein levels in carp. *Sensors, 9*(6), 4789-4803. doi:10.3390/s90604789

Krissanakriangkrai, O., Supanpaiboon, W., Juwa. S., Chaiwong, S., Swaddiwudhipong W., & Anderson, K. A. (2009). Bioavailable cadmium in water and fish, in highly contaminated area on the Thai-Myanmay border. *Thammasat International Journal of Science and Technology*, *14*(4), 60-68.

Kumar, & Singh. (2010). Cadmium toxicity in fish: An overview. *GERF Bulletin of Biosciences*, *1*, 41-47.

Langston, W., Chesman, B., Burt, G., Pope, N., & Mcevoy, J. (2002). Metallothionein in liver of eels *Anguilla anguilla* from the Thames Estuary: an indicator of environmental quality? *Marine Environmental Research, 53*(3), 263-293. doi:10.1016/s0141-1136(01)00113-1

Larsson, A., Bengtsson, B. E., & Haux, C. (1981). Disturbed ion balance in flounder, *Platichthys flesus L.* exposed to sublethal levels of cadmium. *Aquatic Toxicology, 1*(1), 19-35. doi:10.1016/0166-445x(81)90004-7

Laurie, A. D. (2004). Quantitation of metallothionein mRNA from the New Zealand common bully (*Gobiomorphus cotidianus*) and its implications for biomonitoring. *New Zealand Journal of Marine and Freshwater Research, 38*(5), 869-877. doi:10.1080/00288330.2004.9517286

Linde, A., Sánchez-Galán, S., Vallés-Mota, P., & García-Vázquez, E. (2001). Metallothionein as Bioindicator of Freshwater Metal Pollution: European Eel and Brown Trout. *Ecotoxicology and Environmental Safety, 49*(1), 60-63. doi:10.1006/eesa.2001.2042

Liu, J., Qu, W., & Kadiiska, M. B. (2009). Role of oxidative stress in cadmium toxicity and carcinogenesis. *Toxicology and Applied Pharmacology, 238*(3), 209-214. doi:10.1016/j.taap.2009.01.029

Liu, K., Chi, S., Liu, H., Dong, X., Yang, Q., Zhang, S., & Tan, B. (2015). Toxic effects of two sources of dietborne cadmium on the juvenile cobia, *Rachycentron canadum* L. and tissue-specific accumulation of related minerals. *Aquatic Toxicology,165*, 120-128. doi:10.1016/j.aquatox.2015.05.013

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods, 25*(4), 402-408. doi:10.1006/meth.2001.1262

Lizardo-Daudt, H. M, & Kennedy, C. (2008). Effects of cadmium chloride on the development of rainbow trout *Oncorhynchus mykiss* early life stages. *Journal of Fish Biology, 73*, 702–718.

Malakootian, M., Tahergorabi, M., Daneshpajooh, M., & Amirtaheri, K. (2011). *Sacha International Academic Journals, 1*, 94-100.

Martelli, A., Rousselet, E., Dycke, C., Bouron, A., & Moulis, J. (2006). Cadmium toxicity in animal cells by interference with essential metals. *Biochimie, 88*(11), 1807-1814. doi:10.1016/j.biochi.2006.05.013

McGeer, J.C., Niyogi, S., Smith, D.S. (2012). Cadmium. In Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), *Homeostasis and toxicology of non-essential metals, fish physiology 31B* (pp. 125–184). New York: Elsevier.

Mishra, A. K., & Mohanty, B. (2008). Acute toxicity impacts of hexavalent chromium on behavior and histopathology of gill, kidney and liver of the freshwater fish, *Channa punctatus* (Bloch). *Environmental Toxicology and Pharmacology, 26*(2), 136-141. doi:10.1016/j.etap.2008.02.010

Mumford, S., Heidel, J., Smith, C., Morrison, J., MacConnell, B., & Blazer, V. (2007). *Fish Histology and Histopathology*. Retrieved January 25, 2016, from https://training.fws.gov/resources/course-resources/fish-histology/Fish_Histology_Manual_v4.pdf.

National Resources Canada. (2008). *Canadian minerals yearbook*. Canada: Mineral and Metal Sector Natural Resources Canada.

Netpae, T., Suckley, S., & Phalaraksh, C. (2015). Cadmium tolerance fungi isolated from polluted sites in the Mae Tao creek, Thailand. *Advanced Studies in Biology, 7*, 29-37. doi:10.12988/asb.2015.41050

Nico, L., Sharp, P., & Collins, T. (2011). Imported Asian swamp eels (Synbranchidae: Monopterus) in North American live food markets: Potential vectors of non-native parasites. *Aquatic Invasions, 6*(1), 69-76. doi:10.3391/ai.2011.6.1.08

Nordberg, M., & Nordberg, G. F. (2009). Metallothionein: Historical development and overview. In Sigel, A., Sigel, H.,and Sigel, R. K. O. Metallothionein and Related Chelators, *Met. Ion Life. Sci.* (pp. 1-30). Chichester, UK: John Wiley & Sons, Ltd.

Ognjanović, B.I., Marković, S.D, Pavlović, S.Z., Zikić, R. V., Stajn, A.S., & Saicić, Z.S. (2008). Effect of chronic cadmium exposure on antioxidant defense system in some tissues of rats: protective effect of selenium. *Physiological Researchs, 57*(3), 403-11.

