การใช้กบหนอง (*Fejervarya limnocharis*) เป็นตัวเฝ้าระวังการปนเปื้อนของแคดเมียมในจังหวัดตาก ประเทศไทย

โมห์ด ชาม บิน โอทมาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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USING THE RICE FROG (*FEJERVARYA LIMNOCHARIS)* AS A SENTINEL SPECIES FOR CADMIUM CONTAMINATION IN TAK PROVINCE, THAILAND

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MOHD SHAM BIN OTHMAN: USING THE RICE FROG (*FEJERVARYA LIMNOCHARIS*) AS A SENTINEL SPECIES FOR CADMIUM CONTAMINATION IN TAK PROVINCE, THAILAND. THESIS ADVISOR: NOPPADON KITANA Ph.D., THESIS CO-ADVISOR: PROFESSOR MARK GREGORY ROBSON, Ph.D.,204 pp.

By utilizing the cadmium contamination history in Mae Sot District, Tak Province, Thailand, this research was developed to use a common native amphibian species, the rice frog (*Fejervarya limnocharis*) as a sentinel species for the problem. The objective of this research was to determine the suitability of this species as a sentinel for cadmium contamination. Frogs were collected on monthly basis during November 2007 and October 2008 from several rice fields in contaminated site (Mae Tao) and reference site (Mae Pa) in Mae Sot District, Tak Province. Frog samples were subjected to analyses for cadmium contamination level, hepatic biomarker, morphometry and gravimetry, and biological and ecological effects. The contaminant analysis showed that Mae Tao frogs had higher hepatic, renal, testicular and whole organismal cadmium than Mae Pa frogs, with the kidney as the greatest accumulator. This was mirrored by the bioconcentration factor data that showed the same trend. The biomarker analysis showed that frogs from the contaminated site had higher hepatic metallothionein and hepatic glutathione-S-transferase levels than those from the reference site. However, only glutathione-S-transferase showed a strong stressor-response correlation with hepatic cadmium. The morphometric and gravimetric analysis revealed that only condition factor, renosomatic index and female gonadosomatic index showed significant differences between Mae Pa and Mae Tao frogs. However, albeit being insignificant, Scaling coefficient and hepatosomatic index showed similar trends. In the biological and ecological effect study, it was found that Mae Tao frogs had higher macro-melanophage count in the liver. Hepatocyte swelling, possible necrotic and apoptotic hepatocytes, renal tumor-like aggregation, renal hemorrhage and more testicular ovarian follicle were also observed in Mae Tao frogs. The results showed that cadmium contamination may, either directly or indirectly, cause an increase in cadmium accumulation in the liver, kidney, testis and whole organism; an increase in hepatic metallothionein and hepatic glutathione-S-transferase; a decrease in condition factor, renosomatic index and female gonadosomatic index; and histopathological changes in the liver, kidney and testis of the rice frog. Therefore these results were able to justify the use of *Fejervarya limnocharis* as a sentinel species for cadmium contamination.

Field of Study : Environmental Management Student's Signature .....

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

โมห์ด ชาม บิน โอทมาน : การใช้กบหนอง (*Fejervarya limnocharis*) เป็นตัวเฝ้าระวังการปนเปื้อนของแคดเมียมในจังหวัดตาก ประเทศไทย. (USING THE RICE FROG (*FEJERVARYA LIMNOCHARIS*) AS A SENTINEL SPECIES FOR CADMIUM CONTAMINATION IN TAK PROVINCE, THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.นพดล กิตนะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร.มาร์ค เกรกอรี ร็อบสัน 204 หน้า.

จากประวัติการปนเปื้อนแคดเมียมในพื้นที่ อ.แม่สอด จ.ตาก ประเทศไทย การศึกษานี้ได้เลือกใช้กบหนอง (*Fejervarya limnocharis*) ซึ่งเป็นสัตว์สะเทินน้ำสะเทินบกที่พบได้ทั่วไปในพื้นที่มาเป็นตัวเฝ้าระวัง โดยมีวัตถุประสงค์เพื่อตรวจสอบความเหมาะสมของกบหนองในการนามาใช้เป็นตัวเฝ้าระวังผลกระทบจากการปนเปื้อนแคดเมียม เมื่อเก็บตัวอย่างกบเดือนละครั้งในช่วง พฤศจิกายน พ.ศ. 2550 ถึง ตุลาคม พ.ศ. 2551 จากพื้นที่เกษตรกรรมในพื้นที่ปนเปื้อน (ต.แม่ตาว) และ พื้นที่อ้างอิง (ต.แม่ปะ) ใน อ.แม่สอด จ.ตาก แล้วนำมาตรวจวิเคราะห์ด้านต่าง ๆ คือ การปนเปื้อนแคดเมียม ตัววัดทางชีวภาพในตับ ขนาดลักษณะสัณฐานและน้ำหนักอวัยวะ ตลอดจนผลทางชีวภาพและนิเวศวิทยา พบว่า กบหนองจากพื้นที่แม่ตาวมีปริมาณแคดเมียมสะสมในตับ ไต อัณฑะ และในร่างกายสูงกว่ากบหนองจากพื้นที่แม่ปะอย่างมีนัยสำคัญ โดยมีไตเป็นอวัยวะที่เก็บสะสมแคดเมียมไว้สูงที่สุด และเมื่อนำข้อมูลเหล่านี้มาคำนวณหาค่าการเพิ่มความเข้มข้นแคดเมียมในสิ่งมีชีวิตเทียบกับสิ่งแวดล้อมพบว่ามีแนวโน้มในทางเดียวกัน การตรวจสอบตัววัดทางชีวภาพในตับแสดงให้เห็นว่ากบหนองจากพื้นที่ปนเปื้อนมีปริมาณโปรตีนเมทัลโลไทโอนีน และระดับเอ็นไซม์กลูตาไทโอนเอสทรานสเฟอเรสมากกว่ากบหนองจากพื้นที่อ้างอิงอย่างมีนัยสำคัญ โดยที่เอ็นไซม์กลูตาไทโอนเอสทรานสเฟอเรสในตับแสดงสหสัมพันธ์กับปริมาณแคดเมียมที่พบในตับอย่างชัดเจน ในการตรวจสอบขนาดลักษณะสัณฐานและน้ำหนักอวัยวะ พบว่ามีความแตกต่างอย่างมีนัยสำคัญระหว่างพื้นที่ในด้าน ค่าปัจจัยความสมบูรณ์ของร่างกาย ดัชนีน้ำหนักไตเทียบกับน้ำหนักตัว และดัชนีน้ำหนักรังไข่เทียบกับน้ำหนักตัว และพบแนวโน้มความแตกต่างในแบบเดียวกันกับค่าสัมประสิทธิ์สเกลิง และดัชนีน้ำหนักตับเทียบกับน้ำหนักตัว เมื่อศึกษาผลกระทบทางชีวภาพและนิเวศวิทยาที่เกิดขึ้น พบว่ากบจากพื้นที่แม่ตาวมีลักณะทางจุลพยาธิวิทยาสูงกว่ากบจากพื้นที่แม่ปะ คือ จำนวนเซลล์แมโครเมลาโนฟาจในตับ การบวมและการตายของเซลล์ตับ การเกิดเนื้องอกในไต การมีเลือดออกในไต และ การพบฟอลลิเคิลในอัณฑะ ผลการศึกษาแสดงให้เห็นว่าการปนเปื้อนแคดเมียมอาจส่งผลกระทบทางตรงหรือทางอ้อมต่อกบหนอง ทำให้มีการสะสมแคดเมียมในตับ ไต อัณฑะ และร่างกายในปริมาณสูงขึ้น พร้อมกับการเพิ่มขึ้นของโปรตีนเมทัลโลไทโอนีนและเอ็นไซม์กลูตาไทโอนเอสทรานสเฟอเรสในตับ และส่งผลให้เกิดการลดลงของค่าปัจจัยความสมบูรณ์ของร่างกาย ดัชนีน้ำหนักไตเทียบกับน้ำหนักตัว และ ดัชนีน้ำหนักรังไข่เทียบกับน้ำหนักตัว และยังพบการเปลี่ยนแปลงทางจุลพยาธิวิทยาในตับ ไต และ อัณฑะ ซึ่งข้อมูลเหล่านี้แสดงให้เห็นถึงความเหมาะสมที่จะนำกบหนองมาใช้เป็นตัวเฝ้าระวังผลกระทบจากการปนเปื้อนแคดเมียมในสิ่งแวดล้อม

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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ABBREVIATIONS

ANOVA	analysis of variance
anti-VTG	Anti-vitellogenin
apo-MT	Apo-metallothionein
BCF	Bioconcentration factor
CCFAC	Codex committee on food additives and contaminants
Cd	Cadmium
CF	Condition factor
CITES	Convention on international trade in endangered species
DNA	Deoxyribonucleic acid
EMT	epithelial to mesangial transition
EPA	Environmental protection agency
GSH	Glutathione
GSI	Gonadosomatic index
GST	Glutathione-S-transferase
HCl	Hydrochloric acid
holo-MT	Holo-metallothionein
HSI	Hepatosomatic index
IARC	International Agency for Research on Cancer
IUCN	International union of conservation of nature
KCl	Potassium chloride
LC	Least concern

MMC	Macro-melanophage center
MPL	Maximum permissible limit
mRNA	Messenger ribonucleic acid
MT	Metallothionein
NTEL	non toxic effect level
OECD	Organization for economic co-operation and development
PBS	Phosphate buffer solution
ROS	Reactive oxygen species
RSI	Renosomatic index
TOFs	testicular ovarian follicles
USNRC	United states national research center
VTG	Vitellogenin
WI	Weekly intake

CHAPTER 1

GENERAL INTRODUCTION

1.1 Cadmium in the Environment and Its Bioavailability

According to Jamil (2001), cadmium is a divalent cation reported to be one of the most toxic metal pollutant. Cadmium level in the aquatic ecosystem showed remarkable variation between the media's concentration. Many pollutants, especially cadmium do not stay in the aquatic phase because they have high sorption to the sediment. Sorption of the pollutants to the sediment often results in the very low concentration of the pollutant in the liquid phase. Hence, most of the time, the concentration is not high enough to produce toxicity to aquatic organisms. In water, the concentration is usually very low. Chen and Liu (2006) stated that majority of the metals in the cadmium laden soil will be retained in the soil phase (soil and sediment), and the remaining, if released into aquatic ecosystem cannot reach concentration high enough to produce toxicity to aquatic animals. Francis et al. (1984) reported that high sorption capacity of stream sediment for cadmium, as predicted by Langmuir isotherm, was reflected by the small quantities of cadmium mobilized from the metal enriched sediments. This means that most cadmium in the aquatic ecosystem will be sorbed onto soil and sediment and not in the dissolved form. Therefore, Chen (2005) stated that

cadmium has the lowest risk onto aquatic species. However high level of cadmium in freshwater environment may occur as a result of natural weathering of minerals in sediments and bedrocks or as a result of anthropogenic activities such as mining (Olsvik et al., 2000)

1.2 Introduction to Cadmium Contamination in Tak Province, Thailand

Cadmium contamination in Tak province was reported by Simmons et al. (2005) who studied the cadmium concentration in areas along the flow of Mae Tao Creek which is used for irrigation. While the cadmium levels in the creek itself were below the permissible level, there were elevated levels in the soils around the creek. Pongsakul and Attajarusit (1999) reported that the Thailand background total soil cadmium ranges from 0.002 to 0.141 mg/kg. On the other hand, the Thai investigation level for cadmium in soil is 0.15 mg/kg (Zarcinas et al., 2003). In the study by Simmons et al. (2005), it was found that soil cadmium ranged from 0.5 to 214 mg/kg. The study suggested that irrigation waters withdrawn from Mae Tao Creek were the primary source of the observed elevated levels of cadmium. Hence it was inferred that contamination is associated with the deposition of sediments through irrigation waters.

Simmons et al. (2005) also reported that 85 percent of the rice-fields sampled produced rice grain that has cadmium level that exceeded the Codex Committee on Food Additives and Contaminants (CCFAC) draft Maximum Permissible Level

for rice grain of 0.2 mg/kg They also calculated the estimated Weekly Intake (WI) of cadmium from rice which ranged from 20 to 82 µg Cd per kg body weight. This posed a significant public health risk to local community.

1.3 The Concept of Sentinel Species

Over the years, sentinel species has been used by scientists as one of the tools in environmental monitoring and risk assessment. Jamil (2001) stated that the use of bioindicators and biomonitors is complementary to the more usual monitoring involving the determination of residue levels of toxicants in the environment. The biological data obtained from the sentinel species is used in complement with the physical and chemical environmental data. Sentinel species is defined as any biological monitors that are able to accumulate a pollutant in tissue without significant adverse effects (Beeby, 2001). This definition emphasized that there are two main facets of a sentinel species. The first is the species' ability to accumulate a pollutant and the second is its ability to do so without causing adverse effects. However, throughout the years, many other elements have been added to the concept of sentinel species. Therefore instead of limiting to accumulators only, the concept of a sentinel species now includes bioindicators, integrators and biomarkers. In a workshop titled "Using Sentinel Species Data to Address the Potential Human Health Effects of Chemicals in the Environment", animal sentinel is defined as any non-human organism that can react to an environmental contaminant before the contaminant impacts humans

and can offer the possibility of expanding our understanding and response to environmental health concerns (van der Schalie et al., 1999). Using a sentinel as an indicator of human health hazard has several advantages: a) a sentinel may provide early warning of potential risks before disease develops in human populations; b) for some toxicants, the biomarkers of exposure and toxic effects are similar in humans and the sentinel animals; and c) the exposure conditions may be comparable under some circumstances, such as for people and their companion animals. According to van der Schalie et al. (1999), the application of the sentinel species science includes monitoring applications, regulatory compliance, complex mixture characterization, evaluation of treatment efficacy or restoration success, identification of deleterious ecosystem changes and public health decision making

The choice of organisms to be used as sentinel species is vastly varied. They range from invertebrates such as earthworms and mussels to vertebrates such as amphibians and reptiles. Their degrees of usability and effectiveness vary from one species to another. The use of any particular species as a sentinel species depend on the type of pollutants and xenobiotics studied. Choice of a species also depends on the type of effects these pollutants and xenobiotics exert on the species. Jamil (2001) mentioned that sentinels are biomonitors acting as early alarm signals. Sentinel species should exist on the site under study, being present either naturally or because they were deliberately introduced to the site.

Ji et al. (2006) reported that mussels and oysters had been used as sentinel organisms. Amphibians (Loumbourdis et al., 2007) and their tadpoles (Loumbourdis et al., 1999) could be considered as good bioindicators for various xenobiotics, especially sediment-borne pollutants. Loumbourdis & Wray (1998) stated that *Rana ridibunda* is a good indicator species for heavy metal pollution. Kitana et al. (2007) cited that using wildlife as sentinel species not only provide information on exposure and bioavailability, but also on its response to and the effects of the pollutant.

There are many ways how pollutants and xenobiotics can interact with the sentinel species. Bioindicators reflect on the effect of pollutants on the presence of the species. The effects are often observed on the population and organismal level. Some sentinels are able to accumulate pollutants in tissues while some others are integrators. Accumulation and integration of pollutant in the sentinel can be used as a measure of exposure (Beeby, 2001). Biomarker, on the other hand, is defined as functional measures of exposure to stressors expressed at sub-organismal, physiological or behavioral level (Galloway, 2006). Linde-Aries et al. (2008) stated that biomarkers are sensitive tools for biological effect measurement in environmental quality assessment and concurrent use of several biomarkers/parameters is important to minimize misinterpretation. According to Martín-Díaz et al. (2008), ecotoxicological data obtained in laboratory studies is often difficult to translate into accurate prediction of possible effects in the fields.

To add, Henry (2000) suggested that it may be productive that toxicological studies evaluate the contaminants' effects on the organisms and their adaptation within the environment. Therefore laboratory results are best validated by field research because overestimation and underestimation of effects may occur in laboratory oriented studies.

The effectiveness of a sentinel in providing relationship between the species and the pollutant has always been as a source of debate. Some quarters would even question the rational and practicality of using sentinels in any risk assessment. However, Beeby (2001) emphasized that sentinels offer the prospect of providing a simple and rapid means of quantifying complex pollution signals. While physical and chemical environmental data can provide hard information on the extent of the pollution in the environment, biological data is able to show the effects of the pollutant studied on living organisms, either directly or indirectly. Linde-Arias et al. (2008) reported that prior to death or overt sickness, organism may respond to stress by changing molecular, physiological or behavioral responses. Consequently, the ability to recognize and measure these changes may provide an early warning of later, much more serious consequences.

Burger et al. (2007) reported that for any indicator to be biologically relevant, it must exhibit changes in response to a stressor, but not be so sensitive that changes occur when there is no cause for concern. The changes should also be attributable to a particular stressor and be reflective of impairment to other

population or species. Beeby (2001) has listed three desirable attributes for a species to be an effective sentinel. Firstly, the sentinel should be insensitive to ambient concentration. Secondly, the sentinel should be able to integrate pollution signal over area or over time interval. Finally, a good sentinel should show simple correspondence between tissue and ambient level. Olsvik et al. (2000) stated that some species may become acclimatized to elevated levels, but this is usually manifested by altered uptake, altered elimination rate or by changes in sequestration and immobilization.

As stated before, response of a sentinel towards the pollutant can be seen not only at the population and species level, but also at the suborganismal level. For some species, there were positive correlations between pollutants and their response (Burger et al., 2007) while others may show a different trend. For some metals, especially cadmium, accumulation may not reach steady state because they are accumulated throughout life (Beeby, 2001). Galloway (2006) has listed the classification of biomarkers into four categories of endpoints according to the US National Research Council. They include internal dose, biologically effective dose, preclinical biological effect and susceptibility. For example, blood and tissue contaminant concentration or metabolites may be used to estimate exposure. Altered enzyme activities may indicate effective dose, susceptibility or pre-clinical effects while the presence of pathological lesion may indicate adverse consequences. These endpoints have been incorporated as priority endpoints in the new OECD test guidelines for environmental monitoring (Hutchinson and

Pickford, 2002) and they include vitellogenin and gonad histology. The choice of endpoints depends on what level of biological organization a researcher chooses. These biological organizations are summarized in Table 1.1.

While many species may be able to fulfill all three desirable attributes of a sentinel, some species are chosen in favor of others. The main reason for this is the practicality of using that particular species. Some characters of a favored sentinel species include ubiquitous, abundant, easy to identify and large enough for analysis (Beeby, 2001) and has wide geographic distribution (Ji et al., 2006). Jamil (2001) also listed a few properties suitable for a good sentinel. Ease to capture is one of the main criteria in favoring a species as a sentinel. The easier the species is captured, the more desirable the species is to be chosen as sentinel species. Prior knowledge about the distribution area of the species is also an advantage. However, one of the most important criteria that made a species more favorable than others is its known avenue of exposure to the contaminant.

Many species has been used as sentinel species for cadmium exposure. They include *Carrasius auratus*, *Rana pipiens* and *Micropterus salmoides* (Francis et al., 1984), *Bufo arenarum* (Pérez-Coll et al., 1997), *Rana ridibunda* (Loumbourdis and Wray, 1998; Loumbourdis and Vogiatzis, 2002), *Gastero aculeatus* (Bervoets and Blust, 2003; Bervoets et al., 2001), *Nerodia* spp. (Burger et al., 2007) and *Ruditapes philippanarum* (Ji et al., 2006).

Table 1.1: Representative indicators which can be measured at various levels of biological organization (Jamil, 2001)

Biochemical	Physiological	Histo-pathological	Individual	Population	Communities
• Enzymes • Metabolites • DNA integrity • Stress proteins • Antioxidant enzymes	• Creatinine & other enzymes • Transaminase enzymes • Cortisol • Triglycerides HSPs & Metallothionein • Steroid hormones	• Necrosis • Macrophage aggregate • Parasitic lesions • Functional parenchyma, tissues etc • Carcinomas	• Growth • Total body lipids • Organo-indices • Genetic disorders • Behavior changes • Gross anomalies (lesions)	• Abundance • Size & age distribution • Sex ratio & susceptibility • Bioenergetic parameters • Reproductive health	• Index of Biotic Integrity • Intolerant/resistant species • Genotypes • Locomotive parameters • Stressed & weak types

1.4 Amphibian as Sentinel Species

One group of animal that has been used widely as sentinel species is the amphibian. Loumbourdis et al. (1999) reported that amphibians are good indicators of pollution due to the high permeability of their skin, both in aquatic and terrestrial. Due to the high permeability, pollutants are able to be absorbed into the body dermally. This is in addition to the more usual route of pollutant exposure which includes oral route and inhalation. Therefore, there are more than one significant ways on how xenobiotics are able to enter the body.