Padungtod, C. & Swaddiwudhipong, W. (2007). *Health risk management for cadmium contamination in thailand: Are challenges overcome?*. Retrieved January 25, 2016, from http://www.who.int/ifcs/documents/forums/forum5/

Perera, P. A., Kodithu, S. P., Sundara, T. V., & Edirisingh, U. (2015). Bioaccumulation of Cadmium in Freshwater Fish: An Environmental Perspective. *Insight Ecology, 4*(1), 1-12. doi:10.5567/ecology-ik.2015.1.12

Ponsen, S., Narkkong, N., Pamok, S., & Aengwanich, W. (2009). Comparative hematological values, morphometric and morphological observation of the blood cell in capture and culture asian eel, *Monopterus albus* (Zuiew). *American Journal of Animal and Veterinary Sciences, 4*(2), 32-36. doi:10.3844/ajavsp.2009.32.36

Powell, D. B. (2000). Immune system. In Ostrander [redacted]. *The Laboratory fish* (pp. [redacted]

Pronina, S. V., Batueva, M. D., & Pronin, N. M. (2014). Characteristics of melanomacrophage centers in the liver and spleen of the roach *Rutilus rutilus* (Cypriniformes: Cyprinidae) in Lake Kotokel during the Haff disease outbreak. *Journal of Ichthyology, 54*(1), 104-110. doi:10.1134/s003294521401010x

Quirós, L., Piña, B., Solé, M., Blasco, J., López, M. Á, Riva, M. C., . . . Raldúa, D. (2007). Environmental monitoring by gene expression biomarkers in *Barbus graellsii*: Laboratory and field studies. *Chemosphere, 67*(6), 1144-1154. doi:10.1016/j.chemosphere.2006.11.032

Raskovic, B., Stankovic, M., Markovic, Z., & Poleksic, V. (2011). Histological methods in the assessment of different feed effects on liver and intestine of fish. *Journal of Agricultural Sciences, Belgrade, 56*(1), 87-100. doi:10.2298/jas1101087r

Rhee, J., Raisuddin, S., Hwang, D., Lee, K., Kim, I., & Lee, J. (2009). Differential expression of metallothionein (MT) gene by trace metals and endocrine-disrupting chemicals in the hermaphroditic mangrove killifish, *Kryptolebias marmoratus. Ecotoxicology and Environmental Safety, 72*(1), 206-212. doi:10.1016/j.ecoenv.2008.06.001

Rose, S., Vincent, S., Meena, B., Suresh, A., & Mani, R. (2014). Metallothinein induction in fresh water catfish *Clarias gariepinus* on exposure to cadmium. *International Journal of Pharmacy and Pharmacetical Science, 6*(1), 377-383.

Rumahlatu, D., Corebima, A. D., Amin, M., & Rachman, F. (2012). Kadmium dan efeknya terhadap ekspresi Protein Metallothionein pada *Deadema setosum* (Echinodea; Echinodermata). *Jurnal Penelitian Perikanan, 1*, 26-35.

Salazar-Lugo, R., Vargas, A., Rojas, L., & Lemus, M. Histopathological changes in the head kidney induced by cadmium in a neotropical fish *Colossoma macropomum. Open Veterinary Journal, 3*(2), 145-150.

Santovito, G., Piccinni, E., Boldrin, F., & Irato, P. (2012). Comparative study on metal homeostasis and detoxification in two Antarctic teleosts. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 155*(4), 580-586. doi:10.1016/j.cbpc.2012.01.008

Scoullos, M. J. (Ed), Vonkeman, G. H., Thornton, I., & Makuch, Z. (2001). *Mercury-Cadmium-Lead: Handbook for Sustainable Heavy Metals Policy and Regulation.* Dordrecht: Kluwer Academic Press.

Shafland, P. L., Gestring, K. B., & Stanford, M. S. 2010. An Assessment of the Asian Swamp Eel (*Monopterus albus*) in Florida. *Reviews in Fisheries Science. 18*(1), 25–39.

Shariati, F., Sari, A. E., Mashinchian, A., & Pourkazemi, M. (2010). Metallothionein as potential biomarker of cadmium exposure in persian sturgeon (*Acipenser persicus*). *Biological Trace Element Research, 143*(1), 281-291. doi:10.1007/s12011-010-8877-9

Silva, A. G., & Martinez, C. B. (2007). Morphological changes in the kidney of a fish living in an urban stream. *Environmental Toxicology and Pharmacology, 23*(2), 185-192. doi:10.1016/j.etap.2006.08.009

Simmons, R., Pongsakul, P., Saiyasitpanich, D., & Klinphoklap, S. (2005). Elevated levels of cadmium and zinc in paddy soils and elevated levels of cadmium in rice grain downstream of a zinc mineralized area in Thailand: Implications for public health. *Environmental Geochemistry and Health, 27*(5-6), 501-511. doi:10.1007/s10653-005-7857-z

Sivaperumal, P., Sankar, T., & Viswanathannair, P. (2007). Heavy metal concentrations in fish, shellfish and fish products from internal markets of India vis-a-vis international standards. *Food Chemistry, 102*(3), 612-620. doi:10.1016/j.foodchem.2006.05.041

Squadrone, S., Prearo, M., Brizio, P., Gavinelli, S., Pellegrino, M., Scanzio, T., . . . Abete, M. (2013). Heavy metals distribution in muscle, liver, kidney and gill of European catfish (*Silurus glanis*) from Italian Rivers. *Chemosphere, 90*(2), 358-365. doi:10.1016/j.chemosphere.2012.07.028

Stohs, S. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine, 18*(2), 321-336. doi:10.1016/0891-5849(94)00159-h

Stori, E., Rocha, M., Dias, J., Santos, C. D., Souza, C. D., Amaral, L., & Dias, J. (2014). Elemental characterization of injuries in fish liver. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 318*, 83-87. doi:10.1016/j.nimb.2013.05.109

Szczerbik, P., Mikolajczyk, T., Sokolowskamikolajczyk, M., Socha, M., Chyb, J., & Epler, P. (2006). Influence of long-term exposure to dietary cadmium on growth, maturation and reproduction of goldfish (subspecies: Prussian carp Carassius auratus gibelio B.). *Aquatic Toxicology, 77*(2), 126-135. doi:10.1016/j.aquatox.2005.11.005

Szebedinszky, C., Mcgeer, J. C., Mcdonald, D. G., & Wood, C. M. (2001). Effects of chronic Cd exposure via the diet or water on internal organ-specific distribution and subsequent gill Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry, 20*(3), 597-607. doi:10.1002/etc.5620200320

Tabassum, H., Ashafaq, M., Khan, J., Shah, M. Z., Raisuddin, S., & Parvez, S. (2016). Short term exposure of pendimethalin induces biochemical and histological perturbations in liver, kidney and gill of freshwater fish. *Ecological Indicators, 63*, 29-36. doi:10.1016/j.ecolind.2015.11.044

Taghipour, H., Mosafer, M., Pourakbar, M., & Armanfar, F. (2012). Heavy metal concentrations in groundwater used for irrigation. *Health Promotion Perpective, 2*(2), 205-210. doi: 10.5681/hpp.2012.024

Tan, S., Li, H., Jin, Y., & Yu, H. (2016). Cadmium exposure effects on the expression of metallothionein 2 gene in gtrass carp (*Ctenopharyn godonidellus*). *Genes Genom, 38*, 127-135. doi: 10.1007/s13258-015-0347-5.

Tekin-Özan, S., & Kir, I. (2008). Seasonal variations of heavy metals in some organs of carp (*Cyprinus carpio* L., 1758) from Beyşehir Lake (Turkey). *Environmental Monitoring and Assessment, 138*(1-3), 201-206. doi:10.1007/s10661-007-9765-4 thai_padungtod.pdf

Thamjedsada, T., & Chaiwiwatworakul, P. (2012). Evaluation of cadmium contamination in the Mae Tao Creek Sediment. *International Conference on Sustainable Environmental on Technologies (ICSET): Implication of Public Health. Environmental Geochemistry and Health,* 27, 501-511.

Thévenod, F. (2009). Cadmium and cellular signaling cascades: To be or not to be?. *Toxicology and Applied Pharmacology, 238*(3), 221-239. doi:10.1016/j.taap.2009.01.013

Timmermans, M. J., Ellers, J., Roelofs, D., & Straalen, N. M. (2005). Metallothionein mRNA expression and cadmium tolerance in metal-stressed and reference populations of the springtail *Orchesella cincta. Ecotoxicology, 14*(7), 727-739. doi:10.1007/s10646-005-0020-x

Unhalekhaka, U & Kositanont, C. (2008). Distribution of cadmium in soil around Zinc mining area. *Thai Journal of Toxicology*, *23*(2), 170-174.

United Nations Environment Programme (UNEP). (2010). *Final review of scientific information on cadmium*. Retrieved November 25, 2015, from drustage.unep.org/chemicalsandwaste/sites/unep.org.chemicalsandwaste/files/publications/GAELP_PUB_UNEP_GC26_INF_11_Add_2_Final_UNEP_Cadmium_review_and_appendix_Dec_2010.pdf.

Vašák, M., & Meloni, G. (2008). Metallothionein structure and reactivity. In Zatta, P (Ed.), *Metallothioneins in biochemistry and pathology* (pp. 3-26.). Singapore: World Scientific.

Velmurugan, B., Selvanayagam, M., Cengiz, E. I., & Unlu, E. (2007). Histopathology of lambda-cyhalothrin on tissues (gill, kidney, liver and intestine) of *Cirrhinus mrigala. Environmental Toxicology and Pharmacology, 24*(3), 286-291. doi:10.1016/j.etap.2007.07.001

Verbost, P. M., Van Rooij, J., Flik, G., Lock, R. A. C., & Wendelaar, B. S. E. (1989). The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *Journal of Experimental Biology, 145*, 185–197.

Vergani, L. (2009). Metallothioneins in aquatic organisms: fish, crustaceans, molluscs, and echinoderms. In Sigel, A., Sigel, H., & Sigel, R. K. O (Eds.), *Metallothionein and related chelators, met. ion life. sci.* (pp. 199-338), Chichester, UK: John Wiley & Sons, Ltd.

Waalkes, M. P. (2000). Cadmium carcinogenesis in review. *Journal of Inorganic Biochemistry, 79*(1-4), 241-244. doi:10.1016/s0162-0134(00)00009-x

Wang, Y., Fang, J., Leonard, S. S., & Rao, K. M. K. (2004). Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radical Biology and Medicine, 36*(11), 1434–1443. doi:10.1016/s0891-5849(04)00223-0

Wang, Y., Ferrari, M. C., Hoover, Z., Yousafzai, A. M., Chivers, D. P., & Niyogi, S. (2014). The effects of chronic exposure to environmentally relevant levels of waterborne cadmium on reproductive capacity and behaviour in fathead minnows. *Archives of Environmental Contamination and Toxicology, 67*(2), 181-191. doi:10.1007/s00244-014-0018-6

Wangsongsak, A., Utarnpongsa, S., Kruatrachue, M., Ponglikitmongkol, M., Pokethitiyook, P., & Sumranwanich, T. (2007). Alterations of organ histopathology and metallolhionein mRNA expression in silver barb, *Puntius gonionotus* during subchronic cadmium exposure. *Journal of Environmental Sciences, 19*(11), 1341-1348. doi:10.1016/s1001-0742(07)60219-8

Weeraprapan, P., Phalaraksh, C., Chantara, S., & Kawashima, M. (2015). Water quality monitoring and cadmium contamination in the sediments of Mae Tao stream, Mae Sot District, Tak Province, Thailand. *International Journal of Environmental Science and Development, 6*(2), 142-146. doi:10.7763/ijesd.2015.v6.577

Wei, Y., Zhang, J., Zhang, D., Tu, T., & Luo, L. (2014). Metal concentrations in various fish organs of different fish species from Poyang Lake, China. *Ecotoxicology and Environmental Safety, 104*, 182-188. doi:10.1016/j.ecoenv.2014.03.001

Wilson, J. M., & Laurent, P. (2002). Fish gill morphology: inside out. *Journal of Experimental Zoology, 293*, 192–213.