Many pollutants, especially cadmium do not stay in the aquatic phase because they have high sorption to the sediment. Sorption of the pollutants to the sediment often results in the very low concentration of the pollutant in the liquid phase. Hence, most of the time, the concentration is not high enough to produce toxicity to aquatic organisms. However, with amphibians this is not the case. According to Selvi et al. (2003), even amphibian eggs and tadpoles have been used as bioindicator because the eggs are not protected be impervious shell and the tadpole's skin is highly permeable both in water and air. Francis et al. (1984) reported that the embryo of leopard frog remained just above the sediment layer, thus there is a possible transport of pollutant from the sediment into the embryo during the pre-hatching period. Even after hatching, the leopard frog's tadpoles situated themselves onto the sediment hence allowing a possible transfer of

pollutant from the sediment to the tadpoles. Therefore it is apparent that the contact between the pollutant and the frog occur during all stages of life cycle, even during developmental stages. Figure 1.1 illustrates the life stages of a frog and its main mode of exposure to pollutant, especially cadmium. To add, Loumbourdis and Wray (1998) reiterated that frogs are able to accumulate pollutant, especially heavy metals in high concentration. Pérez-Coll (1997) stated that amphibians, in general are considered as good indicators of environmental pollution. In another paper, Pérez-Coll et al. (1999) even considered the suggestion that amphibian embryos should be increasingly employed in ecotoxicity studies. This is further supported by Zorita et al. (2008) by stating that bottom dweller organisms tend to concentrate contaminants to a higher degree than other. Since tadpoles generally fall into this category, its ability to concentrate contaminants is suggested to be high. However, the developmental stage of the tadpoles to be used for these studies should also be taken into consideration. This is because, Rao and Madhyastha (1987) stated that variability in the toxicity of heavy metals within age groups of tadpoles may be related to the developmental changes associated with metamorphosis.

Initially, *Bufo melanostictus*, *Microhyla heymonsi*, *Microhyla fissipes* and *Fejervarya limnocharis* have been considered as a candidate for sentinel for this research. However, the first three species has several limitations. While *Bufo melanostictus* has a better size for analysis, this species is an explosive breeder. Because of this, this toad is difficult to be found outside the wet season.

Microhyla heymonsi and *Microhyla fissipes*, on the other hand, can be found all year round. Yet, their small size is a hindrance for them to be used as a sentinel species. Furthermore, initial sampling yielded more *Fejervarya limnocharis* (Figure 1.2) individuals than the other three species. Therefore, in this study, the rice-field frog, *Fejevarya limnocharis* has been chosen to be a representative sentinel species for cadmium contamination. This species falls under the Order Anura and Family Raniidae (IUCN, Conservation International & NatureServe, 2006). The rice-field frog is also known as Indian cricket frog, Boie's wart frog, grass frog, field frog, marsh frog, common pond frog and terrestrial frog (IUCN, Conservation International & NatureServe, 2006). According to AmphibiaWeb (2008), this species has a pointed snout that is projected beyond the mouth and a distinct tympanum. The body of the rice-field frog has small tubercles, sometimes with small longitudinal folds. It has a gray brown color, sometimes with olive above and suffused by bright carmine. Male frog has loose gular region with brown or blackish w-shaped mark. It has stronger forelimb with pad-like subdigital tubercles under the first finger as opposed to female frog. The male throat is also often mottled with dark brown.

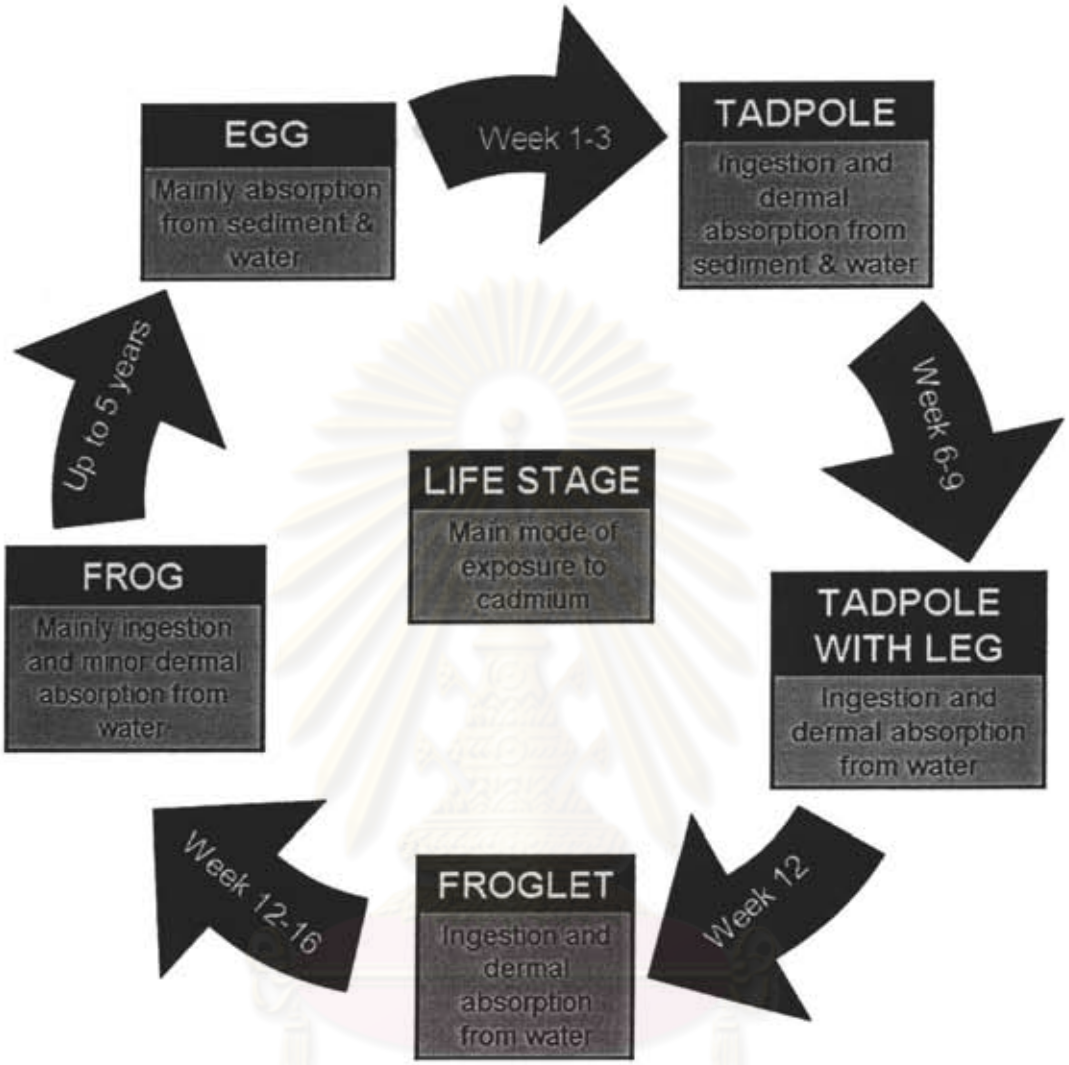

Figure 1.1: A diagram of a generalized frog's life stages and their main mode of exposure to cadmium from the environment

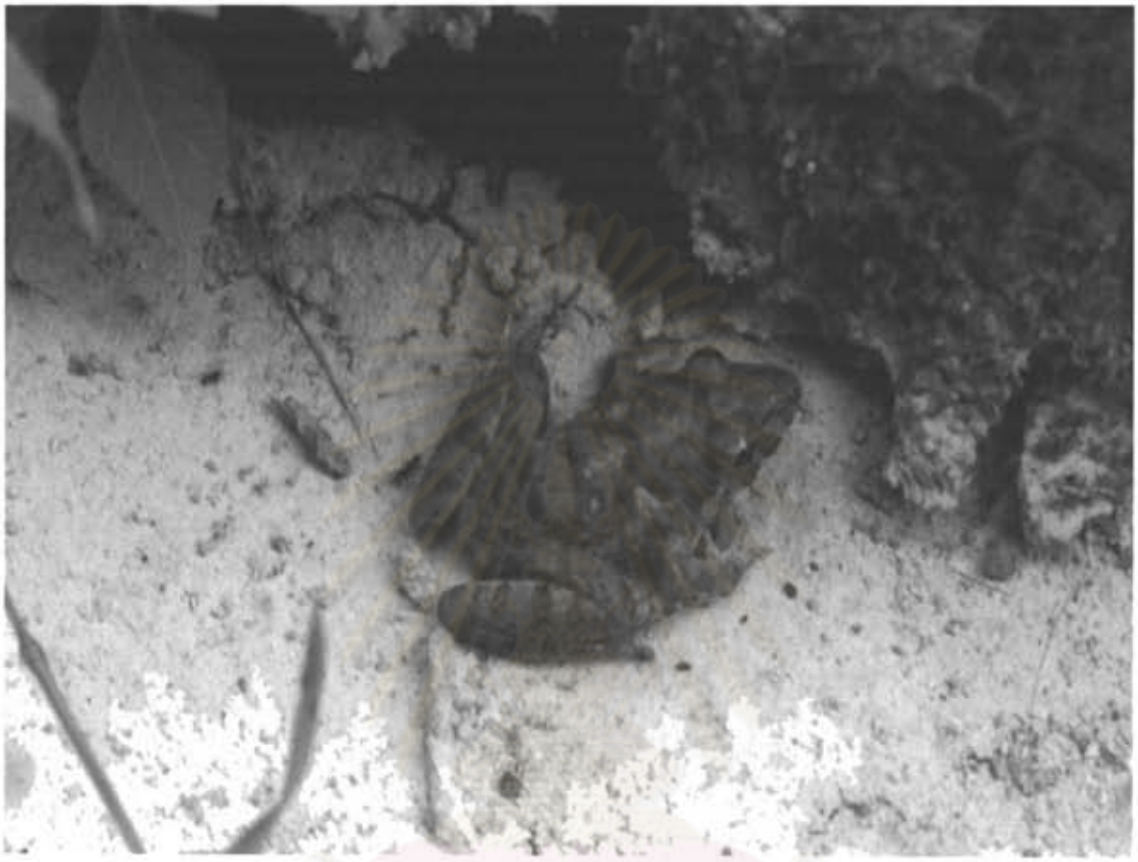

Figure 1.2: A photograph of the rice frog, *Fejervarya limnocharis*.

This species has a wide range of distribution, covering Southeast Asia, South Asia and part of East Asia (Figure 1.3), inhabiting most open wet habitats. Its distribution range includes Bangladesh, Cambodia, China, Hong Kong, India, Indonesia, Japan, Laos, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Singapore, Sri Lanka, Taiwan, Thailand and Vietnam (AmphibiaWeb, 2008). Under IUCN, the conservation status of *Fejevarya limnocharis* is classified as "Least Concern" or LC (AmphibiaWeb, 2008; IUCN, Conservation International & NatureServe, 2006). It is also not listed under the CITES. The LC status is given to the species because of its very wide distribution and its tolerance of broad range of habitats. It is reported to have a large population and the population is very stable.

According to AmphibiaWeb (2008), this species is very common in Southeast Asia especially in the rice fields. The breeding of the frog is often triggered by rain and it is usually the first species to come to the calling sites. *Fejevarya limnocharis* is also utilized as human food in some countries (IUCN, Conservation International & NatureServe, 2006), including Thailand.

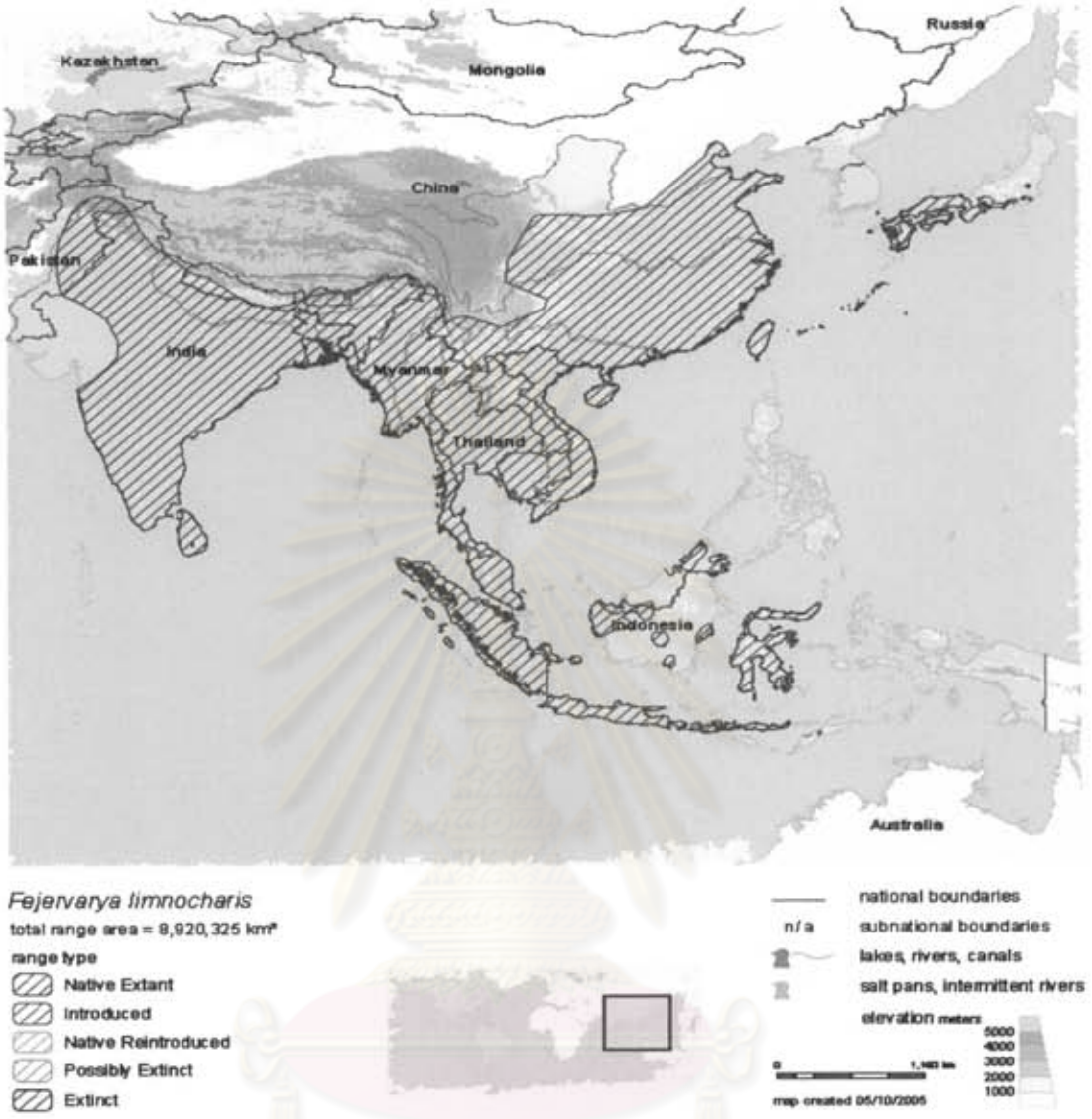

Figure 1.3; The Geographic Distribution of *Fejevarya limnocharis* (IUCN, Conservation International & NatureServe, 2006))

1.5 Research Objectives

General Objective

To study the suitability of using *Fejevarya limnocharis* as a sentinel species for cadmium contamination

Specific Objectives

- To compare the contaminant analysis parameters of *Fejervarya limnocharis* from contaminated site with those from reference site.
- To compare the biomarker study parameters of *Fejervarya limnocharis* from contaminated site with those from reference site.
- To compare the morphometric and gravimetric indices of *Fejervarya limnocharis* from contaminated site with those from reference site.
- To compare the biological and ecological indicators of *Fejervarya limnocharis* from contaminated site with those from reference site

1.6 Research Hypothesis

H1: There are significant differences in the

- contaminant analysis parameters,
- biomarker study parameters,
- morphometric and gravimetric indices and
- histological and skeletochronological parameters

between *Fejervarya limnocharis* from contaminated site with those from reference site.

H2: *Fejervarya limnocharis* is a suitable candidate as a sentinel species for cadmium contamination

1.7 Research Scopes and Approaches

The approach of the study is outlined as in the flowchart in Figure 1.4 below. The thesis statement of this study is to determine whether *Fejervarya limnocharis* is a good candidate as a sentinel species for cadmium contamination.

In order to address the thesis statement, four parts of research activities has been devised (Figure 1.5). A battery of analyses has been chosen instead of concentrating on one specific parameter. This is because Schmitt et al. (2007) stated that any biomarker or sentinel studies should incorporate a battery of biomarkers to show the sentinel's respond to environmental conditions. Furthermore, a study by Linde-Arieas et al. (2008) on *Oreochromis niloticus* showed that a multibiomarker or multiparameter approach was able to reveal the differences in the health of the species among reference and contaminated sites. Zorita et al. (2008) reiterated that in any ecotoxicological studies, the use of a battery of bioresponses is recommended since single biomarker is not able to reflect the impairment of organism's health and/or its adaptation to the impaired environmental condition. To add, this research is not designed around standardized laboratory conditions. Instead, this study attempts to look at the changes that are already occurring in the natural population of a sentinel species. In this case, the sentinel species under scrutiny is the rice frog, *Fejervarya limnocharis*. This is because Henry (2000) suggested that it may be productive

that toxicological studies evaluate the contaminants' effects on the organisms and their adaptation within the environment.

The first part of the research activities noted above is contaminant analysis. In this part, cadmium levels in the environmental samples and in tissues were compared. The tissues selected in this part were liver, kidney, ovary and testis. Apart from that whole organismal cadmium accumulation were also compared. At the end of this part tissue and organismal bioconcentration factors were determined. The second part of this research dealt with biomarker study where metallothionein, glutathione-S-transferase and vitellogenin levels were compared. The third part of the study was the morphometric and gravimetric study where morphometric indices were compared. In this part, weight-length relationship and condition factor were also determined. The final part of this research was the biological and ecological indicators where histopathology and age determination were done.

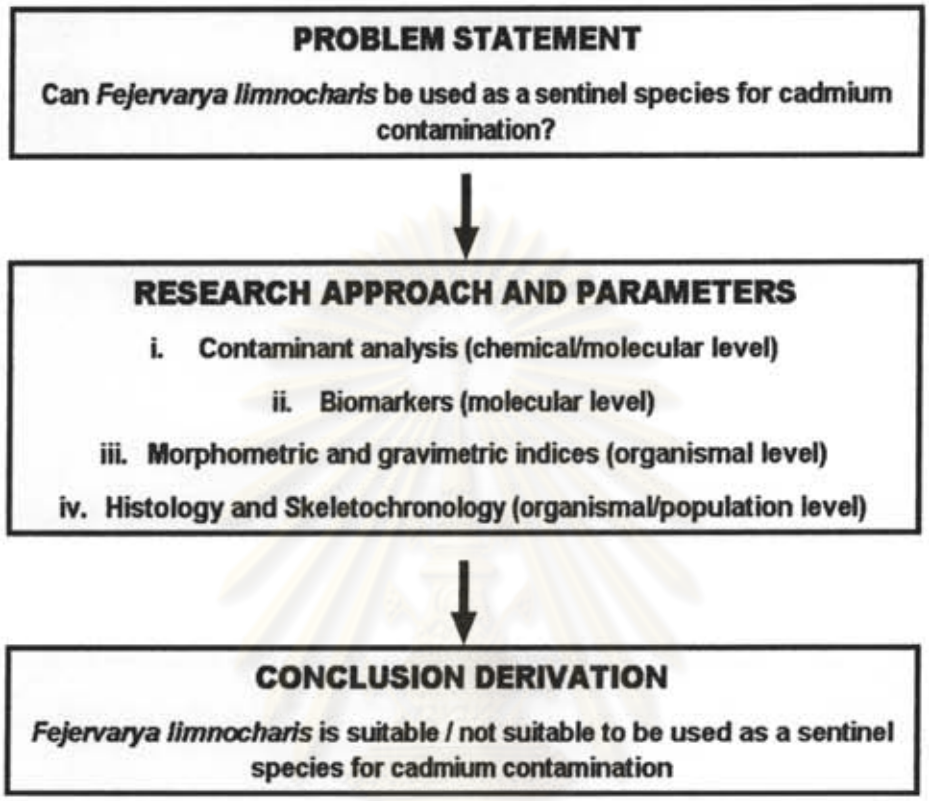

Figure 1.4: Research approach of this study

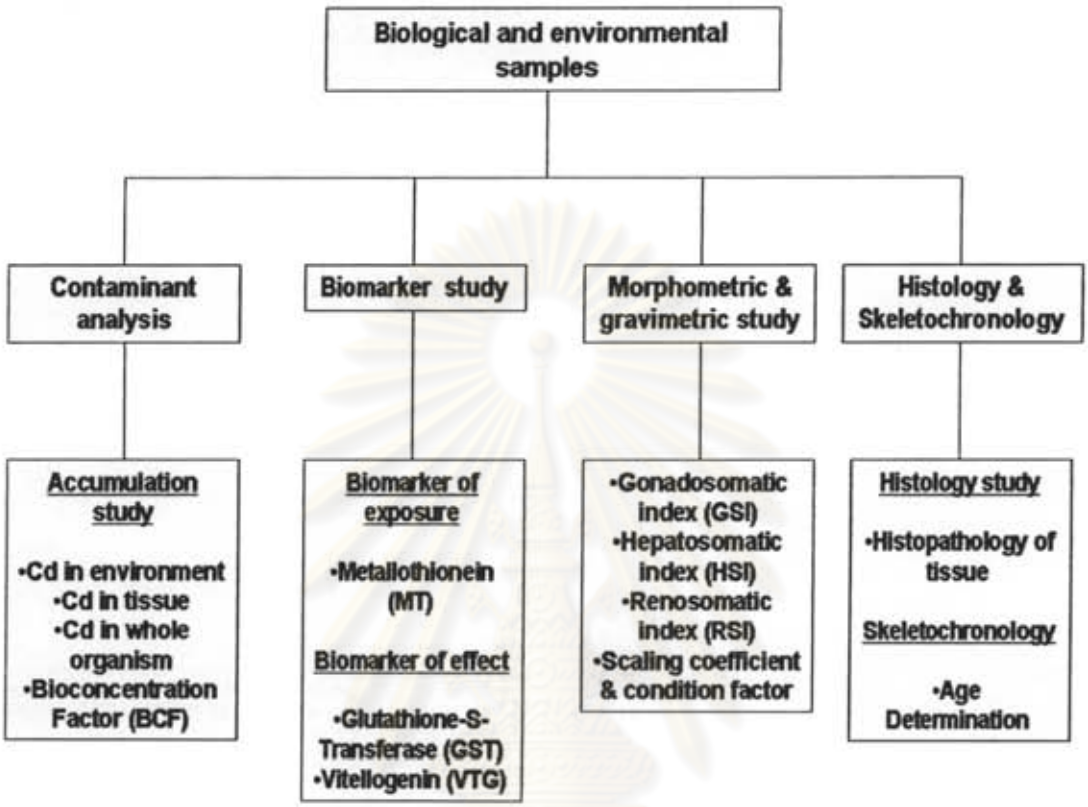

Figure 1.5 : Research scope of this study

1.8 Introduction to Research Location and Research Timeframe

Samples were collected from Mae Pa and Mae Tao in Mae Sot District, Tak Province (Figure 1.6). The reference site, Mae Pa, is located at 16°40'43"N; 98°35'36"E and the area is irrigated by Huay Luek Creek. The contaminated site, Mae Tao, is located 8.4 km south of the reference site at 16°45'13"N; 98°35'25"E. This area is irrigated by the Mae Tao Creek. Simmons et al. (2005) reported that there were elevated cadmium levels in the paddy soils and rice grain in vicinity of Mae Tao Creek downstream of a zinc mining area. Preliminary analysis showed that the cadmium concentrations were 0.0007 mg/L (water) and 0.0988 mg/kg (sediment) at the reference site. The concentration at the contaminated site was 0.0015 mg/L (water) and 1.0110 mg/kg (sediment).