Włostowski, T., Krasowska, A., & Bonda, E. (2008). Joint effects of dietary cadmium and polychlorinated biphenyls on metallothionein induction, lipid peroxidation and histopathology in the kidneys and liver of bank voles. *Ecotoxicology and Environmental Safety, 69*(3), 403-410. doi:10.1016/j.ecoenv.2007.03.014

Wolf, J. C., & Wolfe, M. J. (2005). A brief overview of Nnonneoplastic hepatic toxicity in fish. *Toxicologic Pathology, 33*(1), 75-85. doi:10.1080/01926230590890187

Wu, H., & Liu. C. K. (1940). The buccopharyngeal epith-lium as the principal respiratory organ in *Monopterus javanicus*. *Sinensia, 11*, 221-239.

Wu, S. M., Jong, K. J., & Lee, Y. J. (2006). Relationships Among Metallothionein, Cadmium Accumulation, and Cadmium Tolerance in Three Species of Fish. *Bulletin of Environmental Contamination and Toxicology, 76*(4), 595-600. doi:10.1007/s00128-006-0961-7

Yang, M., Pan, C., Bo, J., & Wang, K. (2014). Regulation of metallothionein gene expression in response to benzo[a]pyrene exposure and bacterial challenge in marine cultured black porgy (*Acanthopagrus schlegelii*). *Chinese Journal of Geochemistry, 33*(4), 404-410. doi:10.1007/s11631-014-0705-z

Yeşilbudak, B., & Erdem, C. (2014). Cadmium accumulation in gill, liver, kidney and muscle tissues of common carp, *Cyprinus carpio*, and *Nile Tilapia*, *Oreochromis niloticus*. *Bulletin of Environmental Contamination and Toxicology, 92*(5), 546-550. doi:10.1007/s00128-014-1228-3

Yin, S. A., Ismail, A., & Zulkifli, S. Z. (2012). Heavy metals uptake by Asian swamp eel, *Monopterus albus* from paddy fields of Kelantan, Peninsular Malaysia: Preliminary study. *Tropical Life Sciences Research, 23*(2), 27–38.

Younis, E. S. M., Abdel A. W., Asgash N. A. Ebaiad, H., & Mubarak M. (2013). Histological changes in the liver and intestine of Nile Tilapia, *Oreochromis niloticus*, exposed to sublethal concentrations of cadmium. *Pakistan J. Zool, 45*, 833-841.

Zalups, R. K., & Ahmad, S. (2003). Molecular handling of cadmium in transporting epithelia. *Toxicology and Applied Pharmacology, 186*(3), 163-188. doi:10.1016/s0041-008x(02)00021-2

APPENDIX

APPENDIX A VECTOR

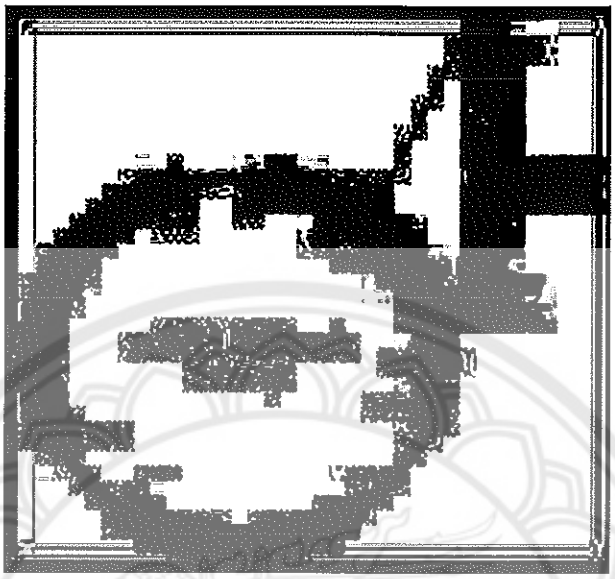

Figure 18 pJET 1.2/blunt vector map

APPENDIX B STANDARD CALIBRATION

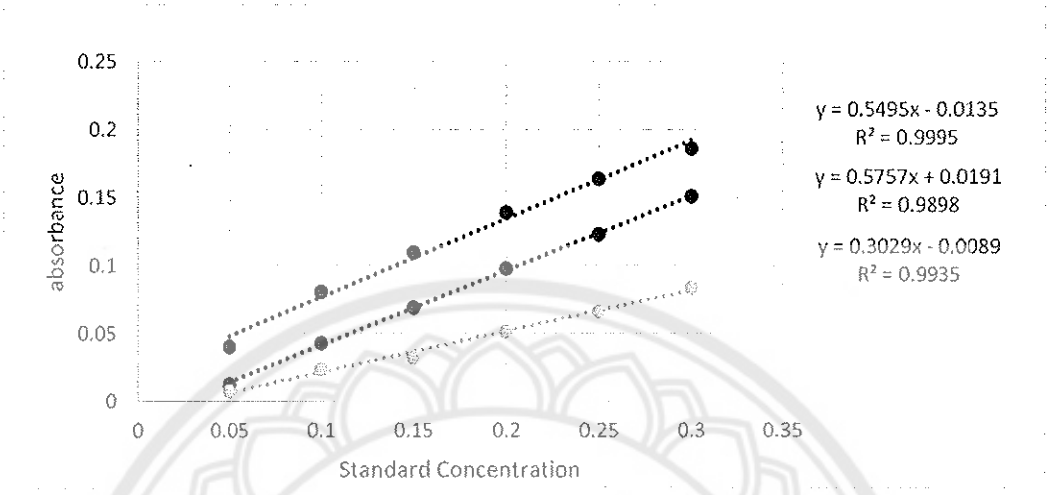

Figure 19 Standard curve comprimising six standard calibration of 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/L of Cd

APPENDIX C CHEMICALS, SOLUTIONS AND PROTOCOLS

PART I: CHEMICAL AND SOLUTIONS

1. 10% Neutral Buffer Formalin (NBF) (1000 ml)

1.1 Formalin	100 ml
1.2 H_2O	900 ml
1.3 NaH_2PO_4	4 g
1.4 Na_2HPO_4	6.5 g

1.5 Mixed all the ingredients and stored at room temperature.

2. Harris's Hematoxylin Solution (1000 ml)

2.1 Hematoxylin	5 g
2.2 Ethanol	50 ml
2.3 KAI$(SO_4)2$ (Potassium alum)	100 g
2.4 DI water	1000 ml
2.5 $NaIO_3$ (Sodium Iodate)	1 g
2.6 CH3COOH (Acetic acid)	40 ml

2.7 Dissolved hematoxylin in Ethanol and was then added to the potassium alum which has been previously dissolved in 1000 ml of warm DI water. Brought the mixture to a boil rapidly, removed from heat and added sodium iodate slowly and carefully. The solution was then brought to boil for 2-3 minutes and then was removed from heat. Cooled the solution by plunging into cold water or ice bath. After cooling down, filter using filter paper. Store the stock in brown bottle. For working Harris Hematoxylin, every 100 ml of hematoxylin stock was added with 4 ml acetic acid.