Sample collections were done for 12 months starting from November 2007 to October 2008. Even though samples were collected on monthly basis, all data were analyzed on a tri-monthly basis. Data from all twelve months were pooled into four groups based on the average total rainfall of each groups as follows.

- Early dry season : November 2007 – January 2008
- Late dry season : February 2008 – April 2008
- Early rainy season : May 2008 – July 2008
- Late rainy season : August 2008 – October 2008

Seasonal average rainfall was obtained from World Meteorological Association (2007) and shown in Figure 1.7

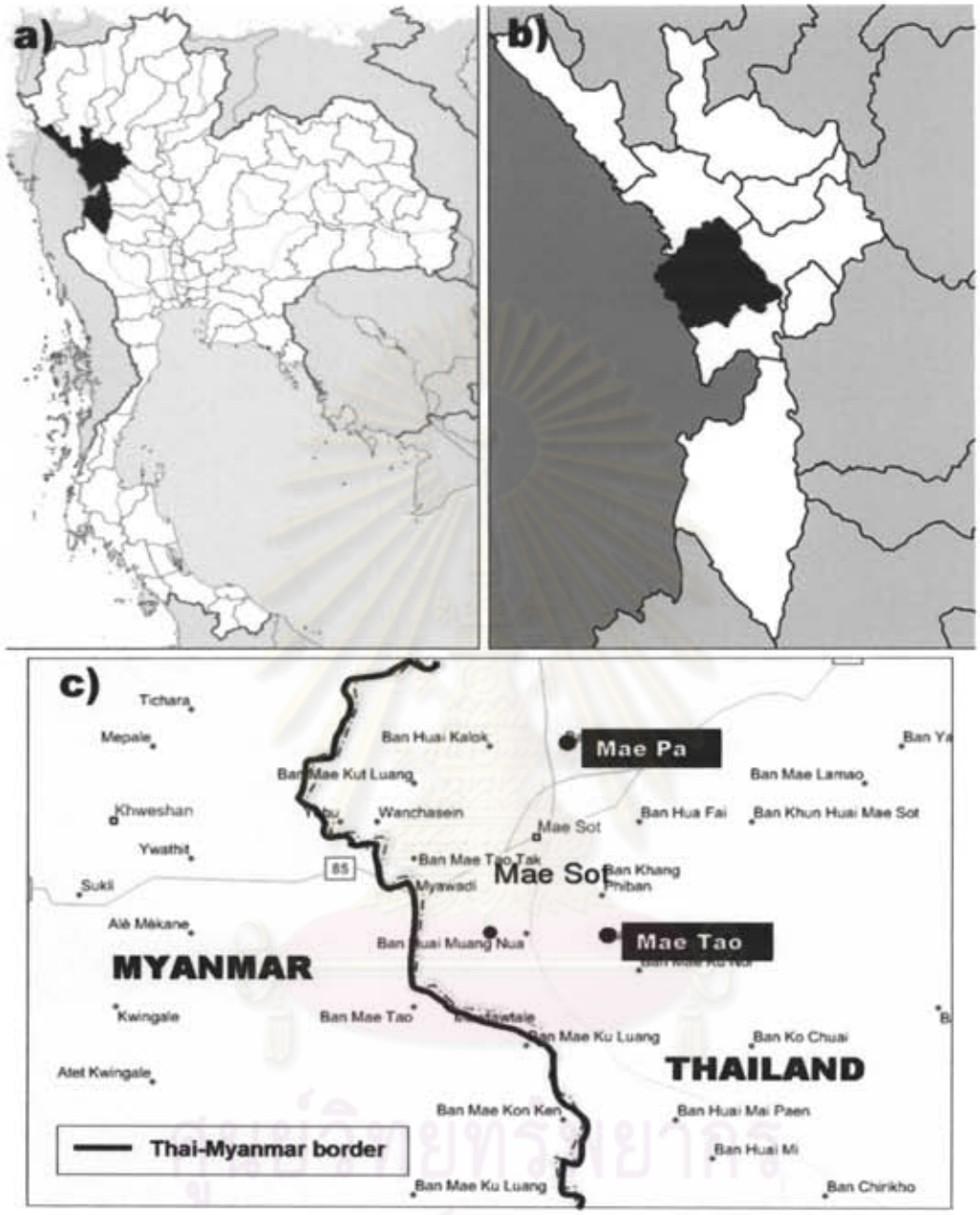

Figure 1.6: A map showing the geographic location of a) Tak Province; b) Mae Sot district; and c) sampling sites of Mae Pa (reference site) and Mae Tao (contaminated site)

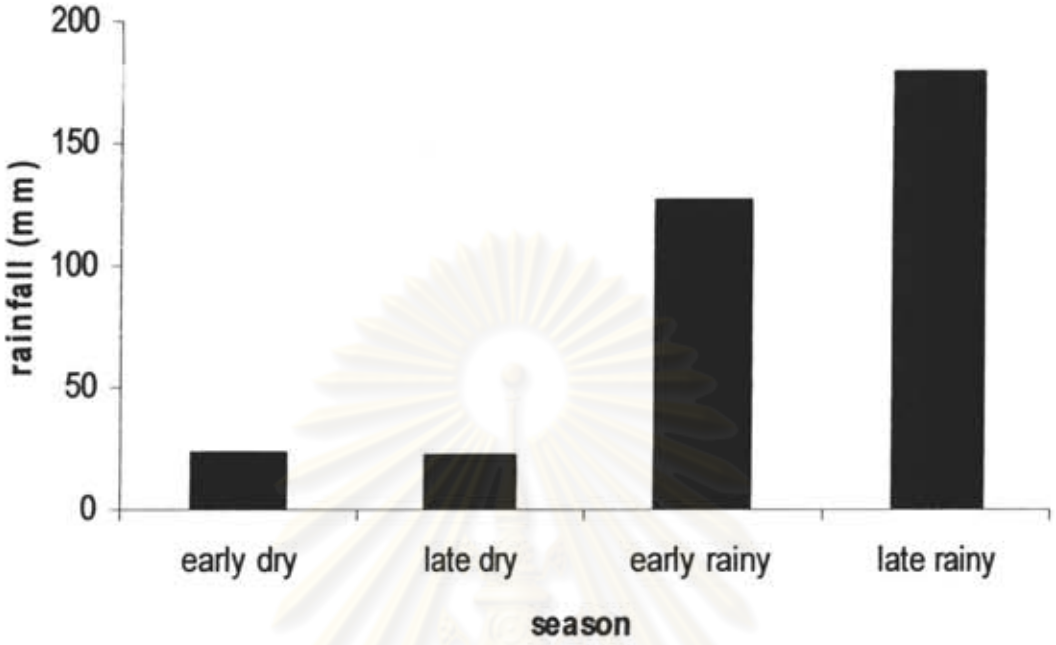

Figure 1.7: Seasonal average rainfall of Mae Sot from November 2007 to October 2008. (Data extracted from World Meteorological Association (2007))

CHAPTER 2

LITERATURE REVIEW

2.1 Contaminant Analysis

2.1.1 Uptake and Transport of Cadmium

Cadmium is not an essential metal for animals (Isani et al., 2008) but it has the ability to be taken and accumulated into the body from the environment. Bervoets et al. (2001) reported that the levels of accumulated metals in the tissue were related to metal levels in sediment, water and food. This is further supported by Bervoets and Blust (2003) saying that it is more likely that tissue level reflects environmental level because metal concentration in tissue follows concentration in the environment. This is reflected by the result of their study on *Gobio gobio* that showed both environmental and tissue cadmium and zinc followed a clear concentration gradient of the river. Francis et al. (1984) reported that *Carrasius auratus*, *Rana pipiens* and *Micropterus salmoides* showed strong correlations between cadmium concentration in water and tissue, sediment and tissue and water and sediment. A study by Pérez-Coll et al. (1997) found that cadmium uptake by the *Bufo arenarum* liver represents about 26% of the total cadmium administered to the experimental animals. However, it need to be

emphasized that most results concerning the effect of cadmium accumulation in animal or tissues are often contradictory and are generally obtained from experiments performed for short period of time with high concentration of cadmium (Isani et al., 2008). Studies on wild population of animals naturally exposed to different levels of environmental cadmium have been quite limited, especially on frogs.

Cadmium level in the aquatic ecosystem showed remarkable concentration variation between the media. Many pollutants, especially cadmium do not stay in the aquatic phase because they have high sorption capacity to the sediment. Sorption of the pollutants to the sediment often results in the very low concentration of the pollutant in the liquid phase. Hence, most of the time, the concentration is not high enough to produce toxicity to aquatic organisms. In water, the concentration is usually very low. Chen and Liu (2006) stated that majority of the metals in the cadmium laden soil will be retained in the soil phase, and the remaining, if released into aquatic ecosystem cannot reach concentration high enough to produce toxicity to aquatic animals. Francis et al. (1984) reported that high sorption capacity of stream sediment for cadmium, as predicted by Langmuir isotherm, was reflected by the small quantities of cadmium mobilized from the metal enriched sediments. This means that most cadmium in the aquatic ecosystem will be sorbed onto soil and sediment and while only a small percentage will stay in the dissolved form. Therefore, Chen (2005) stated that cadmium has the lowest risk onto aquatic species. However high level of

cadmium in freshwater environment may occur as a result of natural weathering of minerals in sediments and bedrocks or as a result of anthropogenic activities such as mining (Olsvik et al., 2000)

Cadmium uptake into the body may follow various routes. Since the concentration of cadmium in the water is low, dermal route is often not the most significant route of exposure to cadmium. On the other hand, most cadmium will enter the body via the oral route. The modes of oral route include direct ingestion from water and soil and also through the food chain. However, amphibians may be able to accumulate cadmium through the dermal route as significant as the oral route. Loumbourdis et al. (1999) reported that amphibians have high permeability to of their skin, both in aquatic and terrestrial ecosystems, to pollutants. Due to the high permeability, pollutants are able to be absorbed into the body dermally. This is in addition to the more usual routes of pollutant exposure which include oral route and inhalation. Therefore, there are more than one significant ways on how xenobiotics are able to enter the body.

Sediment-borne cadmium also may be transported from the environment to the organism. Francis et al. (1984) found that the embryo larval stage of the leopard frog in their study showed a high affinity for sediment borne cadmium. This is because the embryo of leopard frog remained just above the sediment layer, thus there is a possible transport of pollutant from the sediment into the embryo during the pre-hatching period. Even after hatching, the leopard frog's tadpoles situated

themselves onto the sediment hence allowing a possible transfer of pollutant from the sediment to the tadpoles. Therefore it is apparent that the contact between the pollutant and the frog occur during all stages of life cycle, even during developmental stages.

2.1.2 Final Location and Accumulation Pattern

According to Isani et al. (2008), pollutants, including cadmium are rarely distributed uniformly in body tissues. Different tissues have the ability to accumulate metals differently. This is exhibited in studies on *Rana ridibunda* (Loumbourdis and Wray, 1998; Loumbourdis and Vogiatzis, 2002), *Gastero aculeatus* (Bervoets and Blust, 2003; Bervoets et al., 2001), *Nerodia* spp. (Burger et al., 2007) and *Ruditapes philippanarum* (Ji et al., 2006). Olsvik et al. (2001) reiterated that tissue accumulation is dependent on ambient metal concentrations and speciation and whether the metals are taken up via the water or via the diet. Isani et al. (2008) supported this notion by saying that distribution of metal accumulation in different organs can vary depending on the source of uptake and also on the species of the sentinel. The selection of particular suitable tissue or organ would increase the accuracy of the determination of metal bioavailability and contamination (Yap et al. 2009)

Loumbourdis and Vogiatzis (2002) reported that liver is one of the main target organs of cadmium accumulation in *Rana ridibunda* and the increase of cadmium

concentration is distinct and significant. In another study, Loumbourdis and Wray (1998) found that *Rana ridibunda* liver has higher cadmium concentration than carcass. To add, Pérez-Coll et al. (1997) found out that 26% of the cadmium uptake is deposited into the liver. In another study Foran et al. (2002) stated that cadmium can accumulate and be retained in the liver. A study by Bervoets et al. (2001) found that cadmium level was the highest in the liver and the lowest in muscle. However, the study also reported that cadmium accumulation from fish feed was the highest in the gut, followed by kidney, liver and muscle respectively. All the above-said statements showed how liver is one of the major sites for cadmium accumulation in the body. To explain how liver could be a main accumulation site, Loumbourdis et al. (2007) reported that cadmium may gain entry into hepatocytes via endocytosis mediated by iron binding protein for example ferritin. Endocytosis of cadmium-ferritin complex may serve an entry route from cadmium into liver. Isani et al. (2008) stated that cadmium accumulation in the liver was more evident only at longer exposure period.

Generally, after absorption, metal is transferred to liver, then to blood and finally accumulated in kidney (Isani et al., 2008). Therefore, in a long term exposure, renal cadmium will be higher than hepatic cadmium. Loumbourdis et al. (2007) stated that cadmium from liver is then released through the bile duct into gut lumen. Part of cadmium is removed by feces. However, most of the cadmium enters again into the liver via enterohepatic circulation. Via the blood circulation, cadmium will be transported to the various target organs. While the liver is one of

the first sites of metal accumulation, the kidney often showed the higher accumulation levels. Earlier on it was stated that according to Isani et al. (2008), following treatment, cadmium accumulation in the liver was more evident only at longer exposure period. The same was also true to cadmium accumulation in the kidney. Bervoets and Blust (2003) reported that it is possible to describe variation in metal level in liver, gills and kidney of fish where the highest cadmium level is in kidney followed by gills. A study done on *Salmo trutta* revealed that accumulation of cadmium was highest in the kidney and liver respectively (Olsvik et al., 2001). In water snakes, Burger et al. (2007) reported that different tissues accumulated metals differently. A study by Loumbourdis et al. (2007) found out that when *Rana* sp. was exposed to cadmium, it started to be deposited in liver, kidney and the gut studied. However, the kidney is found to be the main site of accumulation. According to Isani et al. (2008), cadmium concentration in kidney is higher than the liver because renal metallothionein induction is lower. Flament et al. (2003) reported that cadmium is also readily incorporated in the kidneys and reproductive tissues of *Pleurodeles waltl* larvae. Lee (1983) supported this notion by saying that cadmium directly targets testis. In addition, adult *Chrysemys picta* from impacted sites has higher cadmium concentration in liver, kidneys and gonads (Rie, 2000)

The pattern and magnitude of cadmium accumulation also differ depending on various factors. Ji et al. (2006) studied *Ruditapes philippanarium* and found that there was an observable linear increase in cadmium accumulation with body

size. Therefore they suggested that linear accumulation of cadmium in the species is an inherent trait. Apart from that, life stage also influences the magnitude of cadmium accumulation and thus its toxicity to the organism. For instance, Loumbourdis et al. (1999) found that adult *Rana ridibunda* accumulated cadmium differently than their tadpoles. When exposed to 200 ppm cadmium for thirty days, adult *Rana ridibunda* recorded about 900 ppm dry weight in their body. On the other hand, the tadpoles of the same species recorded lower concentration in their bodies.

2.1.3 Effects of Cadmium Accumulation

The toxicity of cadmium on an organism differ vastly according species, stage of development, and type of organ or tissue affected. A study on amphibians revealed that lowest non-toxic effect level (NTEL) for amphibians on this group of animals is 9 µg Cd^{2+}/L. In one study by Freda (1991), it was reported that in *Rana temporaria*, the concentration of 4 µg of cadmium at pH 4.0, 4.5 and 6.0 posed no serious effect on their embryo. However, the concentration was toxic to their larvae. Therefore, it can be concluded that *Rana temporaria* larvae are more susceptible to the toxicity of cadmium than their embryos. When the comparison was made between adult and tadpoles, the toxicity of cadmium also exhibited different trend and effect. Loumbourdis et al. (1999) reported that tadpoles had better cadmium detoxification mechanism than adult. After the initial cadmium shock, the tadpoles were able to establish

effective detoxification mechanism that was capable to neutralize the excess cadmium. As a result, the tadpoles accumulated less cadmium than adult *Rana ridibunda*. Hence, *Rana ridibunda* tadpoles can withstand higher concentration of cadmium before it exerts its toxic effect. However, Flament et al. (2003) reported that metamorphosis is strongly delayed in the presence of 10.9 uM of cadmium.

According to Herkovits et al. (1998), at the early stages of development there was a rather low uptake of cadmium. The low cadmium uptake obtained at early developmental stage could be related to the protective coat of the vitelline membrane and the cortex, preventing high toxicity exerted by cadmium. However, as development advances, cadmium uptake increases. In a different study on *Oryzias lattipes*, Foran et al. (2002) concluded that the early stage eggs are more sensitive to cadmium toxicity than other developmental stages. A study on *Rana ridibunda* revealed that exposure to sub-lethal concentration of cadmium may increase susceptibility of eggs and larvae to disease or retard the growth and metamorphosis of larvae (Loumbourdis et al., 1999). In *Salmo trutta* Hansen et al. (2006) found that cadmium-resistant fish (fish living in the cadmium-polluted areas) showed a longer time to accumulate lethal levels. However, it was also shown that cadmium-resistant fish has smaller sized offspring, decreased fecundity, smaller brood size, longer time to reach maturity and shorter female life expectancy compared to reference fish. These instances showed how for different organism, the differences in life stage and exposure could give different toxicity-response outcomes.

Alvarez et al. (2004) reported that cadmium has been classified by the International Agency for Research on Cancer as category I (human) carcinogen while Dudley and Klaasen (1984) reported that cadmium is hepatotoxic. This is apparent when the ability of the liver to sequester free forms of metals is exceeded. In this case, during chronic toxicity of with heavy metals, including cadmium, liver damage could occur (Urena et al., 2007). Martin et al. (2002) reported that low concentrations of cadmium were shown to mimic the effects of androgen and stimulate the proliferation of prostate epithelial cells. Therefore, the ability of cadmium to mimic the effects of androgens may, in part, explain the risk of prostate cancer associated with exposure to the metal.

Cadmium also is able to exert its effects on the physiological and immunological systems of the organism. Nordberg et al. (2005) reported that cadmium may cause renal damage, and human co-exposure to cadmium and arsenic may give rise to more pronounced renal damage. A study by Loumbourdis and Vogiatzis (2002) reported that cadmium interacts with vessels in vertebrate, implicating processes such as arteriosclerosis and hypertension. It also causes rupture of small vessels, resulting in discharge of red blood cells in the surrounding tissues. The same study also reported that dietary cadmium lead to iron depression in the liver. This would exacerbate oxygen to liver cells thus leading to anemia and tissue iron depletion. Cadmium also affect metabolism in the liver by interfering

with critical enzymatic reactions and leading to the decrease in liver lipid content and the increase liver glycogen content.

According to Loumbourdis and Vogiatzis (2002) cadmium accumulation could result in the depression in the immune system. This leads to massive invasion of parasites and foreign materials which results in the production of macro-melanophage centers (MMC). Macro-melanophage centers, being originated from Kupffer cells, may accumulate cadmium and become scavenger of cadmium. Macro-melanophage centers production may also be accelerated by the presence of red blood cells into the tissue. Since Loumbourdis and Vogiatzis (2002) reported that cadmium may cause the rupture of small vessels that lead to the discharge of red blood cells in the surrounding tissues, consequently, this would increase the production of macro-melanophage centers. Therefore, it can be concluded that cadmium-related production of macro-melanophage centers in the tissues can be caused either through the presence of red blood cells in the tissue or via parasitic invasion caused by depression in the immune system.