3. Eosin (200 ml)

3.1 Eosin Y	2 g
3.2 H_2O	40 ml
3.3 95% EtOH	160
3.3 80 % EtOH	600 ml
3.4 Acetic acid	4 ml

3.4 eosin Y stock was prepared by mixing Eosin Y with H_2O until dissolved then added 95 % EtOH. The stock was kept in room temperature. For the working

eosin Y, every 200 ml of eosin Y stock was added to 600 ml of 80% EtOH and mixed it well. Then, 4 ml of acetic acid was added to the mixture. The solution was kept in room temperature.

4. Isopropanol (50 ml)

5. Chloroform (50 ml)

6. 80% Ethanol (100 ml)

6.1 Absolute ethanol	80 ml
6.2 Distilled water (DW)	20 ml

6.3 Mixed all ingredients.

7. 2 % SDS (50 ml)

7.1 SDS	1 g
7.2 H_2O	50 ml

7.3 Dissolved SDS in 50 ml of H_2O, stored at room temperature.

8. 0.4 N NaOH (50 ml)

8.1 NaOH	0.8 g
8.2 H_2O	50 ml

8.3 Transferred to NaOH volumetric flask and make up to 50 ml, autoclaved at 121°C for 30 minutes, cooled down and stored at room temperature.

9. 2 M NaCl (50 ml)

9.1 NaCl	5.84 g
9.2 H_2O	50 ml

9.3 Transferred to NaOH volumetric flask and make up to 50 ml, autoclaved at 121°C for 30 minutes, cooled down and stored at room temperature.

10. 0.5 M EDTA, pH 8.0 (50ml)

10.1 EDTA	9.306 g
10.2 H_2O	40 ml

10.3 Mixed the EDTA and H_2O, stirred vigorously on a magnetic stirrer. Dissolve all EDTA by adjusting pH to 8.0 with NaOH, made it up to 50 ml. Autoclaved at 121°C for 30 minutes, cooled down and stored at room temperature .

11. 10 mM EDTA (50 ml)

11.1 0.5 M EDTA	1 ml
11.2 H_2O	49 ml

11.3 Mixed all ingredients.

12. 2 M (D+) Glucose (10 ml)

12.1 D(+) Glucose	3.6 g
12.2 H_2O	10 ml

12.3 filter sterile into sterile tubes. Aliquot in 2 ml tubes and store at -20°C.

13. 1 M MgSO4.7H2O (10 ml)

14. 1 M MgCl2.6H2O (10 ml)

15. Cracking buffer (10 ml)

20% sucrose, 50 mM NaOH, 0.5% SDS

15.1 Sucrose	2 g
15.2 0.4 M NaOH	5 ml
15.3 2%SDS	2.5 ml

15.4 Mixed all ingredients

16. 1 % agarose gel (50 ml)

16.1 Agarose	0.5 g
16.2 0.5X TBE buffer	50 m

16.3 Mixed all ingredients and heat by putting in the microwave until all agarose dissolved.

17. 100 mM $CaCl_2$ (100 ml)

17.1 $CaCl_2$	1.47 g

17.2 Transfer to volumetric flask and make up to 100 ml, sterile filter, and store at 4°C.

18. 85% mM $CaCl_2$, 15 % glycerol (50 ml)

18.1 100 mM $CaCl_2$	42.5 ml
18.2 Glycerol	7.5 ml

18.3 Transfer and filter to sterile polyethylene tube and store at 4°C.

19. SOB (60 ml)

19.1 Tryotone	1.2 g
19.2 Yeast extract	0.3 g
19.3 NaCl	0.0351

19.4 Transferred to volumetric flask and make up to 20 ml, autoclave at 121°C for 30 minutes, cool down and store at 4°C, added with:

1 M $MgSO_4.7H_2O$	600 µl
1 M $MgCl_2.6H_2O$	600 µl

20. SOC (20 ml)

20.1 Tryptone	0.4 g
20.2 Yeast extract	0.1 g
20.3 NaCl	0.0117 g
20.4 KCl	0.0037 g

20.5 Transferred to volumetric flask and make up to 20 ml with H_2O, autoclaved at 121°C for 30 minutes, cooled down and stored at 4°C

21. LB plate (100 ml)

21.1 Tryptone	1 g
21.2 Yeast extract	0.5 g
21.3 NaCl	1g
21.4 Agar	2 g

21.5 Transferred to volumetric flask and made up to 100 ml with H_2O, autoclaved at 121°C for 30 minutes, cooled down and store at 4°C

22. 2X YT broth (100 ml)

22.1 Tryptone	1.6 g
22.2 Yeast extract	1 g
22.3 NaCl	0.5 g

22.4 Transferred to volumetric flask and made up to 100 ml with H_2O, autoclaved at 121°C for 30 minutes, cooled down and stored at 4°C.

23. 2X YT broth (100 ml)

23.1 Tryptone	1.6 g
23.2 Yeast extract	1 g
23.3 NaCl	0.5 g
23.4 Agar	1.5 g

23.5 Transferred to volumetric flask and made up to 100 ml with H_2O, autoclaved at 121°C for 30 minutes, cooled down and stored at 4°C.

PART II: PROTOCOLS

1. Measuring level of cadmium

1.1 Preparation of the standard Cd solutions

Begin with the 0.05 ppm standard using stock standard of 10 ppm. The desired volume of calibration standard is 50 mL. Therefore, the volume of stock standard must be computed as shown below:

Cstock = 10 ppm

Ccalibration = 0.05 ppm

Vcalibration = 50 mL

Vstock = Vcalibration x Ccalibration / Cstock

Vstock = (50 mL) x (0.05 ppm) / (10 ppm) = 0.25 mL. This value was then added with 49.75 mL of DI water (total volume is 50 mL). Similarly, for the 0.1, 0.15, 0.2, 0.25 and 0.3 ppm standard.

1.1.1 0.05 ppm standard = 0.25 mL of 10 ppm stock standard + 49.75 mL DI water.

1.1.2 0.10 ppm standard = 0.5 mL of 10 ppm stock standard + 49.50 mL DI water.

1.1.3 0.15 ppm standard = 0.75 mL of 10 ppm stock standard + 49.25 mL DI water.

1.1.4 0.20 ppm standard = 1.0 ml of 10 ppm stock standard + 49.0 mL DI water.

1.1.5 0.25 ppm standard = 1.25 ml of 10 ppm stock standard + 48.75 mL DI water.

1.1.6 0.30 ppm standard = 1.5 ml of 10 ppm stock standard + 48.5 mL DI water.

1.2 Preparation of the sample solutions using acid digestion method for fish organs (kidney, liver and gills) samples

1.2.1 Dissected the fish and take the organs (gill, liver and kidney)

1.2.2 Dried and oven at 150 °C until constant weight

1.2.3 Homogenized and ground to powder

1.2.4 Took 0.5 g of dry tissue and transfer to polyethylene tubes

1.2.5 Added 5 ml of freshly prepared nitric acid-perchloric acd (10:4)

1.2.6 Left overnight at room temperature

1.2.7 Put digestion tube in water bath set boiling water temperature 100 °C and boil for 2 hours until tissue dissolve

1.2.8 Allowed to cool, filtered using Whatman filter paper and transferred to 25 ml volumetric flask made it up to mark with DI water

1.2.9 Kept it in the plastic bottles for analyzed by using atomic absorption spectrophotometer (AAS)

1.3 Preparation of the sample solutions using acid digestion method for sediment samples

1.3.1 Dried and oven at 150 °C until constant weight

1.3.2 Homogenized, ground and sieved through a 2 mm sieve.

1.3.3 Took 0.5 g of dry tissue and transferred to volumetric flask

1.3.4 Added 10 ml of freshly prepared nitric and left overnight at room temperature for pre-digestion process

1.3.5 Put sample at 130 °C for 24 hours

1.3.6 Allowed to cool, filtered using Whatman filter paper and transferred to 25 ml volumetric flask made it up to mark with DI water

1.3.7 Kept it in the plastic bottles for analyzed by using atomic absorption spectrophotometer (AAS)

2. Tissues processing and H&E staining

2.1 Fixation: Analyzed tissues (liver, kidney and gills) were put in pre-labelled and placed into formalin (histological fixative) as soon the tissues were removed from fish body.

2.2 Decalcification (optional, for gills only), after fixation, the bone in the gills must be decalcified, or else it would not cut on the microtome, the steps are as follows:

2.2.1 Immersed the gills in 5% nitric acid and check it every 10 minutes until it become soft.

2.2.2 Rinse in running water for 15- 30 minutes until the smell disappeared.

2.3 Dehydration: It aimed to displace the residual aqueous fixative as well as cellular water. This was done with a series of alcohols, i.e. 30%, 40%, 50%, 60%,70%, 80%, 90 %, 95% and 100%.

2.3.1 The specimens were placed on concentration 30%-95% for 1 hour per each and on concentration 100% alcohol for overnight.

2.4 Clearing: It aimed to remove dehydrant with a substance that will be miscible with the embedding medium (paraffin)

2.4.1 Immersed sample in xylene solution overnight

2.5 Infiltration: Once the tissues become transparent, they are transferred to the paraffin wax contained in the wax bath for embedding (temperature 58°C).

2.6 Embedding and sectioning: Once the tissues were embedded with the paraffin wax, tissue blocks were prepared with the help of brass moulds and section cutting of 5 µm was done with the help of rotary microtome. Thin ribbons are transferred to floatation bath. Glass slides coated with egg albumin were used to take out the thin sections from the floatation bath.

2.7 H&E staining steps:

2.7.1 Deparaffinize:

2.7.1.1	Xylene	for 10 minutes
2.7.1.2	Xylene	for 10 minutes

2.7.2 Rehydration

2.7.2.1	Absolute alcohol	for 5 minutes
2.7.2.2	Absolute alcohol	for 5 minutes
2.7.2.3	95% alcohol	for 2 minutes
2.7.2.4	70% ethyl alcohol	for 2 minutes
2.7.2.5	Washed in running tap water	for 1 minute

2.7.3 Stain

2.7.3.1	Hematoxylin solution	for 6-7 minutes
2.7.3.2	Washed in running tap water	for 1 minute
2.7.3.3	Bluing in lithium carbonate	for 30 seconds to 1 minute
2.7.3.4	Washed in running tap water	for 1 minute
2.7.3.5	95% alcohol	for 10 dips
2.7.3.6	Eosin Y solution	for 30 seconds to 1 minute

2.7.4 Dehydration

2.7.4.1	95% alcohol	for 5 minutes
2.7.4.2	Absolute alcohol	for 5 minutes
2.7.4.3	Absolute alcohol	for 5 minutes

2.7.5 Clearing

2.7.5.1	Xylene	for 5 minutes
2.7.5.2	Xylene	for 5 minutes
2.7.5.3	Xylene	for 5 minutes

2.7.6 Mounting: Mount the slides with 1-2 drops of permount and cover with cover slides, avoid bubbles.

2.8 Observed under microscope

3. Gene cloning

3.1 RNA extraction

3.1.1 Added 1 ml of TRIzol™ Reagent per 50--100 mg of tissue to the sample and homogenize using a homogenizer

3.1.2 Incubated for 5 minutes

3.1.3 Centrifuged the sample for 10 minutes at 10.000 rpm at 4°C

3.1.4 Transferred the supernatant into new tube

3.1.5 Added 200 µL of chloroform

3.1.6 Vortexed and let sit for 3 minutes

3.1.7 Centrifuged the sample for 5 minutes at 10.000 rpm at 4°C. The mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.