Cadmium is a known reproductive toxicant. Flament et al. (2003) reported that cadmium could pose the effect of steroid pathway modification and potentially modify differentiation of the gonad (gonadogenesis). This is confirmed by Foran et al. (2002) who reported that cadmium has the documented ability to act centrally to disrupt steroidogenesis at all level of the pituitary gland. This disruption has the effect of causing alterations in the testis and liver, which can

lead to impaired reproduction. Cadmium also can reduce liver function and this reduction may either impair the trafficking, release or production of vitellogenin and/or the metabolism of estradiol. The study by Foran et al. (2002) also found that parental or *in ovo* exposure to cadmium may impact the endocrine function of the next generation of adults. Apart from that cadmium is also an androgen mimic (Martin et al., 2002) which has effect on the proliferation of prostate epithelial cells. On the other hand, Garcia-Morales et al. (1994) stated that cadmium has an estrogen mimetic effect which cannot be extended to other heavy metal. These observations may lead the conclusion that cadmium is one of the environmental pollutants that can cause endocrine disruption.

Cadmium also may exert its effect on the developmental biology of an individual. Cervera et al. (2005) summarized that reproductive and developmental disorders have frequently been associated with cadmium exposure in different organisms. In a study on *Pleurodeles waltl*, Flament et al. (2003) found that exposure of the larvae of to cadmium does not affect the primary step of gonadogenesis,. Nevertheless, it strongly inhibits metamorphosis and consequently further gonadal development. The study also found that metamorphosis is strongly delayed in the presence of cadmium. This might be the effect of cadmium in cell division via the antagonistic action of cadmium on cell cycle. The study concluded that while there is a lack of evidence on the effect of cadmium on sex differentiation, cadmium exerts a great inhibitory effect on metamorphosis.

Canton and Sloof (1982) reported that *Xenopus laevis* show same sensitivity to cadmium as *Oryzias latipes* based on inhibition of the larval development

2.1.4 Bioconcentration Factor

Cadmium bioconcentration factor (BCF) is the ratio of its concentration in tissue versus in the environment (Herkovits et al., 1998; Flament et al., 2003). However, Olsvik et al. (2001) reported that bioconcentration factor is expressed as the ratio of metal concentration in organism to metal concentration in water. This is confirmed by Illinois General Assembly (2008) and EPA (2008) who fine-tuned the definition by specifically defining bioconcentration factor as the concentration of substance in all or part of an aquatic organism divided by the concentration of the substance in the water. A distinction is made between bioconcentration and bioaccumulation. According to European Center for Ecotoxicology and Toxicology of Chemicals (2009), bioconcentration is defined as the net result of the uptake, distribution, and elimination of a substance in an organism due to water-borne exposure, whereas bioaccumulation includes all routes of exposure (i.e. air, water, soil, food). An accumulation study done by Canton and Sloof (1982) on fish and amphibians found that cadmium concentration ratios (C_{org}/C_{water}) of 280 and 130 respectively could be determined. It was also found that although cadmium accumulated more quickly to a higher level in *Poecilia* sp. than in *Xenopus* sp., the concentration ratios are in the same order of magnitude. Since cadmium bioconcentration factor is higher

in larvae exposed to the lower cadmium concentration, Flament et al. (2003) confirms that amphibians could play an important role in the biomagnification process even when they are exposed to very low cadmium concentration in the environment. A bioconcentration value of more than 1000 indicates that the organism has a very high potential to bioaccumulate pollutants (Jean-Louis, 1998). The value of less than 30 is considered to be low potential, while the values in between 30 and 100 is termed as average or high potential.

2.2 Biomarker Study

2.2.1 Introduction to Biomarkers

Biomarkers, as defined by Galloway (2006) are functional measures of exposure to stressors expressed at various levels. Jamil (2001) described biomarker as a variation in cellular or molecular constituents, processes, structures or functions. This variation may be induced by a xenobiotic and is measurable in a biological system or a sample. According to Zorita et al., (2007), the measurements of biological response might be used to identify sources of pollution and biological effects of a wide range of pollution. To reiterate, the authors defined biomarkers as measurements at the molecular, biochemical or cellular level, which indicate that the organism has been exposed to pollutants (biomarker of exposure) and/or the magnitude of the organism's response to the pollutants (biomarker of effect).

Biomarkers can be classified into several groups. According to Monserrat et al. (2007), biomarkers can be classified as specific and non-specific. Specificity, in this sense, refers to its response to a specific toxicant. Stress proteins, enzymes and other bio-molecules have been shown to be affected by metal pollutions and they can be regarded as either specific or non-specific biomarkers. The use of toxicant-specific biomarkers such as metallothionein has been widely employed to indicate presence of heavy metal. On the other hand, the effect on non-specific biomarker cannot be attributed directly to one cause. In most cases, the effect of a toxicant on a non-specific biomarker is indirect. For example, since pollutants can directly or indirectly modify the balance between the concentration of pro-oxidants and antioxidants, the determination of oxidative stress (DNA damage, protein oxidation, lipid peroxidation) and/or antioxidant responses in aquatic species is commonly employed as a non-specific biomarker.

According to Linde-Arias et al. (2008), biomarkers are sensitive tools for the assessment of biological effects of pollutants. However, the employment of one single biomarker is not recommended. This is because Schmitt et al. (2007) stated that any biomarker or sentinel studies should incorporate a battery of biomarkers to show the sentinel's respond to environmental conditions. Furthermore, a study by Linde-Arias et al. (2008) on *Oreochromis niloticus* showed that a multibiomarker or multiparameter approach was able to reveal the differences in the health of the species among reference and contaminated sites.

Zorita et al. (2007a) reiterated that in any ecotoxicological studies, the use of a battery of bioresponses is recommended since single biomarker is not able to reflect the impairment of organism's health and/or its adaptation to the impaired environmental condition

2.2.2 Metallothionein

One of the direct responses of metal uptake is the production of metallothionein. Metallothioneins are a family of protein molecules that are induced by free cytosolic metal ions especially cadmium (Hansen et al., 2006; Baykan et al., 2007). These molecules have low molecular weight of about 7 Kd (Hansen et al., 2006). The most significant character of metallothionein is its high cysteine amino acid content (Loumbourdis et al., 2007; Isani et. al., 2008) and its lack of aromatic amino acids (Sutherland and Stillman, 2008). According to Monserrat et al. (2007), the behavior of metallothionein is dominated by the chemistry of the thiol (-SH) group of the molecule. Within this thiol group, metals are sequestered and bound. The metal-thiolate clusters allow rapid intra-metallothionein exchanges of metallic ions between clusters as well as changes with other metallothionein molecules. The binding of metallothionein during an excess of harmful metals protect the organism against its toxicity effect. This is because sequestration of cationic metals by metallothionein limits the availability of these cations at undesirable sites.

Binding mechanism of metals to the metallothionein molecules are still being investigated. However, Sutherland and Stillman (2008) mentioned that there are two overall mechanisms of cadmium binding to metallothionein. The first mechanism is through cooperative mechanism in which only metal free metallothionein or apo-metallothionein (apo-MT) and the metal saturated metallothionein or holo-metallothionein (holo-MT) are significantly populated in the tissue. In this mechanism, cadmium ions would first bind completely to one metallothionein molecule. After the metallothionein molecule is completely saturated, then only the rest of the cadmium ions bind to the next metallothionein molecule. Therefore, a large number of metallothionein molecules present are either apo-metallothionein or holo-metallothionein while the population of partially metallated metallothionein in the tissue is not significant. The second mechanism suggested that the binding between cadmium and metallothionein is non cooperative. This mechanism allows the presence of a significant amount of partially metallated metallothionein, along with apo-metallothionein and holo-metallothionein in the tissue. Sutherland and Stillman (2008) suggested that the presence of these partially metallated specimen could potentially serve a biological function.

The functions of metallothionein are generally twofold. Firstly, it plays a role as a metal binding protein. Within this function, metallothionein serves two purposes. The first one is its essential role in regulation of intracellular essential metal concentration such as zinc. Therefore, Olsvik et al. (2001) stated that

metallothioneins are important in metal homeostasis. On the other hand, apart from regulatory purposes, the role of metallothionein is also on detoxification (Jamil, 2001). A number of researches found that metallothionein plays a central role in the detoxication of non-essential metals including cadmium (Pérez-Coll et al., 1997; Loumbourdis et al., 2007). According to Olsvik et al. (2001), metallothioneins are able to sequester excessive metals, thus reduces the toxic effect of metals on proteins and enzymatic processes. This is confirmed by Monserrat et al. (2007) by stating that the binding of metallothionein during an excess of harmful metals protects the organism against toxicity by limiting the availability of these cations at undesirable sites. Consequently, Olsvik et al. (2001) mentioned that metallothionein molecules are assumed to be acute phase protein molecules involved in detoxification of metal. One important statement that Rosa et al. (2008) made was that cadmium bound to metallothionein is non-toxic and therefore is unable to exert negative effects on the surrounding tissues. Mouchet et al. (2006) reported that each metallothionein molecules are able to chelate and sequestrate seven metal ions in mammals. Therefore, metallothionein is important in cadmium acclimatization (Hansen et al., 2006) and is predominant in cadmium depuration (Mouchet et al., 2006). To summarize, Isani et al. (2008) stated that metallothionein is involved in accumulation, detoxification, transport and homeostasis of metals.

Apart from its role in the metal-binding process, the high cysteine content in metallothionein allows it to function as anti-oxidant (Hansen et al., 2006).

Kuroshima (1995) reported that metallothionein synthesis may occur in response to oxidative stress. Therefore, metallothionein is suggested to play an antioxidative role to protect cells from radicals in similar fashion to glutathione. According to Loumbourdis et al. (2007) and Isani et. al. (2008), metallothionein was shown to play a protective role in radical scavenging and may act as scavengers of reactive oxygen species (ROS)

In an organism, metallothionein can be found in various tissues. According to Pérez-Coll et al. (1997), metallothionein have been identified in liver, kidney, pancreas and intestine of vertebrates. To add, Loumbourdis et al. (2007) stated that metallothionein concentration increases in the liver, kidney and gut after exposure to cadmium and about 60% of cadmium in the mucosa of the small intestine are metallothionein-bound. However, the liver is often employed in major protective mechanisms by metallothionein (Pérez-Coll et al., 1997; Povlsen, 1990). According to Mouchet et al. (2006), the sensitivity of different tissues or cells to cadmium appears to be related, at least in part, to metallothionein biosynthesis and expression of metallothionein genes. It is also appears that the liver and kidney are more sensitive to cadmium accumulation than any other tissues. This explains the prevalence of higher levels of metallothionein in the liver than other tissues. Intraspecific comparison of hepatic metallothionein was made by Henry et al. (1994) and it was found that hepatic metallothionein levels in non-mammals (chicken and frog) were slightly higher than rodents, while humans and higher mammals had much higher levels of

metallothionein. Human and higher mammals need higher level of metallothionein because it plays a role in homeostasis of metals, detoxification of metals and free radical scavenging. Within one species, metallothionein is also produced in different stages of life cycle. Pérez-Coll et al. (1997) stated that cadmium binding fractions of metallothionein are reported in both the adults and embryos of *Bufo arenarum*.

Metallothionein molecules have strong affinity towards metal ions. Henry et al. (1994) reported that metallothionein's unique cysteine content allows it to have a high affinity for metals such as cadmium. However, according to Baykan et al. (2007), metallothionein molecules have higher affinity to cadmium as compared to zinc. Therefore, metallothionein has a higher preferential tendency of binding to cadmium than to zinc. It is also reported that cadmium is a strong inducer of metallothionein (Kuroshima, 1995) and are known to be a better inducer of metallothionein than other metals (Hansen et al., 2006).

According to Zorita et al. (2008) metallothionein molecules are metal-responsive protein especially induced at mRNA level and expressed at the protein level. The expression pattern of metallothionein was characterized by an early and strong induction. The strong induction occurs regardless of the contamination level of cadmium, and this strong induction can be interpreted as acclimatization (Brulle et al., 2007). The increased resistance to cadmium the in last embryonic stage of *Xenopus laevis* is also attributable to the enhancement of defense mechanism

via metallothionein induction and expression. However, researches also showed that an increase in the transcription of metallothionein gene is almost always followed by the reduction in the expression of other gene. Brulle et al., (2007) hypothesized that the increase in quantity of transcripts of metallothionein and decrease in other proteins are due to two factors. Firstly, cadmium has an effect on the expression of these effectors and the synthesis of metallothionein, which chelate cadmium could allow a return to basal level. Secondly, there is a preferential energy allowance for metallothionein synthesis to allow a faster and more effective detoxification and to rapidly acquire a metal resistance.

The correlation between metallothionein and heavy metal concentrations is a positive one. Urena et al. (2007) stated that metallothionein synthesis is one of the best known biochemical responses to metal exposure and there is a strong positive association between metallothionein levels and metal concentration. Metallothionein synthesis is induced under condition of elevated metal concentration (Linde-Arias et. al., 2008). This would provide more binding sites for metals and ions and thus limiting latent damage by the metal. This is confirmed by Brulle et al. (2007) that stated that exposure to cadmium would lead to the development of detoxification mechanism including production of metallothionein in order to allow tolerance of individuals to metallic contaminants. Acclimatization and resistance to metals, especially cadmium has always been related to metallothionein and Herkovits et al. (1998) stated that a relationship between metallothionein-cadmium and tissue cadmium has been accepted by

many researchers. Therefore, higher level of metallothionein and metallothionein-like protein indicated exposure to cadmium (Kitana et al., 2007). This has been documented in researches by Baykan et al. (2007) and Hansen et al. (2006) who found that metal exposed fish and cadmium-acclimatized trout has higher metallothionein-like protein.

According to Isani et al. (2008) the absorbed cadmium is transferred to liver. There, cadmium would induce the synthesis of hepatic metallothionein. Therefore upon exposure to cadmium, there would be an increase in the metallothionein concentration in the liver. This is demonstrated by studies on *Rana ridibunda,* (Loumbourdis et al., 2007), *Platichthys flesus* (Povlsen, 1990), *Anguilla anguilla* (Bird et al., 2008), *Xenopus laevis* (Mouchet et al., 2006) and *Sparus aurata* (Isani et al., 2008).

However, there is a limitation to the ability of metallothionein to sequester cadmium ions and detoxify them. According to Rosa et al. (2008) when high concentration of cadmium in tissue are reached, the protective effects of metallothionein are overwhelmed. This would to lead to spillover, where the overwhelmed defense capacity would lead to insufficient metallothionein production, hence insufficient cadmium-metallothionein binding (Mouchet et al., 2006). This phenomenon would leave a high concentration of cadmium in its free from in the tissue. Rosa et al. (2008) stated that these unbound cadmium can become potentially toxic.

Nevertheless, a number of studies have indicated that metallothionein is a good candidate in the study of biomarkers. Martín-Díaz et al. (2008) mentioned that when assessing exposure to variety of contaminants in the aquatic environment, biotransformation enzymes and metal-binding proteins such as metallothionein play an important role in organic and metal pollutant contamination respectively. Loumbourdis et al. (2007) suggested that metallothionein is a good biomarker of exposure for heavy metal pollution while Baykan et al. (2007) reiterated that metallothionein may be a useful biochemical marker in assessing metal toxicity and predicting the potential hazardous effects by metals. The use of metallothionein as a toxicant-specific biomarker has been widely employed to indicate the presence and the effect of heavy metal pollution (Monserrat et al., 2007). Mouchet et al (2006) reported that numerous studies have been carried out to use metallothionein as biomarkers for cadmium contamination. A study by Choi et al. (2006) found that the amount of metallothionein-like protein in *Laternula elliptica* increased linearly with exposure time and the amount of cadmium accumulated in the tissue. This suggests that there is a potential utility of metallothionein-like proteins as a biomarker for exposure to cadmium. To add, the quick response to cadmium, after only a few days of exposure further indicates the suitability of metallothionein as a biomarker providing an early warning signal. Therefore (Bird et al., 2008) concluded that the strong relationship between metallothionein and heavy metal concentration

demonstrates that it is a potentially good biomarker for heavy metal contamination.

2.2.3 Oxidative Stress and Glutathione-S-Transferase

According to Valdivia et al. (2006), oxidative stress refers to the imbalance between production of reactive oxygen species (ROS) and the antioxidant defenses in organism. Monserrat et al. (2007) then stated that oxidative stress can be defined as the disturbances between reactive oxygen species concentration and the antioxidant defenses concentration, favoring the first. In the body, reactive oxygen species are produced naturally during metabolic processes (Hansen et al 2006). In normal circumstances, the negative effects of reactive oxygen species on the cell or tissue are normally prevented from causing toxic effects by antioxidants. However, when the balance between reactive oxygen species concentration and the antioxidant levels is tipped towards the reactive oxygen species, this would lead to oxidative stress. This may eventually lead to oxidative damages and general disturbances of cellular redox balance. According to Monserrat et al. (2007) reactive oxygen species can induce several deleterious effects at cellular level.

As stated above, the presence of reactive oxygen species in the body is normal. However in the intracellular environment, the presence of many enzymatic and non-enzymatic antioxidant defenses keeps reactive oxygen species at a low

concentration (Monserrat et al., 2007). Valdivia et al. (2006) stated that superoxide dismutase, catalase and glutathione-S-transferase are some of the more important and the better studied antioxidants as an indicator of defense mechanism against reactive oxygen species. To add, Hermes-Lima and Zenteno-Savin (2002) reiterated that glutathione-S-transferase is one of the key enzymatic players in the defense mechanism against reactive oxygen species. Martín-Díaz et al. (2008) reported that glutathione transferases are a family of enzymes that utilize glutathione (GSH) as a substrate in reaction which permit the biotransformation and disposal of a wide range of exogenous compound. These enzymes are phase II type enzymes which catalyze the synthetic conjugation reactions of xenobiotic parent compound and their metabolites with reduced glutathione to facilitate the excrement of chemicals. The overall metabolic pathway and the role of glutathione-S-transferase is illustrated in Figure 2.1.

Various studies have linked cadmium with oxidative stress and antioxidants. Hansen et al. (2006) stated that cadmium is able to generate reactive oxygen species, therefore increasing the activity of antioxidants. According to Mouchet et al. (2006), cadmium can increase reactive oxygen species formation and generate oxidative stress. This would lead to lipid peroxidation and generate conditions that promote apoptosis and necrosis. To add, cadmium is also known to promote alterations in proteins, DNA and membrane structure and functions (Isani et. al., 2008) and generate DNA damage such as DNA breaks via

production of reactive oxygen species (Mouchet et al., 2006). Alvarez et al. (2004) stated that the induction of oxidative stress and its resulting increased level of lipid peroxidation would lead to the modified activity of the enzymes in the antioxidant defense system. Studies on *Salmo trutta* found that there was a significant higher superoxide dismutase mRNA levels in the gills and liver of cadmium exposed individuals as compared to the reference trout. Therefore Hansen et al (2006) concluded that the activity levels of antioxidant enzymes were higher in metal exposed trouts those in reference trouts. Many studies also emphasized that exposure to cadmium would lead to the change in the concentration of glutathione. Loumbourdis et al. (1999) reported that a relatively high glutathione concentration was observed in *Rana ridibunda* exposed to 200 ppm of cadmium for 30 days. Therefore, it was concluded that cadmium caused an increase in glutathione concentration (Loumbourdis et al., 2007). Kuroshima (1995) explained that the increase in glutathione may be a biochemical response of living cells to protect themselves from heavy metal toxicity.

Despite the various studies on the effect of cadmium on oxidative stress, the mechanism of this activation is poorly understood (Hansen et al., 2006). It has been proposed that cadmium inhibit the complex III of electron transport chain in mitochondria, thereby, facilitating the production of superoxide. Isani et al. (2008) stated that cadmium does not go to redox cycling and its involvement in lipid peroxidation is indirect due to displacement of redox metal ions or reduction in glutathione content. To counteract, reactive oxygen species are detoxified by

antioxidant enzymes and scavenger molecules, hence explaining the up-regulation of antioxidant systems as a result of cadmium contamination. The up-regulation of antioxidant system also include the increase in glutathione-S-transferase enzyme production and activities. This is confirmed by Martín-Díaz et al. (2008) who reported that glutathione-S-transferase activities in *Carcinus maenas* and *Ruditapes philippanarum* was found to be significantly induced by the presence of cadmium.

The instances illustrated above show how the determination of antioxidant activities can be used as a biomarker for heavy metal pollution, especially cadmium despite it being a non-specific biomarker and a biomarker of effect (as opposed to biomarker of exposure). Valdivia et al. (2006) stated that the use of oxidative stress indicators as a biomarker system to assess health status would be a useful tool, especially which could detect early exposure to contaminants and other environmental stressor. This is agreed by Larose et al. (2008) who stated that some of the most sensitive markers of toxicant effects are alterations in the activities of biotransformation enzymes such as glutathione-S-transferase and previous research has shown that heavy metal may induce glutathione-S-transferase's activity.

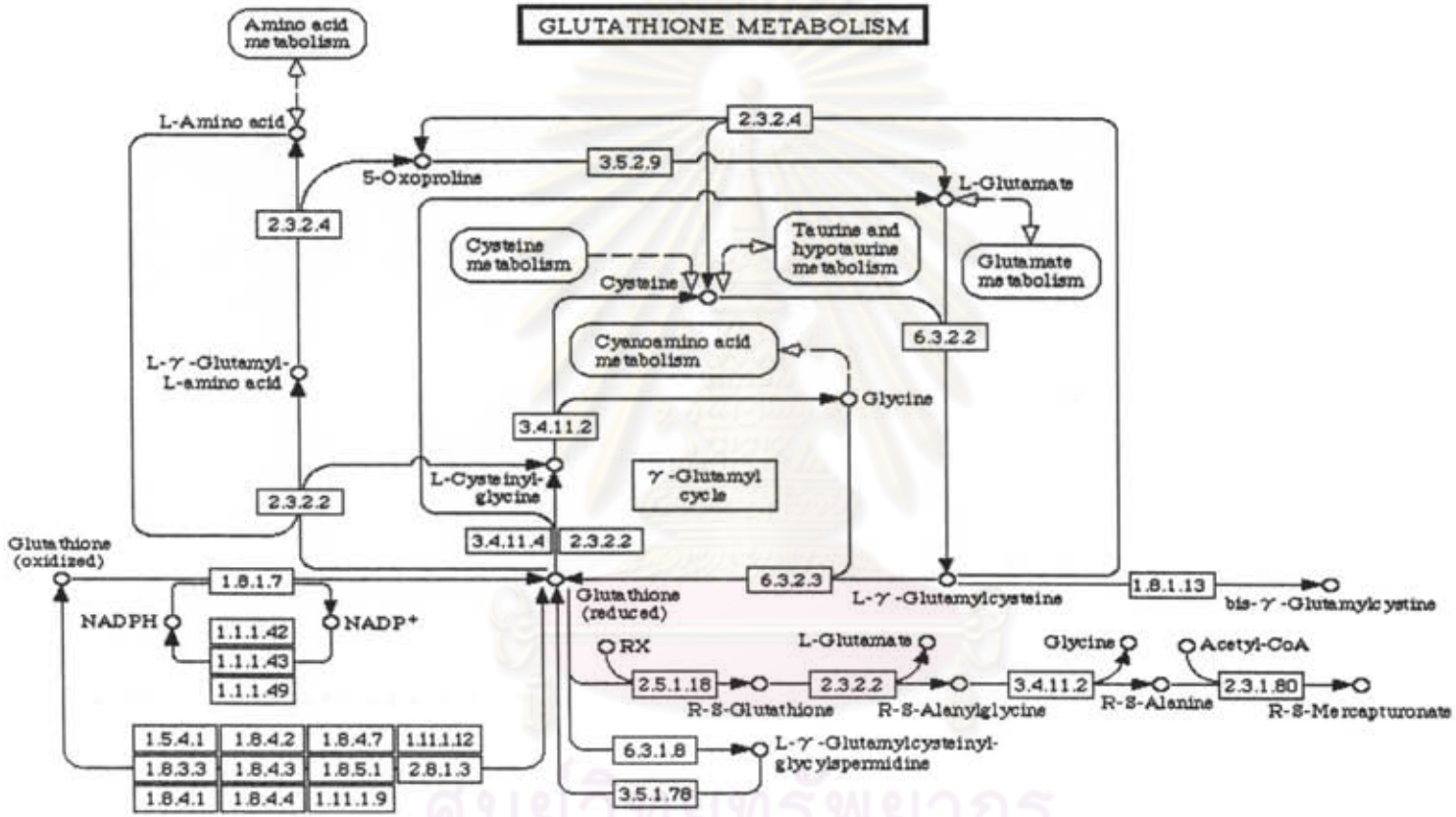

Figure 2.1: Metabolic pathway of glutathione, including the role of glutathione-S-transferase enzyme (code number 2.5.1.18), obtained from KEGG Bioinformatics technology (Kanehisa and Goto, 2000)

2.2.4 Vitellogenin

Vitellogenin is the female-specific precursor of vitellin (Cervera et al., 2005) or yolk protein which is normally synthesized by the liver in female individuals (Cargouët et al., 2007). According to Bon et al. (1997), the vitellogenin of rainbow trout detected under SDS-PAGE showed that it was composed of 2 molecular forms with the sizes of 170 Kd and 390 Kd. The 170 Kd molecule is a monomeric form while the 390 Kd molecule is a dimeric form. Goodwin et al. (1992) stated that after synthesis in the liver, the vitellogenin produced circulates in the blood stream and is transported to the ovary. Activated by the induction of endogenous estrogen, vitellogenin is cleaved into the yolk protein (Irwin et al., 2001) and then incorporated by endocytosis in the growing oocytes (Povlsen, 1990). Vitellogenin synthesis is called vitellogenesis and it has a large metabolic demand on the liver of the female. Being estrogen dependent (Li et al. 2006), vitellogenin production and incorporation into the oocytes is regulated by estrogen (Irwin et al. 2001). While vitellogenin is known to be female-specific, Goodwin et al. (1992) contradicted this by stating that the presence of vitellogenin in male fish suggest that vitellogenin is not absolutely sex-specific. This is based on their study which concluded that the presence of vitellogenin in the male individuals of *Ictalurus punctatus* may be a consequence of physiologically active level of endogenous or exogenous estrogen. This is also the same conclusion obtained by Wallace (1970) who stated that vitellogenin can be induced in males as well. In another study, Fukada et al. (2001) reported that

male and immature individuals are able to synthesize vitellogenin when they are exposed to exogenous estrogen or to substances that mimic estrogen.

The level of vitellogenin in the blood is cyclical and seasonal and it is influenced by the reproductive activities of the females. According to Bon et al. (1997), during the previtellogenesis period, there is a slight increase in vitellogenin levels in the plasma. Even though Maitra et al. (2007) reported that there is a good correlation in the annual profile of plasma vitellogenin, gonadotropin and gonadosomatic index, the slight increase of vitellogenin level during the previtellogenesis period is not followed by an increase in gonadosomatic index. However, during the vitellogenesis period, a surge of vitellogenin levels can be observed in the plasma. During this period, there is a great correlation between vitellogenin levels and gonadosomatic index. With increased vitellogenin levels, Irwin et al. (2001) reported that larger eggs would be produced. This would give rise to larger hatchlings with higher fitness. Higher vitellogenin levels may also alter reproductive fitness of the population by changing energy allocation, physiology and egg production.

A number of researches have discussed the relationship between cadmium and vitellogenin levels in an organism. A study on *Oncopeltus fasciatus* revealed that the levels of ovarian vitellogenin polypeptides, especially vitellogenin 1 (VTG1) and vitellogenin 2 (VG2), were decreased in cadmium-exposed females (Cervera et al., 2005). Another study by Haux et al. (1988) showed that fish exposed to

sub-lethal level of cadmium often demonstrate marked decrease in total plasma calcium. This hypocalcemic response may impair vitellogenesis. Cadmium exposure was also found to induce a decrease in plasma vitellogenin. Povlsen (1990) offered an explanation regarding the mechanism on how cadmium could affect vitellogenesis and vitellogenin level. Cadmium may interfere with protein synthesizing apparatus at the transcriptional level, thereby inhibiting vitellogenin synthesis. Moreover cadmium treatment and the resulting metallothionein synthesis are expected to compete with vitellogenin synthesis in liver cells, hence affecting its synthesis. It is hypothesized that cadmium inhibits estradiol-induced vitellogenin synthesis but also estradiol may stimulate the induction of metallothionein.

The use of vitellogenin as a biomarker for xenobiotic contamination has been established in various literatures. Irwin et al. (2001) stated that since vitellogenin is regulated by estrogen, therefore it can serve as an excellent model for studying estrogen-mimicking compounds. To add, Cargouët et al. (2007) reported that in vivo induction of vitellogenin in juvenile or male fish is widely used as a sensitive and reliable biomarker of exposure to estrogenic compounds. Fukada et al. (2001) reiterated that vitellogenin has been used as biomarker of exposure (as opposed to biomarker of effects) of fishes to environmental estrogens or estrogenic compounds. Furthermore, vitellogenin is proven to be a sensitive and simple biomarker for assessing exposure to environmental stressor (Bulukin et al., 2007) and it is an established and sensitive endpoint for analysis

to exposure to (anti-) estrogens and their mimics (Eidem et al., 2006). Therefore Li et al. (2006) concluded that vitellogenin is an ideal biomarker for screening estrogenic activity in aquatic environment.

2.3 Morphometric and Gravimetric Study

2.3.1 Morphometry and Gravimetry

Morphometric and gravimetric data has always been a good indicator of the effect of pollution on an organism. Often, pollutant has a direct effect on growth of an organism. According to Monserrat et al. (2007), morphological and physiological alterations have been reported in individuals exposed either naturally or experimentally to different pollutants. Kitana et al. (2007) reported that morphometric analysis was used to compare site-related differences in body size. For instance, in a study by Norris et al. (2000), it was found that the hepatosomatic index of trout living in uncontaminated site was greater than those living in sites contaminated by cadmium and zinc. The same study also reported that there was an association between high renal cadmium with low hepatosomatic index in many fish species.

Larose et al. (2008) reported that hepatosomatic index is a physiological biomarker that reflects responses following chemical and cellular interactions hence are indicative of irreversible damage. A study by Norris et al. (2000)

suggested that excessive lipid peroxidation following exposure to metals may result in cell membrane damage and cell death in livers. These will then lead to the reduction of liver weight, hence the reduction in hepatosomatic index. Wolf and Wolfe (2005) stated that a decrease of liver size as a consequence of a loss of hepatic glycogen and/or lipid is a common morphologic response of liver to metal toxicity. Reduced hepatosomatic index may be viewed as liver atrophy which is caused by reduction in the size of liver cells, lipid peroxidation/depletion or nuclear and cytoplasmic inclusions. These suggest that reduced hepatosomatic index may be used as a biomarker for heavy metal exposure. Maes et al. (2005) reported that recent studies have related metal toxicity with changes in morphometric indices, especially hepatosomatic index. In a study on farmed *Anguilla anguilla,* Urena et al. (2007) found that the inverse (1/x) of cadmium concentrations in liver and kidney are significantly correlated with hepatosomatic index. This shows that as cadmium content increases, the hepatosomatic index decreases. Apart from that, there is also a relationship between metal toxicity and growing condition. Urena et al. (2007) stated that growing conditions (food and water chemistry) may determine metal composition in eel tissues as well as the response that these fish exhibit to metal toxicity. A study by Bird et al. (2008) on *Anguilla anguilla* found that, even though the regression slopes relating all the parameters studied are very shallow, cadmium concentration in the liver were strongly correlated with both length and weight. Therefore, the significance of assessing biometric response (weight, condition,

growth) has been suggested as an important measure of pollution impact on an organism (Van Straalen and Timmermans, 2002)

Not many researches had been focused on what is the effect of cadmium accumulation on the renosomatic index. However, Barbier et al. (2005) stated that when the liver's capacity to sequester heavy metal, including cadmium, is exceeded, the metal-metallothionein complex is released into the blood and circulated to the kidneys where it is reabsorbed and accumulated in the renal epithelium. Most probably, the effect of cadmium accumulation on the kidney size is similar to its effect on liver size. However, more studies are needed to find the relationship between cadmium and renosomatic index.

Kitana et al. (2007), in a study on *Chrysemys picta* reported that morphometric analysis was used to compare site related differences in body size, which is often associated with other traits that directly affect reproductive success. Certain changes in reproduction and reproductive structures maybe considered to be a response to environmental contaminant especially in the site where high cadmium level prevails.

The relationship between cadmium accumulation and gonadosomatic index is an interesting one. In a study on *Oncorhynchus mykiss*, Bon et al. (1997) found out that gonadosomatic indices of individuals remained a minimum of 0.5% during the pre-vitellogenesis period. On the other hand, during the period of

endogenous vitellogenesis and exogenous vitellogenesis, there is a great correlation between vitellogenin levels with female gonadosomatic indices. In the presence of cadmium, Povlsen (1990) stated that vitellogenin production (vitellogenesis) is inhibited. This is because cadmium treatment and the resulting metallothionein synthesis compete with vitellogenesis in the liver cells. Apart from that, Haux et al. (1998) reported that sub-lethal exposure to cadmium is often demonstrated by a marked a hypocalcaemic response (decrease in total blood plasma calcium) which may impair vitellogenesis. Therefore a decline in vitellogenin level may be linked, although not exclusively, to cadmium exposure. Therefore, if cadmium can impair vitellogenesis and there is a great correlation between vitellogenin levels with female gonadosomatic indices, it can be inferred that cadmium exposure may be able to result in the reduction of female gonadosomatic indices.

2.3.2 Weight-length Relationship, Scaling Coefficient and Condition Factor

Weight-length relationship and condition factor has always been used to assess the well-being of fish species. The use of these parameters in *Fejervarya limnocharis* is novel because it has never been used in amphibians before. The weight-length relationship can be used to reflect the impact of the environment on the growth of an organism. This is based on a formula reported by LeCren (1951), which stated the relationship between weight (W) and length (L) as

$$W = aL^b$$

This relationship can been transformed into its logarithmic mode to obtain linear graph, hence transforming the equation into

$$\log W = b \log L + \log a$$

Eastwood and Couture (2002) termed the weight-length relationship, *b*, which is the slope of the linear graph, as Scaling coefficient which is a descriptor of the growth pattern of a population determined from length and weight measurement. The values of *a* and *b* were derived empirically from the log-transformed equation above (Larose et al., 2008). It has been proposed that the Scaling coefficient be used as a bioindicator of long term stress in populations subjected to environmental pollution (Eastwood and Couture, 2002) The use of Scaling coefficient is not limited to providing the ratio between weight and length of an organism. Provided that the b value is constant within one species, the Scaling coefficient can be used to derive condition factor. Condition factor is basically a gross index that indicated general effect of pollution on the species (Linde-Arias et al., 2008). According to Larose et al. (2008), condition factor is obtained from this formula

$$CF = (W / aL^b) \times 100$$

In the formula, W represents the weight of the individual and L refers to the length. Bervoets and Blust (2003) stated that condition factor is used to express the overall well being of an individual. According to Linde-Arias et al. (2008), condition of the whole body or condition factor is a provider of information on the health status of an organism. Eastwood and Couture (2002) concluded that the condition factor is a simple measurement that consistently showed lower condition for the more metal-contaminated fish. Hence, the utilization of condition factor may become a useful tool to assess the effect of pollution on individuals when variation in other environmental factors is limited.

Bird et al. (2008) stated that accumulation of heavy metals by organisms can lead to reduction in growth. However, such detrimental effects are unlikely to become apparent until after changes have already occurred at the molecular and cellular level. It was also found that above a certain metal load, the values of condition factor are always low (Bervoets and Blust, 2003). This suggests that if metal load is higher than a specific threshold level, the weight-length ratio will be reduced, hence the reduction in condition factor. In addition, Urena et al. (2007) mentioned that condition factors are indicative of overall health and therefore, make them good candidate to be considered when studying the effect of metal exposure. A study by Hansen et al. (2006) on fish revealed that the condition factors are lower in metal exposed population as compared to reference population. This indicates that exposure to metals may affect the fitness and

condition of the effect of metal exposure on fitness of an individual. To add, Maes et al. (2005) also stated that there is a clear relationship between increased heavy metal content with lower condition factor. A study by Norris et al. (2000) also established similar trend. They reported that female *Salmo trutta* at uncontaminated site tend to have a higher condition factor than females at the contaminated site. According to Linde-Arias et al. (2008), although condition factor is not specific or sensitive and may be affected by non-pollutant factors, it serves as an initial screening biomarker or endpoint to indicate exposure and effects of contaminants and also to provide information on energy reserves. To add, the use of condition factor in an environmental impact assessment is also favored because of its low cost, ease and rapidity. Furthermore, it was reported that condition factor of animals living in the most degraded areas was the lowest as compared to other less degraded areas.

2.4 Histology and Skeletochronology

2.4.1 Histology and Histopathology of Cadmium Accumulation

Histological analysis is one of the important parameters often included in the study of sentinels. Even though histological analysis offers no direct cause-effect relationship between a pollutant and its histopathological changes, Hutchinson and Pickford (2002) stated that histology has been included as priority endpoints in the new OECD test guidelines for environmental monitoring.

According to van Dyk et al. (2007), often the initial effects of heavy metal pollution are evident only at the cellular or tissue level thus leading to observable changes in histological structures. These changes are evident even before significant changes can be identified at higher levels such as organ, individual, population, external and behavioral levels. Therefore, histological and histopathological changes in the tissue are often regarded as one of the early indicators of change for heavy metal pollution. However, while histopathological changes often offer indirect explanation to the causation of a specific pathology by a specific agent such as heavy metals, the result should be approached with great care. This is because, according to Thijssen et al. (2007), histopathological changes caused by cadmium vary considerably from one animal to another and from one individual cell to another. However, one common observation that can be seen is that exposure to environmentally relevant cadmium concentrations often elicited only minor changes while damage become serious after a high cadmium exposure or over long term chronic exposure. Rosa et al. (2008) reported that the toxicity of cadmium may manifest itself in many clinical forms. The same authors also reported that with the exception of recent studies, there have been few attempts to investigate elemental concentrations and histopathological changes. Some of the histological effects of cadmium exposure to a tissue include fibrosis, apoptosis and necrosis (Habeebu et al., 1998; Thijssen et al., 2007; Rosa et al., 2008), hyalinization, vacuolation, and cellular swelling (van Dyk et al., 2007) and cadmium deposition (Itokawa et al., 1978).

One of the most reported effects of cadmium is the development of tumor cells in tissues. According to Junqueira et al. (1995) tumor refers to localized swelling caused by inflammation or abnormal cell proliferation. It is synonymous with neoplasm which is abnormal mass of tissue formed by uncoordinated cell proliferation. It was found that low concentrations of cadmium were shown to mimic the effects of androgen. This leads to the stimulation of prostate epithelial cells proliferation which would lead to the formation of tumor and stimulate the proliferation of prostate epithelial cells (Martin et al. 2002). With this finding, Martin et al. (2002) concluded that the ability of cadmium to mimic the effects of androgens may, in part, explain the risk of prostate cancer associated with exposure to the metal. To add, Alvarez et al. (2004) reported that cadmium has been classified by the International Agency for Research on Cancer as category I (human) carcinogen.

Cadmium is also responsible for the formation of macro-melanophage centers in various tissues such as liver, testis and kidney. According to Loumbourdis and Vogiatzis (2002) cadmium accumulation could result in the depression in the immune system. This leads to massive invasion of parasites and foreign materials which results in the production of macro-melanophage centers (MMC). Apart from its response to parasite invasion, macro-melanophage centers, being originated from Kupffer cells, may also accumulate cadmium and become scavenger of cadmium. Macro-melanophage centers production may also be accelerated by the presence of red blood cells into the tissue. Since Loumbourdis

and Vogiatzis (2002) reported that cadmium may cause the rupture of small vessels that lead to the discharge of red blood cells in the surrounding tissues, this would increase the production of macro-melanophage centers.

According to van Dyk et al. (2007), exposure to heavy metals may cause histological changes in the liver and a histological investigation of exposed specimens may therefore produce meaningful results. Dudley and Klaasen, (1984) reported that in the liver, cadmium induced pathological changes include diffused hepatocyte and eosinophilic cytoplasm. There is also evidence of moderate cell swelling where the shape of hepatocyte becomes polygonal. Apart from that, reduced sinusoidal space and a decrease in the area of the hepatocyte occupied by the nucleus can also be observed. Necrosis is also evident with most hepatocytes around the necrotic area are swollen and the majority of nuclei are pyknotic. A study on *Oreochromis mossambicus* revealed that the most prevalent histological characteristics in cadmium-exposed individuals include hyalinization of hepatocytes, increased vacuolation associated with lipid accumulation, congestion of blood vessels and cellular swellings (van Dyk et al., 2007). Hyalinization refers to dense eosinophilic inclusions while vacuolation refers to excessive accumulation of fat in cytoplasm. On the other hands cellular swelling/hydropic degeneration is manifested by cloudy, granular and enlarged cells due to water influx.

Kidney is one of the main target organs of cadmium toxicity. Alvarez et al. (2004) reported that DNA fragmentation and histopathologically observed changes characteristics of apoptosis are found in the kidney, after cadmium exposure. According to Thijssen et al. (2007), it is known that cadmium exposure may lead to renal interstitial fibrosis. To add, when the kidney is injured by cadmium, glomerular or interstitial infiltrated inflammatory cells become activated and produce reactive oxygen species and fibrogenic and inflammatory cytokines. This in turn stimulates several cellular pathways, including mesangial and fibroblast activation as well as tubular epithelial-to-mesenchymal transition (EMT) leading to over production of extracellular matrix component. Apart from that an increase in vacuolization and an increased amount of lysosomes were observed in kidneys exposed to cadmium. Rosa et al. (2008) reported that the toxicity of cadmium may manifest itself in many clinical forms including renal diseases as cadmium has been found to affect renal proximal tubule function and has been related to renal fibrosis. The study found that moderate and severe thickening of Bowman's capsule and interstitial fibrosis were noted in one third of *Balaena mysticetus* kidney tissues examined and these were found to be cadmium related. In addition, there were morphological changes in kidney include tubular cell degeneration in the initial stages, progressing to interstitial inflammatory reaction and fibrosis.