3.1.8 Transferred the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out

3.1.9 Added 500 µL of isopropanol to the aqueous phase and mixed by inverting the tube.

3.1.10 Incubated for 30 minutes.

3.1.11 Centrifuged for 30 minutes at 12,500 rpm at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.

3.1.12 Discarded the supernatant by aspiration

3.1.13 Resuspended the pellet in 1 mL of 80% ethanol

3.1.14 Centrifuged for 5 minutes at 12.500 rpm at 4°C

3.1.15 Discarded the supernatant

3.1.16 Vacuum or air dry the RNA pellet for 5–10 minutes.

3.1.17 Resuspended the pellet in 20–50 µL of Nuclease-free water by pipetting up and down. Store at -80°C.

3.1.18 Determined the RNA yield by measuring the absorbance at 260 and 280 nm using microplate reader. Calculated the A260/A280 ratio. A ratio of ~2 is considered pure.

3. 2 cDNA synthesis

3.2.1 The cDNA synthesis was done using Tetro cDNA Synthesis Kit (Bioline, USA).

3.2.2 Vortexed all solutions and spin down briefly before use

3.2.3 Set up the priming premix on ice and assembled the reaction to final volume of 20 µl as follows:

5 µl of total RNA (1 µg/ µl)

3 µl of dT-UPM

1 µl of 10 µM dNTP

4 µl of 5X RT buffer

1 µl of RiboSafe RNase Inhibitor

1 µl of Tetro Reverse Transcriptase

2 µl of nuclease free water

3.2.4 Mixed gently by pipetting

3.2.5 Incubated samples at 45°C for 30 min within a PCR T100 thermal cycler (Bio Rad) and terminate reaction by incubating at 85°C for 5 min, then chill on ice.

3.2.6 Stored reaction at –20°C for PCR process.

3.3 3' RACE amplification

3.3.1 The 3'RACE amplification was performed using Phusion High-Fidelity DNA Polymerase

3.3.2 Set up and assembled all reaction components on ice to final volume of 20 µl as follows:

4 µl of 5X Phusion HF buffer

0.4 µl of 10 mM dNTPs

2 µl of 10 µM gene specific primer for MT (forward primer)

2 µl of 10 µM UPM-1 (reverse primer)

0.2 µl of Phusion DNA Polymerase

9.4 µl of nuclease free water

2 µl of cDNA template

3.3.3 Samples were mixed and centrifuged (spin down) and performed within a PCR T100 thermal cycler (Bio Rad) with following condition:

Preheat	98°C for 2 mins 30 secs
Denature	98°C for 15 secs
Annealing	60°C for 15 secs
Extension	72°C for 15 secs
Final extension	72°C for 5 mins

3.3.4 Loaded 5 µl of PCR product for electrophoresis to 0.7% agarose gel for 30 minutes.

3.4 Purification

3.4.1 The purification of PCR product was performed using RBC

3.4.2 Cut the band and place it into the tube

3.4.3 Added DF buffer 500 µl

3.4.4 Put in heat block 55 °C for 10-15 minutes. Mix every three minute by gently inverse.

3.4.5 Loaded into column tube

3.4.6 Centrifuged at 5.000 rpm for 2 minutes and discard supernatant

3.4.7 Added wash buffer with EtOH 500 µl

3.4.8 Centrifuged at 12.500 rpm for 1 minute and discard supernatant

3.4.9 Centrifuged at 12.500 rpm for 2 minutes

3.4.10 Moved the column to tube and let sit at room temperature for 5 minutes

3.4.11 Added DI or nuclease free water 30 µl and store at room temperature for 5 minutes

3.4.12 Centrifuged 12.500 rpm for 2 minutes

3.4.13 Checked the purification result by running it on 1% agarose gel

3.4.14 Stored the mixture at -20 °C

3.5 Ligation

3.5.1 The ligation was performed using pJET 1.2/blunt cloning vector

3.5.2 Set up the ligation reaction on ice. The reaction mix was set in a 0.2 ml PCR tube as follows:

10 µl of 2X reaction buffer

2 µl of purified PCR product

1 µl of pJET 1.2 Blunt cloning vector

6 µl of nuclease free water

1 µl of T4 DNA ligase

3.5.3 Vortexed briefly and spin down

3.5.4 Incubate the ligation mixture at room temperature for 30 minutes

3.5.5 The ligation mixture was kept at 4°C to be used in transformation process

3.6 Competent cell preparation and transformation

3.6.1 Aliquot 2 ml SOB (added with MgSO4 and MgCl4) to 15 ml centrifuge tube

3.6.2 Inoculated single colony of *E. coli* from a plate that has been incubated for16-20 hours at 37°C into 2 ml SOB medium.

3.6.3 Incubated vigorous shaking at 200 rpm overnight

3.6.4 Aliquot 0.5 ml the grown culture (*E. coli* and SOB mixture) to 58 ml of SOB

3.6.5 Incubated with vigorous shaking at 200 rpm for 3 hours

3.6.6 Transferred 30 ml SOB into two 50 ml tubes, centrifuged at 3000 rpm for 10 minutes at 4°C and discarded the supernatant

3.6.7 Resuspended the cell pellets in ice-cold 10 ml of 100 mM $CaCL_2$ to both tubes. Incubated and shook the resuspended cells on ice for 10 minutes

3.6.8 Collected the cells after being centrifuged at 3000 rpm at 4°C for 10 minutes. Discarded supernatant.

3.6.9 Added 1 ml of 100 mM $CaCl_2$ + 15% glycerol. Incubated and shook on ice for 10 minutes.

3.6.10 Dispensed the competent cells into aliquots of 100 µl and store them at −80°C for transformation process.

3.7 Transformation by heat shock method

3.7.1 Incubated SOC (added with 200 µl 1 M $MgSO_4$ + 200 µl of 1 M $MgCl_2$ + 200 µl of glucose) at 37°C for 1 hour

3.7.2 Prepared 2X YT plate that has been spread with 25 µl ampicillin

3.7.3 Thaw 100 µl of competent cells carefully on ice

3.7.4 Added 10 µl of plasmid (ligation mixture) and mixed it by using finger.

3.7.5 Incubated on ice for 30 minutes

3.7.6 Quickly transferred the tubes to a water bath previously set at 42 °C. Incubated for 1 min, and then quickly transferred to ice for 5 minutes.