Cadmium may also impose its effects on gonad histology. Zorita et al. (2007) stated that gonad histology was studied as a supporting parameter in order to

establish the reproductive status of the animals and to detect possible pathological alterations. The author's study revealed that alterations in gonad histology were eventually found in specimens although no specific relationship with sampling station or cruise was observed. Alvarez et al. (2004) reported that DNA fragmentation and histopathologically observed changes characteristics of apoptosis are found in the prostate, seminal vesicle, testes and epididymis after cadmium exposure

In a histopathological survey on of *Mullus barbatus*, Zorita et al. (2008) found that the testis showed high prevalence of melano-macrophage centers (MMC). The occurrence of these centers was as a result of parasitic infestations. Macro-melanophage centers, proposed to be non-specific indicator of environmental pollution, have been observed in gonads of fish inhabiting contaminated environment. McDaniel et al. (2008) stated that a subsequent historical survey of cricket frogs revealed hermaphroditic specimens with incidences of occurrences correlating with major trends in contamination. Testicular ovarian follicles (TOFs) refers to the presence of ovarian follicles in the testes; frequencies of the presence TOFS of less than 2% may represent natural background levels. While the origin of TOFS is unclear, there are some suggestions that they may be induced by exposure to pesticide or estrogenic endocrine disrupting compounds, and cadmium is one of the endocrine disrupting compounds. Another study by Mosconi et al. (2005) revealed that histological sections of testis of *Rana lessonae* caught from agricultural area showed sections with detectable

eosinophil leucocytes in the interstitial compartment while vacuolated areas were present within the tubules.

Cadmium may also exert its effect on the ovary. A study by Paksy et al. (1989) reported that when cadmium was given to prepubertal or postnatally androgenized female rats, follicular atresia, inflammation, hemorrhage and necrosis in the ovary were observed. It was also shown that acute cadmium exposure may interfere with the physiologic release of gonadotropic hormones which may be the consequences of direct action of cadmium on the pituitary and/or altered sex hormone level in proestrous animals. Therefore the study concludes that it seemed more plausible that cadmium acts on steroidogenesis at a subcellular level.

2.4.2 Skeletochronology

Skeletochronology is a histological technique to determine the age of an individual. The age data obtained from skeletochronology often provide valuable information on the well being of an organism. According to Patnaik and Behera, (1981), bone histology has been recognized as the most meaningful and practicable method not only to assess individual age, but also the speed of growth, the age of sexual maturity and the longevity of various species. This is further confirmed by Kyriakopoulou-Sklavounou et al., (2008) who stated that age determination in individuals is important in order to obtain information about the

rate of growth, age and size at sexual maturity and about longevity. The basis of skeletochronology it's the presence of line of arrested growth in the cross section of a long bone. According to Tsiora and Kyriakopoulou-Sklavounou (2002) seasonal climate have a major effect on physiological activities of an individual, hence affecting bone growth. In long bones, annual growth appears as a broad layer of periosteal bone whereas the hibernation period is expressed as narrow haematoxylinophilic line called line of arrested growth. Most research on skeletochronology had been focused on temperate species. In temperate species, it was presumed that only one line of arrested growth was laid down during each hibernating period (Kyriakopoulou-Sklavounou et al., 2008). Very few researches have been done on tropical species including research on *Calotes versicolor* (Patnaik and Behera, 1981) and *Rana nigrovittata* (Khonsue et al., 2000). It is also known that variation in growth rate may be caused by fluctuations in the availability and quality of food, or related to habitat as well as variations in the climate and the environment (Tsiora and Kyriakopoulou-Sklavounou, 2002). The use of data derived from skeletochronology has proven to be an important factor in the study of environmental effects on a species. In many cases that involve heavy metal pollution, the toxic effect of heavy metals manifested in an organ is mainly a function of concentration and exposure time, because many toxicants bioaccumulate. Thus in a study on *Balaena mysticetus*, Rosa et al. (2008) stated that age can be an important factor in wildlife toxicology and cadmium concentration was found to be age related. Therefore, Spear et al.

(2009) recommended that skeletochronology is used along with morphometric data to estimate growth as an index of physiological health.

CHAPTER 3

CADMIUM ACCUMULATION STUDIES OF TWO POPULATIONS OF RICE FROG (*Fejervarya limnocharis*) NATURALLY EXPOSED TO DIFFERENT ENVIRONMENTAL CADMIUM LEVELS IN MAE SOT, TAK PROVINCE, THAILAND

3.1 Introduction

Cadmium in the environment has the ability to be taken and accumulated into the body. Bervoets et al. (2001) reported that the levels of accumulated metals in the tissue were related to metal levels in sediment, water and food. This is further supported by Bervoets & Blust (2003) saying that it is more likely that tissue level reflects environmental level because metal concentration in tissue follow concentration in the environment. This is shown by the result of their study on *Gobio gobio* that showed both environmental and tissue cadmium and zinc followed a clear concentration gradient of the river. Francis et al. (1984) reported that *Carrasius auratus*, *Rana pipiens* and *Micropterus salmoides* showed strong correlations between cadmium concentration in water and tissue, sediment and tissue and water and sediment

Cadmium level in the aquatic ecosystem showed remarkable concentration variation between the media. Many pollutants, especially cadmium do not stay in the aquatic phase because they have high sorption to the sediment. Sorption of the pollutants to the sediment often results in the very low concentration of the pollutant in the liquid phase. Hence, most of the time, the concentration is not high enough to produce toxicity to aquatic organisms. In water, the concentration is usually very low. Chen and Liu (2006) stated that majority of the metals in the cadmium laden soil will be retained in the soil phase (soil and sediment), and the remaining, if released into aquatic ecosystem cannot reach concentration high enough to produce toxicity to aquatic animals. Francis et al. (1984) reported that high sorption capacity of stream sediment for cadmium, as predicted by Langmuir isotherm, was reflected by the small quantities of cadmium mobilized from the metal enriched sediments. This means that most cadmium in the aquatic ecosystem will be sorbed onto soil and sediment and not in the dissolved form. Therefore, Chen (2005) stated that cadmium has the lowest risk onto aquatic species. However high level of cadmium in freshwater environment may occur as a result of natural weathering of minerals in sediments and bedrocks or as a result of anthropogenic activities such as mining (Olsvik et al., 2000)

Cadmium uptake into the body may follow various routes. Sindayigaya et al. (1994) stated that according to literature, there are four possible route for substances such as metals to enter the body. They include food ingestion, absorption of metal in ionic form through the gills, water drinking and skin

absorption. Since the concentration of cadmium in the water is low, dermal route is often not the significant route of exposure to cadmium. Most cadmium will enter the body via the oral route. However, amphibians may be able to accumulate cadmium through the dermal route as significant as the oral route. Loumbourdis et al. (1999) reported that amphibians have high permeability of their skin, both in aquatic and terrestrial ecosystems, to pollutants. Due to the high permeability, pollutants are able to be absorbed into the body dermally. This is in addition to the more usual routes of pollutant exposure which include oral route and inhalation. Therefore, there are more than one significant ways on how xenobiotics are able to enter the body. Sediment-borne cadmium also may be transported from the environment to the organism. Francis et al. (1984) found that the embryo larval stage of the leopard frog in their study showed a high affinity for sediment borne cadmium. This is because the embryo of leopard frog remained just above the sediment layer, thus there is a possible transport of pollutant from the sediment into the embryo during the pre-hatching period. Even after hatching, the leopard frog's tadpoles situated themselves onto the sediment hence allowing a possible transfer of pollutant from the sediment to the tadpoles. Therefore it is apparent that the contact between the pollutant and the frog occur during all stages of life cycle, even during developmental stages.

Different tissues have the ability to accumulate metals differently. This is exhibited in studies by Loumbourdis and Wray (1998), Loumbourdis and Vogiatzis (2002), Bervoets and Blust (2003), Burger et al. (2007) and Bervoets et

al. (2001). Loumbourdis and Vogiatzis (2002) reported that liver is one of the main target organs of cadmium accumulation in *Rana ridibunda*. In another study, Loumbourdis & Wray (1998) found that *Rana ridibunda* liver has higher cadmium concentration than carcass. Loumbourdis et al. (2007) reported that Cadmium may gain entry into hepatocytes via endocytosis mediated by Fe-binding protein for example ferritin. Endocytosis of cadmium-ferritin complex may serve an entry route fro cadmium into liver

Loumbourdis et al. (2007) stated that cadmium from liver is then released through the bile duct into gut lumen. Part of cadmium is removed by feces. However, most of the cadmium enters again into the liver via enterohepatic circulation. Via the blood circulation, cadmium will be transported to the various target organs. Bervoets and Blust (2003) reported that it is possible to describe variation in metal level in liver, gills and kidney of fish where the highest cadmium level is in kidney followed by gills. In water snakes, Burger et al. (2007) reported that different tissues accumulated metals differently. A study by Loumbourdis et al. (2007) found out that when *Rana* sp. was exposed to cadmium, it started to be deposited in liver, kidney and the gut studied. However, the kidney is found to be the main site of accumulation. Flament et al. (2003) reported that cadmium is also readily incorporated in the kidneys and reproductive tissues. Lee (1983) supported this notion by saying that cadmium directly targets testis. In addition, adult *Chrysemys picta* from impacted sites has higher cadmium concentration in liver, kidneys and gonads (Rie, 2000)

Cd bioconcentration factor (BCF) is the ratio of its concentration in tissue vs in the environment (Herkovits et al., 1998). However, Illinois General Assembly (2008) and EPA (2008) fine-tuned the definition by specifically defining bioconcentration factor as the concentration of substance in all or part of an aquatic organism divided by the concentration of the substance in the water. A distinction is made between bioconcentration and bioaccumulation. According to European Center for Ecotoxicology and Toxicology of Chemicals (2009), bioconcentration is defined as the net result of the uptake, distribution, and elimination of a substance in an organism due to water-borne exposure, whereas bioaccumulation includes all routes of exposure (i.e. air, water, soil, food). An accumulation study done by Canton and Sloof (1982) on fish and amphibians found that cadmium concentration ratios (C_{org}/C_{water}) of 280 and 130 respectively could be determined. It was also found that although cadmium accumulated more quickly to a higher level in *Poecilia* sp. than in *Xenopus* sp., the concentration ratios are in the same order of magnitude. Flament et al. (2003) confirms that amphibians could play an important role in the biomagnification process even when they are exposed to very low cadmium concentration in the environment.

3.2 Objective and Sub-objectives

The objective of this part of the research is to compare the contaminant analysis parameters of *Fejervarya limnocharis* from contaminated site with those from reference site. In fulfilling this objective, several sub-objectives have been identified. They are as follow

3.2.1 To compare the hepatic cadmium concentration of *Fejervarya limnocharis* from contaminated site with those from reference site

3.2.2 To compare the renal cadmium concentration of *Fejervarya limnocharis* from contaminated site with those from reference site

3.2.3 To compare the testicular cadmium concentration of *Fejervarya limnocharis* from contaminated site with those from reference site

3.2.4 To compare the ovarian cadmium concentration of *Fejervarya limnocharis* from contaminated site with those from reference site

3.2.5 To compare the whole organismal cadmium concentration of *Fejervarya limnocharis* from contaminated site with those from reference site

3.2.6 To compare the Bioconcentration factors (BCF) of *Fejervarya limnocharis* from contaminated site with those from reference site

3.3 Hypothesis

3.3.1 There are significant differences in the hepatic, renal, testicular, ovarian and whole organismal cadmium concentration between *Fejervarya limnocharis* from contaminated site with those from reference site

3.3.2 There are significant differences in the Bioconcentration factors (BCF) between *Fejervarya limnocharis* from contaminated site with those from reference site

3.4 Methodology

3.4.1 Environmental Samples

Water samples (n=48) were collected in plastic bottles and transported back to the lab on ice. In the lab, 50 mL of the water samples were added to the microwave vessels. Then concentrated nitric acid (CARLO ERBA, analysis grade)) was added into each vessels. Samples were then microwaved at 160°C to 165°C with energy level of 980 kW following the recommended temperature profile (Figure 3.1) using EthosPro Microwave Digestion Labstation. The digested samples were cooled to room temperature before they were filtered. Then distilled water was added to the sample to volume. Sample blank (distilled water) was also subjected to the same microwave procedure. Samples were then analyzed for cadmium content with Graphite furnace atomic absorption spectrometer (AAS ZEEnit 700, Analytik Jena AG) with the digested distilled water acting as blank. Cadmium concentrations were determined by Graphite furnace atomic absorption spectrometer in the unit of mg/L. concentration of cadmium in the sample was then calculated into the unit of mg/kg dry weight sample with the following transformation.

Sediment samples (n=48) were collected in plastic bags and put on ice before they were transported to the lab. The sediment samples were dried at room temperature to a constant mass. The dried sediment was homogenized by hand

grinding with mortar and pestle. Extraneous organic matter was removed during grinding and sifting through a 2 mm-mesh size sifter. About 0.5 gram of the dried and sifted sediment was weighed into each of the microwave vessels. Then concentrated nitric acid was added to the samples before the vessels were secured. Samples were then microwaved at 175°C with energy level of 980 kW following the recommended temperature profile (Figure 3.2). The digested sediment samples were cooled to room temperature before they were filtered. Then distilled water was added to dilute the samples to volume. Sample blank (distilled water) was also subjected to the same microwave procedure. Sediment samples were then analyzed for cadmium content with Graphite furnace atomic absorption spectrometer with the digested distilled water acting as blank. Cadmium concentrations were determined by Graphite furnace atomic absorption spectrometer in the unit of mg/L. The concentration of cadmium in the sediment was then calculated into the unit of mg/kg dry weight.

3.4.2 Biological samples

Frogs were caught live at night time during visual encounter survey (Crump and Scott, 1994), and then placed in a plastic aquarium. The frogs were then transported live to the lab. The frogs were individually subjected to cold anaesthesia procedure before sacrificed by double-pith at brain and spinal cord. Each frog was autopsied and their livers (n=206), kidneys (n=206), ovaries

(n=94) and testes (n=111) were removed and weighed. Each tissue was then oven-dried at 80^0C overnight until the weight become constant. The final dry weight of each organ was recorded. Then a mixture of concentrated nitric acid and hydrogen peroxide (MERCK, synthesis grade) in the ratio of 7:1 was added to each sample in microwave vessels. The vessels were secure before they were microwaved at 200^0C and 980 kW following the recommended temperature profile (Figure 3.3). The digested tissue samples were cooled to room temperature before they were filtered. Then distilled water was added to dilute the samples to volume. Sample blank (distilled water) was also subjected to the same microwave procedure. Tissue samples were then analyzed for cadmium content with Graphite furnace atomic absorption spectrometer with the digested distilled water acting as blank. Cadmium concentrations in the tissues were determined by Graphite furnace atomic absorption spectrometer in the unit of mg/L. The concentration of cadmium in the tissues was then calculated into the unit of mg/kg dry weight.

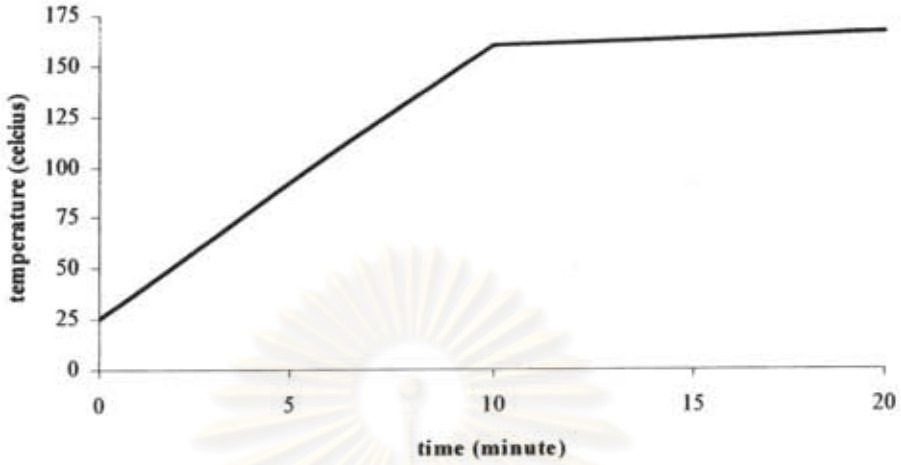

Figure 3.1: Temperature profile for microwave digestion of water sample according to US EPA Method 3015

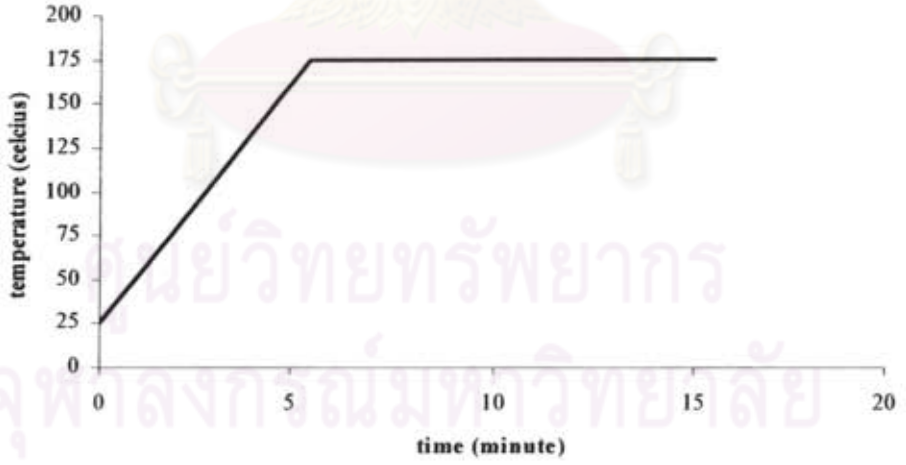

Figure 3.2: Temperature profile for microwave digestion of sediment sample according to US EPA Method 3051

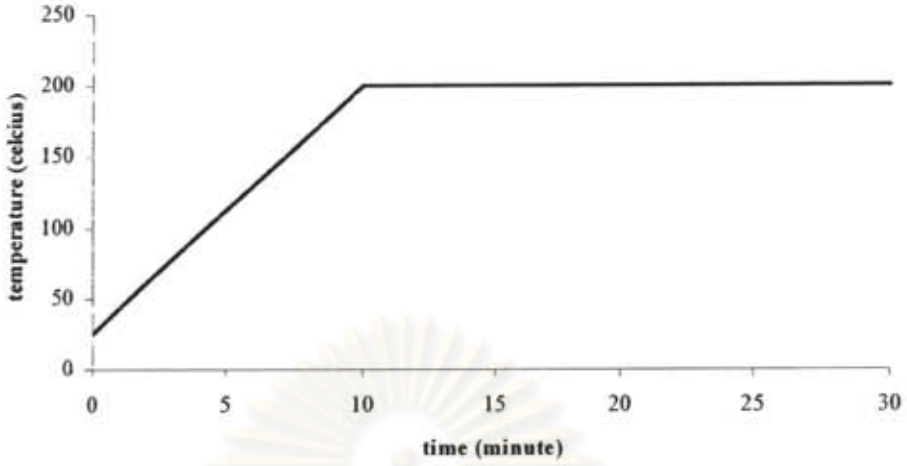

Figure 3.3: Temperature profile for microwave digestion of biological tissue sample

3.4.3 Data and Statistical Analysis

Data on hepatic cadmium, renal cadmium, ovarian cadmium, testicular cadmium and whole organismal cadmium are presented as quarterly average concentration. Hepatic, renal, ovarian, testicular and whole organismal cadmium bioconcentration factor are presented as the ratio between cadmium concentration in the tissue to cadmium concentration in water samples of the respective sampling sites.

All data were statistically analyzed with two-way ANOVA and Student-Newman Keuls test using the SigmaStat 2.0 program.

3.5 Results and Discussion

A summary of cadmium in the water and sediment samples are presented in Figures 3.4 and 3.5. The graphs showed that there were no significant differences of the cadmium in water samples between both Mae Pa and Mae Tao. However, the differences in cadmium concentration in the sediment were significant. For both environmental parameters, the differences between the seasons were not significant.

Figure 3.6 shows the quarterly average hepatic cadmium in *Fejervarya limnocharis* caught from both sites. Hepatic cadmium in *F. limnocharis* caught from Mae Pa ranges from 0.044 mg/kg to 0.592 mg/kg. On the other hand, in *F. limnocharis* caught from Mae Tao, the range is from 0.199 mg/kg to 3.543 mg/kg. The overall average hepatic cadmium concentrations are 0.204 mg/kg for frogs caught from Mae Pa and 1.939 mg/kg for frogs caught from Mae Tao. Further analysis showed that hepatic cadmium concentration in Mae Tao frogs are between 4.5 to 32.2 times higher than Mae Pa frogs. Frogs in both sites show similar fluctuation of hepatic cadmium when compared seasonally. In both sites, hepatic cadmium concentration is the highest during the early rainy season (April, May and June) and the lowest during the late rainy season (July, August and September)

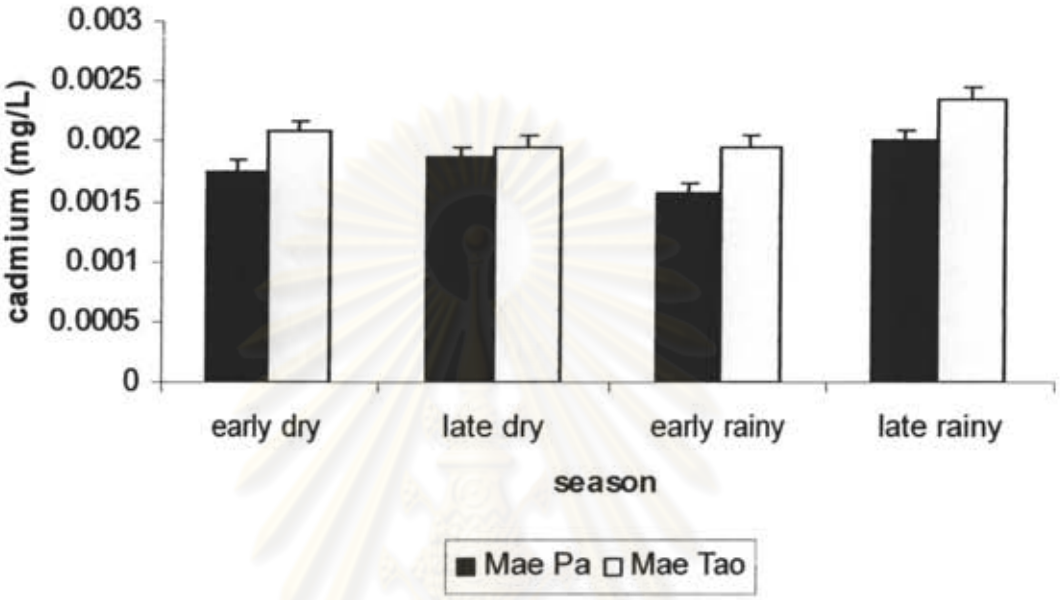

Figure 3.4: Quarterly average cadmium concentration in water samples in Mae Pa (n=24) and Mae Tao (n=24). All mean differences between season and stations are not statistically significant

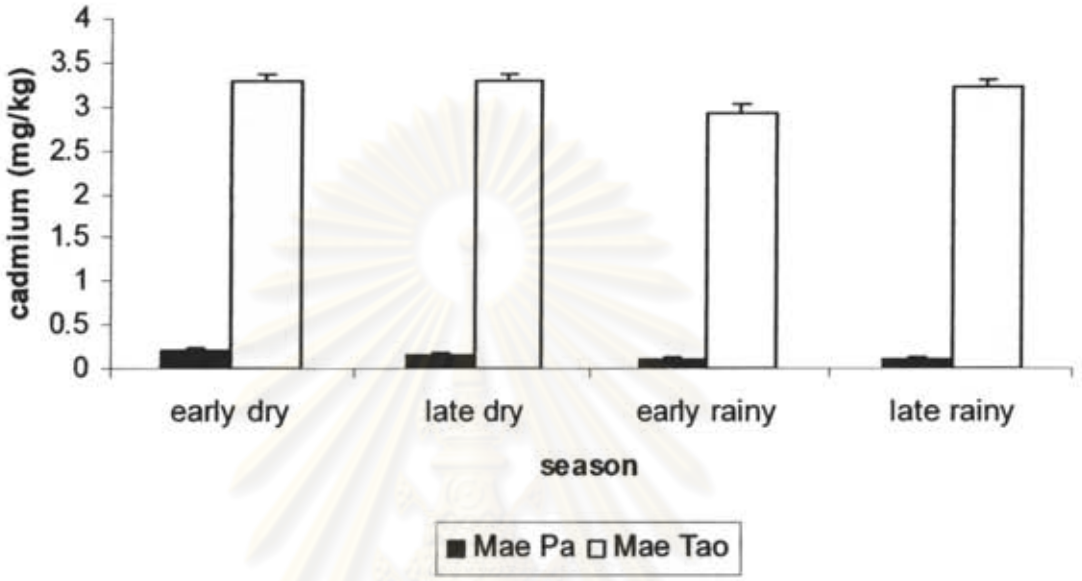

Figure 3.5: Quarterly average cadmium concentration in sediment samples in Mae Pa (n=24) and Mae Tao (n=24). All mean differences between stations are statistically significant ($P<0.05$), but differences between seasons are not significant

For cadmium in kidney, the results are shown in Figure 3.7. It is found that renal cadmium in *Fejervarya limnocharis* caught from Mae Pa ranges from 0.239 mg/kg to 1.715 mg/kg. In *F. limnocharis* caught from Mae Tao, the range is from 1.890 mg/kg to 12.175 mg/kg. The overall average renal cadmium concentrations are 0.786 mg/kg for Mae Pa frogs and 7.253 mg/kg for Mae Tao frogs. The result showed that Mae Tao frogs had renal cadmium concentration of between 5.5 to 16.2 times higher than Mae Pa frogs. Seasonal fluctuation-wise, renal cadmium concentration is the highest during the late early rainy season for Mae Pa frogs and during the late dry season for Mae Tao frog. The lowest renal cadmium concentration in frogs from both sites occurred during the late rainy season

The result for ovarian cadmium in *Fejervarya limnocharis* caught from reference and contaminated sites are shown in Figure 3.8. Ovarian cadmium in *F. limnocharis* caught from Mae Pa ranges from 0.006 mg/kg to 0.041 mg/kg. In *F. limnocharis* caught from Mae Tao, the range is from 0.009 mg/kg to 0.053 mg/kg. The overall average ovarian cadmium concentration in Mae Pa frogs is 0.018 mg/kg while the average ovarian cadmium concentration in Mae Tao frogs is 0.032 mg/kg. When ovarian cadmium concentrations in frogs are compared site-wise, the Mae Tao frogs showed cadmium concentration of between 1.2 to 3.9 times higher than Mae Pa frogs. However, these differences are not statistically significant. When season-wise comparisons are made, ovarian cadmium concentrations in both sites are the highest during the late dry season.

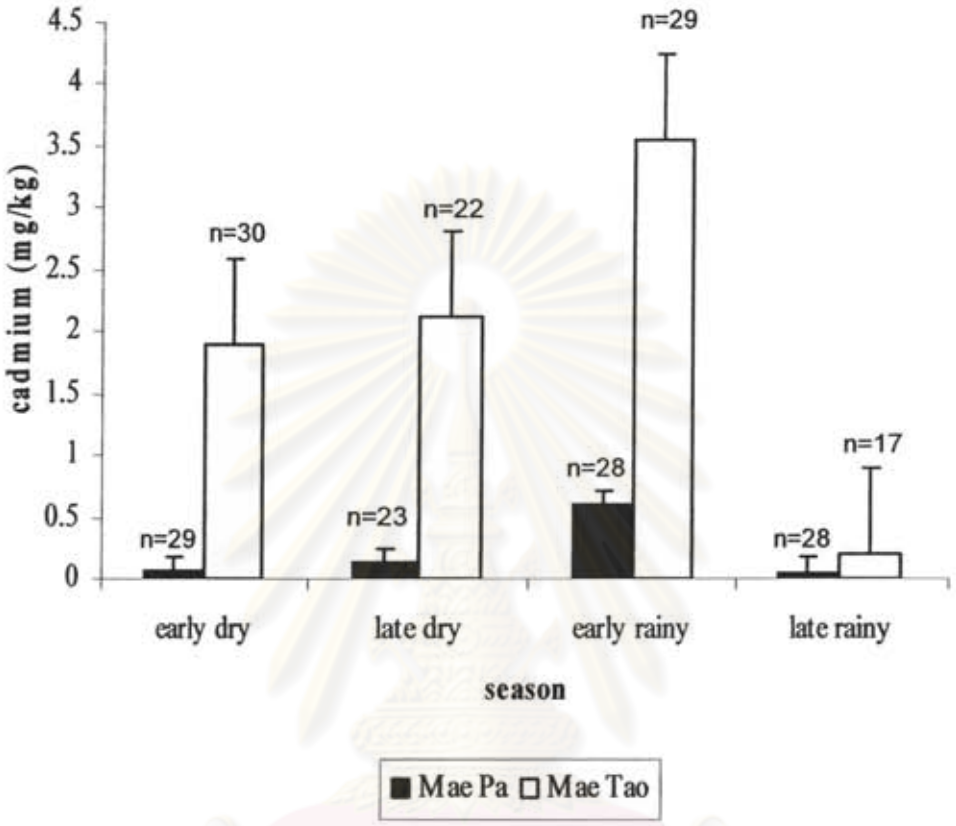

Figure 3.6: Quarterly average hepatic cadmium concentration in *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences between season and stations are statistically significant (P<0.05)

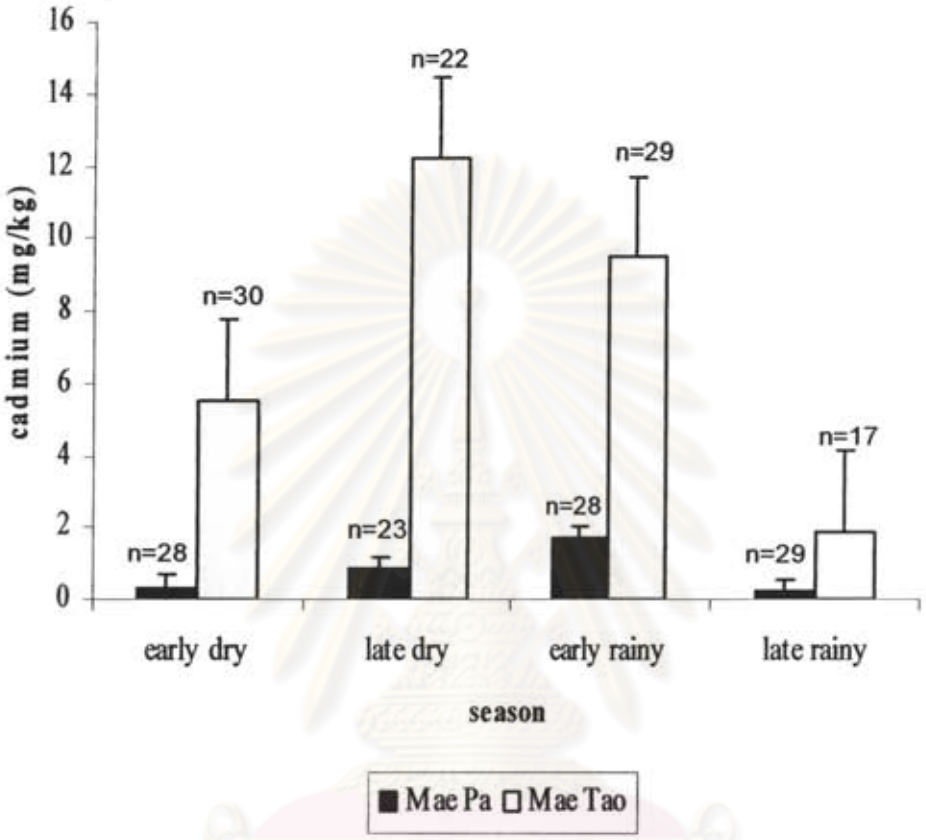

Figure 3.7: Quarterly average renal cadmium concentration in *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences between season and stations are statistically significant (P<0.05)

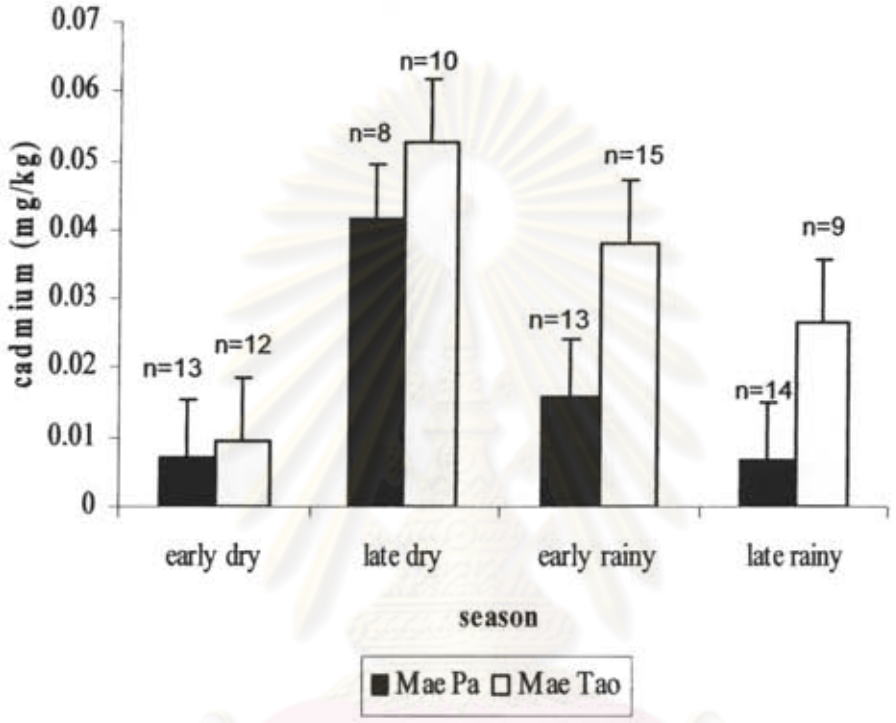

Figure 3.8: Quarterly average ovarian cadmium concentration in *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences between stations are not statistically significant (P<0.05)

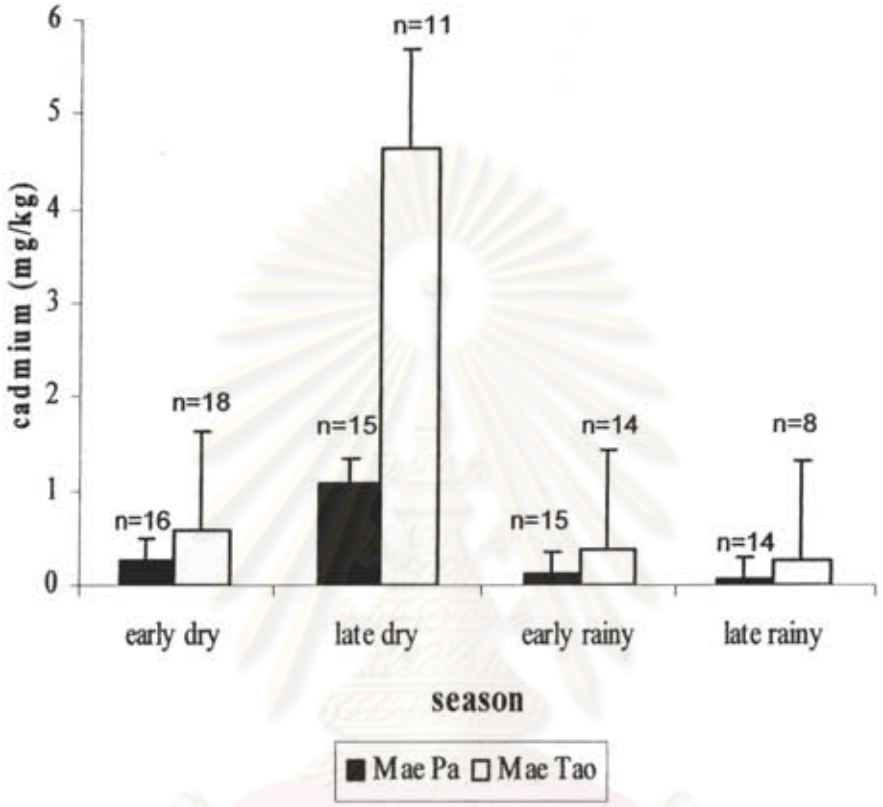

Figure 3.9: Quarterly average testicular cadmium concentration in *Fejervarya limnocharis* caught in Mae Sot, Tak. All mean differences between season and stations are statistically significant (P<0.05)

Figure 3.9 shows the results for quarterly testicular cadmium in *Fejervarya limnocharis* caught from Mae Pa and Mae Tao. The graph showed that testicular cadmium in *F. limnocharis* caught from Mae Pa ranges from 0.044 mg/kg to 1.089 mg/kg. Meanwhile in *F. limnocharis* caught from Mae Tao, the range is from 0.266 mg/kg to 4.626 mg/kg. Mae Pa frogs recorded the overall average testicular cadmium concentration of 0.379 mg/kg while Mae Tao frogs recorded the level of 1.462 mg/kg. Site-related comparison shows that testicular cadmium concentration in Mae Tao frogs are between 2.2 to 6.1 times higher than Mae Pa frogs. For frogs from both sites, the testicular cadmium concentrations are the highest during the late dry season and the lowest during the late rainy season

Cadmium concentration was also determined in whole organism. The results are shown in Figure 3.10. Whole organismal cadmium concentration in *Fejervarya limnocharis* caught from Mae Pa ranges from 0.024 mg/kg to 0.045 mg/kg. On the other hand, in *F. limnocharis* caught from Mae Tao, the range is from 0.180 mg/kg to 0.549 mg/kg. The overall average whole organismal cadmium concentrations are 0.034 mg/kg for frogs caught in Mae Pa and 0.375 mg/kg for frogs caught in Mae Tao. To compare, whole organismal cadmium concentration in Mae Tao frogs are between 7.5 to 14.5 times higher than Mae Pa frogs. Seasonally, Mae Pa frogs caught during the early dry season showed the highest whole organismal cadmium concentration. For Mae Tao, the highest whole organismal cadmium concentration is shown in frogs caught during the late dry

season. The lowest whole organismal cadmium concentrations for both sites are recorded in frogs caught during the late rainy season.

Overall comparisons also show that in both sites, renal cadmium concentrations are higher than hepatic cadmium. Renal cadmium concentration in Mae Pa frogs are between 2.9 to 6.8 times higher than hepatic cadmium concentration. In Mae Tao frogs, the renal cadmium concentrations are between 2.7 to 9.5 times higher than hepatic cadmium concentration. Tissue-by-tissue comparison showed that in both sites, renal cadmium concentration is the highest while ovarian cadmium concentration is the lowest (Figure 3.11). The result is in line with bioconcentration factor analysis where for both sites, kidney showed the highest cadmium bioconcentration factor (Figure 3.12) The average cadmium bioconcentration factor in kidney is 467.75 for Mae Pa frogs and 3672.32 for Mae Tao frogs. Ovary showed the lowest cadmium bioconcentration factor with the average values of 9.97 (Mae Pa) and 15.54 (Mae Tao).

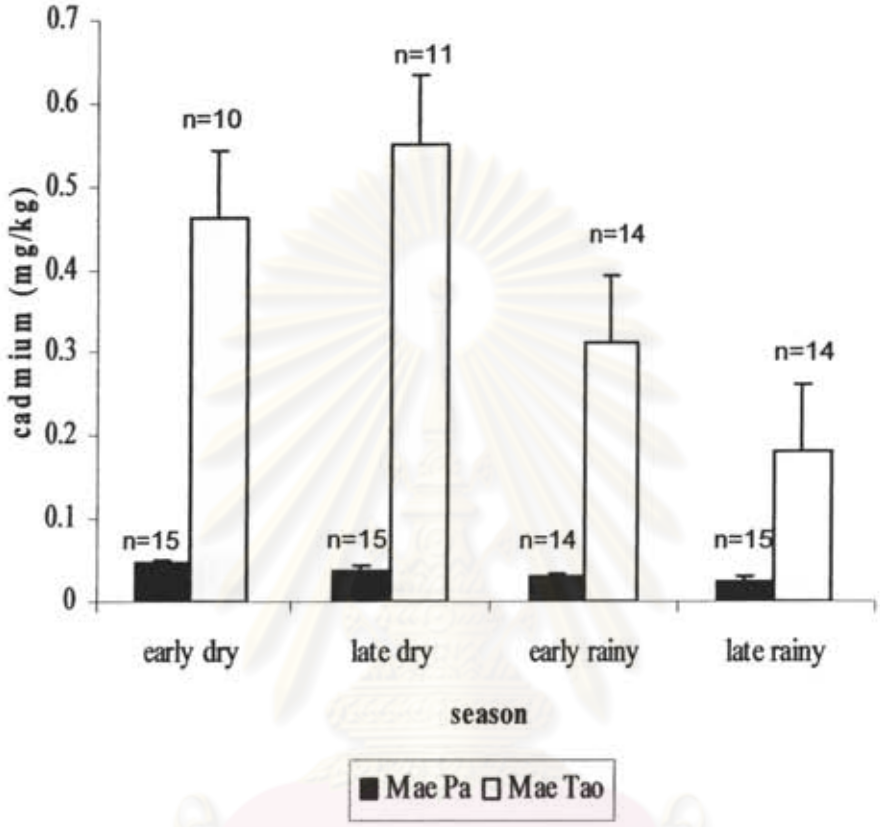

Figure 3.10: Quarterly average whole organismal cadmium concentration in *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences between season and stations are statistically significant ($P<0.05$)

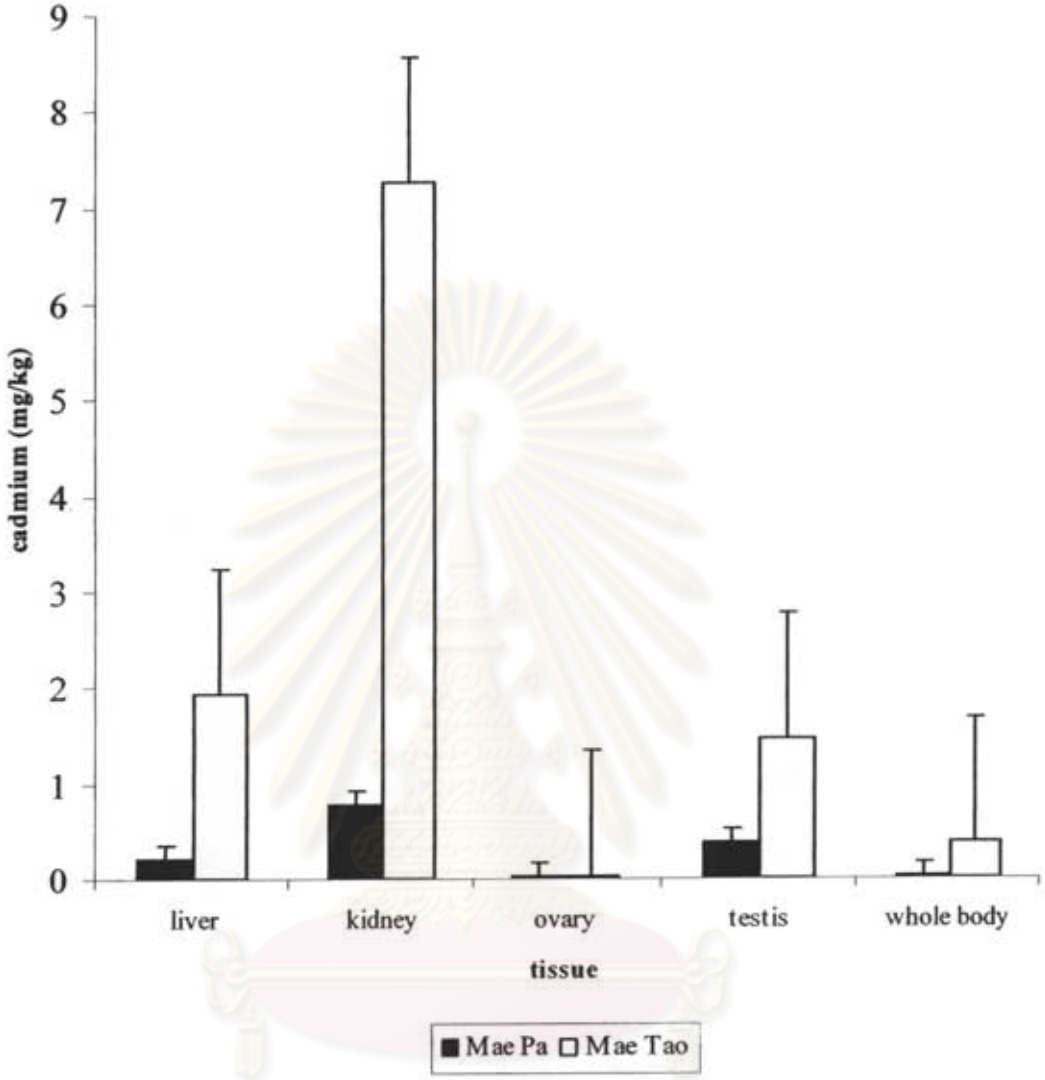

Figure 3.11: Comparison of average cadmium level between the tissues studied in *Fejervarya limnocharis* caught from Mae Sot, Tak.