3.7.7 Added 900 µl of SOC into the tubes

3.7.8 Incubated at 37°C with shaking at 200 rpm for 1 hour

3.7.9 Centrifuged at 3000 rpm for 10 minutes

3.7.10 Removed of 800 µl of medium

3.7.11 Mixed the remaining medium by pipetting

3.7.12 Spread 50-100 µl cell to 2X YT (plus ampicillin)

3.7.13 Incubated at 37°C for 16-18 hours

3.8 Colony cracking and colony PCR

3.8.1 Picked a single colony and streaked on 2X YT plate (20 colonies per plate).

3.8.2 Incubated at 37°C overnight

3.8.3 Streaked the colonies at the bottom of microcentrifuge tubes

3.8.4 Added 7.5 µl of cracking buffer

3.8.5 Added 7.5 µl of 10 µM EDTA and vortexed

3.8.6 Dropped 2 µl of loading buffer on the lid of tubes. Let it sit for 5 minutes at room temperature

3.8.7 Spin the loading buffer down to the bottom and vortexed

3.8.8 Transferred into ice for 5 minutes

3.8.9 Centrifuged at 12.500 rpm for 5 minutes at 4°C

3.8.10 Run 10 µl of the mixture on 0.7% agarose gel.

3.9 Colony PCR

3.9.1 The PCR colony screening was performed using Ready 2X Go kit

3.9.2 Set up and assembled all reaction components on ice to final volume of 10 µl as follows:

5 µl of Ready 2X Go

1 µl of 10 µM gene specific primer for MT (forward primer)

1 µl of 10 µM UPM-1 (reverse primer)

1 µl of nuclease free water

2 µl of template (blue mixture from colony cracking method)

3.9.3 Samples were mixed and spin down and performed within a PCR T100 thermal cycler (Bio Rad) with following condition:

Preheat	98°C for 2 mins 30 secs
Denature	98°C for 15 secs
Annealing	60°C for 15 secs
Extension	72°C for 15 secs
Final extension	72°C for 5 mins

3.9.4 Loaded 5 µl of PCR product for electrophoresis to 0.7% agarose gel for 30 minutes.

3.10 Plasmid extraction

3.10.1 The extraction of plasmid was performed using GeneJET Plasmid Miniprep Kit (Fermentas)

3.10.2 Loaded 1.5 ml of cell suspension into 1.5 ml tubes

3.10.3 Centrifuged at 12.000 rpm

3.10.4 Discarded the medium

3.10.5 Added 250 µl of resuspension solution (with RNase A) and vortexed

3.10.6 Added 250 µl of lysis solution and inverted the tube 4-6 times

3.10.7 Added neutralization solution 350 µl and inverted the tube 4-6 times

3.10.8 Centrifuged at 11.000 rpm for 5 minutes at 4°C

3.10.9 Loaded supernatant to GeneJET spin column

3.10.10 Centrifuged for at 11.000 rpm for 1 minute

3.10.11 Discarded supernatant

3.10.12 Added 500 µl of wash solution, centrifuged at 11.000 rpm for 1 minute at 4°C

3.10.13 Added 500 µl of wash solution, centrifuged at 11.000 rpm for 1 minute at 4°C

3.10.14 Centrifuged empty column for at 11.00 rpm for 1 minute

3.10.15 Moved spin column to new tube

3.10.16 Added with 30 µl of H_2O and centrifuged at 11.00 rpm for 2 minutes at 4°C

3.10.17 Collected the flow-through

3.10.18 Checked the quality of extracted plasmid by electrophoresing it on 1% agarose gel.

4. Real-Time PCR

4.1 RNA Extraction

The RNA extraction is described in part 3.1.

4.2 cDNA synthesis

4.2.1 The cDNA synthesis was done using Tetro cDNA Synthesis Kit (Bioline, USA).

4.2.2 Vortexed all solutions and spin down briefly before use

4.2.3 Set up the priming premix on ice and assembled the reaction to final volume of 20 µl as follows:

2 µl of total RNA (1 µg/ µl)

1 µl of oligo dT_{18}

1 µl of 10 µM dNTP

4 µl of 5X RT buffer

1 µl of Ribosafe RNase Inhibitor

1 µl of Tetro Reverse Transcriptase

10 µl of nuclease free water

4.2.4 Mix gently by pipetting

4.2.5 Incubated samples at 45°C for 30 min within a PCR T100 thermal cycler (Bio Rad) and terminate reaction by incubating at 85°C for 5 min, then chilled on ice.

4.3 Real-Time PCR

4.3.1 Real-Time PCR was performed using 2x SensiFAST SYBR NO-ROX Kit

4.3.2 Set up and assembled all reaction components on ice to final volume of 20 µl for 18S and MT as follows:

10 µl of 2X sensiFAST SYBR
0.3 µl of 10 µM MT/18S (forward primer)
0.3 µl of 10 µM MT/18S (reverse primer)
8.4 µl of nuclease free water
1 µl of cDNA template

4.3.3 Samples are mixed and spin down and performed within Swift SpectrumTM 48 Real Time Thermal Cycler (ESCO Micro Pte. Ltd) with following condition:

Preheat	95°C for 3 mins
Denature	95°C for 15 secs
Annealing	58°C for 10 secs
Extension	72°C for 15 secs

4.3.4 Result were analyzed using $2^{-\Delta\Delta CT}$ method.