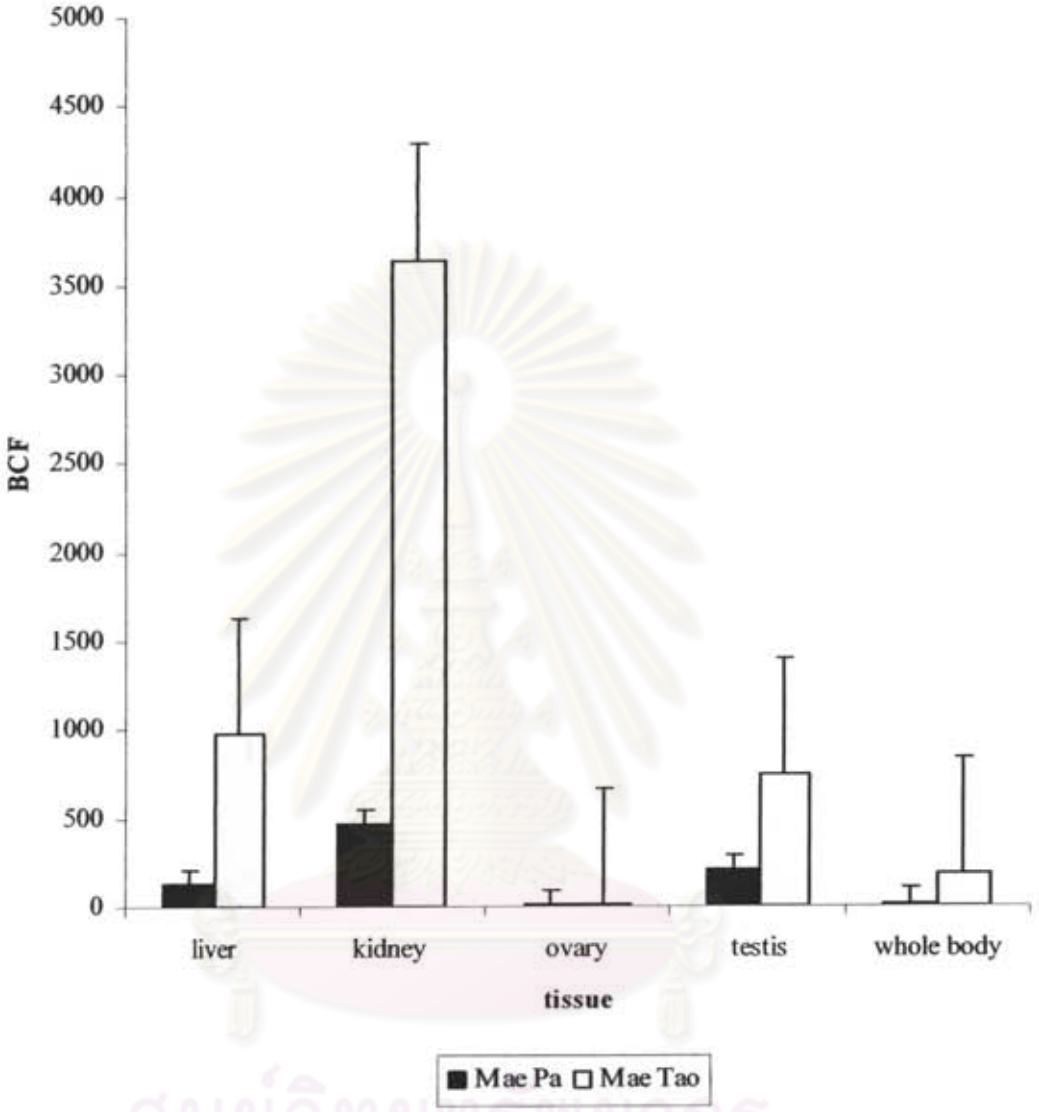

Figure 3.12 : Tissue cadmium bioconcentration factor (BCF) of *Fejervarya limnocharis* caught from Mae Sot, Tak

Loumbourdis and Vogiatzis (2002) reported that liver is one of the main target organs of cadmium accumulation in *Rana ridibunda*. In another study, Loumbourdis and Wray 1998 found that *Rana ridibunda* liver has higher cadmium concentration than carcass. Pérez-Coll et al. (1997) found out that 26% of the cadmium uptake is deposited into the liver. Foran et al. (2002) stated that cadmium can accumulate and be retained in the liver. However, our sets of results showed a different trend. While accumulation of cadmium in the liver is quite high, it is clear that renal cadmium accumulation is even more apparent. This may be because the liver is the primary accumulation site while the kidney is the final accumulation site. Loumbourdis et. al. (2007) reported that cadmium may gain entry into hepatocytes via endocytosis mediated by Fe binding protein for example ferritin. Endocytosis of cadmium-ferritin complex may serve an entry route for cadmium into liver where it will bind with metallothionein or stay as free cadmium in the hepatocytes. Cadmium, especially the free cadmium from liver is then released into the gastrointestinal lumen by the secretion of bile contents in the bile duct. In the gastrointestinal lumen, part of cadmium is removed by feces while most will enter the blood stream through the enterohepatic circulation. Via the blood circulation, cadmium will be transported to the various target organs, especially kidney. Therefore, throughout the life span of the frog, cadmium will be continually accumulated in the liver, and then transported to the kidney. So far, there has been no account on whether cadmium in the kidney is excreted or not. Hence it is assumed that all the cadmium that accumulates in the kidney will be retained there. High cadmium accumulation in the kidney is also reported by

various other studies. In a study by Bervoets and Blust (2003), it was found that the highest accumulation of cadmium in *Gobio gobio* was in kidney (Bervoets and Blust 2003). Studies in other species also revealed that the highest cadmium accumulation was in the kidney. They include *Salmo trutta* (Olsvik et al., 2001), *Rana ridibunda.* (Loumbourdis et al., 2007), *Pleurodeles waltl* (Flament et al., 2003) and *Gasterosteus aculeatus* (Bervoets et al., 2001).

While kidney showed higher cadmium accumulation than liver, we also found out that both are actually suitable indicators for biomonitoring of cadmium accumulation. This is because both hepatic and renal cadmium levels are significantly higher in *Fejervarya limnocharis* caught from the contaminated site as compared to those from the reference site.

Among reproductive tissues ovarian cadmium showed very little accumulation. Furthermore, the differences in ovarian cadmium accumulation between reference and contaminated sites were also not significant. This shows that the ovary probably is not a suitable organ to be used in biomonitoring of cadmium accumulation. However, this study found out that high cadmium accumulation is found in the testis. This is shown by the high testicular cadmium level and high testicular cadmium bioaccumulation factor in *Fejervarya limnocharis* from the impacted site as compared to those from the reference site. This is expected because Lee (1983) stated that cadmium directly targets testis.

In this study, all the frogs used for the analysis of whole organismal cadmium weighed less than two grams. In these frogs, the use of organs, especially kidney and testis was rather difficult. Therefore, for these small frogs, whole body cadmium analyses were performed. We have included the use of rather small frogs in this research because we anticipate in the future, that not all field sampling activities will be able to have a yield of large frogs. In this case, instead of determining cadmium accumulation in organs and tissues, whole organismal cadmium accumulation may be a better choice of analysis. In our study, we found out that there were significant differences in whole organismal cadmium level and whole organismal cadmium bioaccumulation factor between *Fejervarya limnocharis* caught from contaminated site with those from reference site. Frogs from contaminated sites had higher whole organismal cadmium level and whole organismal cadmium bioaccumulation factor. Therefore, in cases where organ cadmium accumulation determination in large frogs is not available, the use of whole organismal cadmium in small frogs is also considered as suitable indicator for biomonitoring of cadmium accumulation.

When we compare cadmium accumulation according to season, we found out that the highest cadmium accumulation occurred either during the late dry season or during the early rainy season. This is because these two seasons are the active seasons when reproductive tissues are developing and when reproduction actually takes place. Zug et al. (2001) stated that rainfall is one of the major determinants of timing of reproduction. AmphibiaWeb (2008) confirmed

this by stating that the breeding of the *Fejervarya limnocharis* is triggered by rain and it is usually the first species to come to the calling sites. In order for the rice frog to be the first species to come to the calling sites during the early rainy season, their ovaries and testes will have to start developing and maturing during the late dry season. Reproductive tissues development and maturation requires energy, hence the frogs would have to increase food and water intake during the late dry season for these purposes. During the early rainy season, the actual breeding occurs, and again these efforts require a lot of energy. Zug et al. (2001) stated that most frogs lay clutches of eggs comprising a large portion of their body mass. Therefore, egg development would constitute a large portion of their overall energy budget. The high energy demand is used for oogenesis and vitellogenesis. Therefore, it is during these two seasons, more food and water were consumed. And with increased food and water consumption, there was also a chance of increased uptake of cadmium along with it. This would explain the high cadmium concentration in the liver, kidney and testes of the frogs during the late dry and early rainy seasons.

3.6 Conclusions

This research found out that rice frog from the contaminated site had higher hepatic, renal and testicular cadmium when compared with rice frogs from the reference site. The results also showed that kidney is the greatest cadmium accumulating organ. We also found out that rice frogs caught during the late dry

and early rainy seasons tend to have higher tissue and organismal cadmium than those caught during late wet and early dry seasons. Therefore, if and when *Fejervarya limnocharis* is used in the biomonitoring of cadmium accumulation, types of tissues used and the season when sampling is performed should be taken into consideration.

CHAPTER 4

BIOMARKER STUDIES OF TWO POPULATIONS OF RICE FROG (*Fejervarya limnocharis*) NATURALLY EXPOSED TO DIFFERENT ENVIRONMENTAL CADMIUM LEVELS IN MAE SOT, TAK PROVINCE, THAILAND

4.1 Introduction

Biomarkers, as defined by Galloway (2006) are functional measures of exposure to stressors expressed at various levels. According to Monserrat et al. (2007), biomarkers can be classified as specific and non-specific. Stress proteins, enzymes and other biomolecules have been shown to be affected by metal pollutions and they can be regarded as either specific or non-specific biomarkers. According to Linde-Arias et al, (2008), biomarkers are sensitive tools for the assessment of biological effects of pollutants.

One of the direct responses of metal exposure is the production of metallothionein. Metallothionein are low molecular weight protein of about 7 Kd that bind and are induced by metals especially cadmium (Hansen et al., 2006; Baykan et al., 2007). Because of this metallothionein production is an important mechanism in cadmium acclimatization and detoxification. Therefore

Loumbourdis et al. (2007) suggested that metallothionein is a good biomarker of exposure for heavy metal pollution. The use of metallothionein as a toxicant-specific biomarker has been widely employed to indicate the presence and the effect of heavy metal pollution (Monserrat et al., 2007). In this research, the traditional method of determining metallothionein concentration is chosen over the more contemporary metallothionein induction. This is because, Schmitt et al. (2007) stated that the traditional method documents cumulative and long term exposure history while metallothionein induction would give an account on the active or recent metallothionein synthesis. Therefore metallothionein mRNA might not be detected in resident organism that have acclimated to contemporary exposure conditions.

Exposure to metal, especially cadmium may also lead to various physiological responses. One of the main physiological effects of cadmium exposure is the increased production of reactive oxygen species, especially the superoxide, that are responsible for oxidative stress in the body (Valdivia et al., 2007). As a result of oxidative stress, the body will produce antioxidants such as superoxide dismutase, catalase and glutathione-S-transferase. Loumbourdis et al. (1999) reported that *Rana ridibunda* exposed to cadmium have relatively high levels of glutathione. Hansen et al. (2006) reported that it has been proposed that cadmium inhibit the complex III of the electron transport chain in mitochondria. This leads to the facilitation of superoxide production, hence increases the production of reactive oxygen species. When this occurs, the cell starts the

detoxification of the reactive oxygen species by antioxidant and scavenger molecules production (Isani et al., 2008). The up-regulation of this antioxidant system includes an increase in glutathione-S-transferase enzyme production (Hermes-Lima and Zenteno-Savin, 2002).

Another biomarker of effect that is included in this study is vitellogenin. Vitellogenin (Vtg) is an egg yolk precursor protein normally synthesized by the liver of female organism (Cargouët et al., 2007). The vitellogenin produced will then be released in blood before it was incorporated in the growing oocytes after induction of endogenous estrogen in sexually mature females (Povlsen, 1990). A study on *Oncopeltus fasciatus* revealed that the ovarian levels of vitellogenin polypeptides were decreased in cadmium exposed females. (Cervera et al., 2005). In the presence of cadmium, Povlsen (1990) stated that vitellogenin production (vitellogenesis) is inhibited. This is because cadmium treatment and the resulting metallothionein synthesis compete with vitellogenesis in the liver cells. Apart from that, Haux et al. (1998) reported that sub-lethal exposure to cadmium is often demonstrated by a marked a hypocalcaemic response (decrease in total blood plasma calcium) which may impair vitellogenesis. Therefore a decline in vitellogenin level may be linked, although not exclusively, to cadmium exposure. To add, Bulukin et al. (2007) emphasized that vitellogenin has proven to be a sensitive and simple biomarker for assessing exposure to environmental stressors and immunochemical biosensor could be used for the analysis of vitellogenin in plasma samples.

4.2 Objective and Sub-objectives

The objective of this part of the research is to compare the contaminant analysis parameters of *Fejervarya limnocharis* from contaminated site with those from reference site. In fulfilling this objective, several sub-objectives have been identified. They are as follow

4.2.1 To compare the hepatic metallothionein concentrations of *Fejervarya limnocharis* from contaminated site with those from reference site

4.2.2 To compare the hepatic Glutathione-S-Transferase activities of *Fejervarya limnocharis* from contaminated site with those from reference site

4.2.3 To compare the plasma vitellogenin concentrations of *Fejervarya limnocharis* from contaminated site with those from reference site

4.3 Hypothesis

4.3.1 There are significant differences in the hepatic metallothionein concentration between *Fejervarya limnocharis* from contaminated site with those from reference site

4.3.2 There are significant differences in the glutathione-S-transferase activities between *Fejervarya limnocharis* from contaminated site with those from reference site.

4.3.3 There are significant differences in the plasma vitellogenin concentrations of *Fejervarya limnocharis* from contaminated site than those from reference site

4.4 Methodology

4.4.1 Metallothionein

Cadmium Haemoglobin Affinity Assay was used to determine metallothionein. Each of the liver tissues (n=97) were pooled according to sampling stations (Baykan et al., 2007) and mixed well. The tissues were subjected to metallothionein preparation steps according to Eaton and Cherian (1991) with modification by Kuroshima (1995). Tissues were homogenized in 0.03M Tris-HCl buffer (pH 8) before the homogenate was centrifuged at 10,000xg for 15 minutes. The supernatant was heated in boiling water bath and

further centrifuged for another 5 minutes. To 0.5 mL of supernatant, about 0.1 mL of cadmium solution and 0.5 mL buffer were added. The mixture was incubated at room temperature before 0.1 mL of 6% bovine hemoglobin solution was added. Then the mixture was heated in boiling water for 2 minutes, cooled and centrifuged at 10,000xg for 5 minutes. The steps of heating, cooling and centrifugation were repeated three times. Then the cadmium in the supernatant was determined with Graphite furnace atomic absorption spectrometer. Metallothionein concentrations were calculated by the assumption that 7g-atoms of cadmium are bound to one mole of metallothionein, and the molecular weight of cadmium-metallothionein complex is 7000.

4.4.2 Glutathione-S-transferase

Total protein concentrations in liver tissue (n=98) were determined by total protein assay (Bradford, 1976). About 0.5 g of liver tissue was homogenized in 1 mL of extraction buffer (50 mM Tris-HCl, 0,15 M KCl, pH 7.4). Then the homogenates were centrifuges at 12,000xg for 30 minutes at 4°C. The supernatant was diluted with phosphate buffer solution (PBS) pH 7.2 at 1:100, 1:1,000 and 1:10,000 ratios. Samples were loaded onto the 96-well protein assay plate. Bradford reagent (Sigma B6916) were added to each well and incubated for 10 minutes. By using a microplate reader, the absorbance of the sampler were read at 620 nm with Bovine serum albumin (Sigma) used as standard. A linear standard curve was plotted where only plots with r^2 value of more than

0.95 were used as the standard curve. After protein concentrations in each sample were obtained, the samples were diluted with phosphate buffer solution to normalize their total protein concentration at 1 mg/mL. The protein concentration was used as a tissue dilution guideline for the determination of the activities of hepatic glutathione-S-transferase. Glutathione-S-transferase activities were determined by glutathione-S-transferase assay (Habig et al., 1974). About twenty microliter of the diluted sample was added to 1 mL of reaction mixture (100 mM 1,2 chlorodinitrobenzene and 200 mM glutathione dissolved in 100 mM Tris-HCl pH 7.4). The activity of glutathione-S-transferase in the sample was determined by reading its absorbance at 340 nm at 25°C. Absorbance was measured after 1 minute lag time, at minute 0 and at minute 5. The specific glutathione-S-transferase enzyme activity was determined by the following formula

$$\text{Specific enzyme activity } (\mu\text{mol//min/mg total protein}) = \frac{\Delta A340 \times V}{E_{Mn} \times V_{ezm}}$$

Where

$$\Delta A340 = \frac{\text{A340 (final)} - \text{A340 (initial)}}{\text{Reaction time (min)}}$$

V = reaction volume (1 mL)

E_{Mn} = The extinction coefficient of CNDB conjugate (9.6 nM^{-1})

V_{ezm} = Volume of enzyme (20 μL)

4.4.3 Vitellogenin

Vitellogenin determination in plasma sample (n=206) of the frog was done by the ELISA (Enzyme Linked Immunosorbent Assay) method (Mylchreest et al., 2003). Since this method is almost always species specific, this research attempted to cross-react rice frog vitellogenin in plasma sample with readily available anti-vitellogenin (anti-VTG) antibodies from other species (*Elliptio complanata* and *Chrysemys picta*). Blood plasma from *Fejervarya limnocharis* were diluted at the ratios of 1:100, 1:1,000 and 1:10,000 with 50 mM carbonate-bicarbonate buffer, pH 9.6. Two sets of dilution were made because one set was used against the antibody of *Elliptio complanata* and the other set was used against the antibody of *Chrysemys picta.* The vitellogenin standards used were known concentration of vitellogenin that ranged from 4.88 to 2500 ng/mL (*Elliptio complanata*) and 156.25 to 10,000 ng/mL (*Chrysemys picta*). In the round bottom 96-well ELISA plate, 100 µL of sample, standard and quality control solution were loaded and incubated overnight at 4°C. The wells were then washed with PBS-Tween three times before they were loaded with 200 µL of 2% gelatin in PBS solution and incubated at room temperature for 2 hours to block nonspecific binding. After 2 hours, the washing steps were repeated and then 100 µL of primary antibody (*Elliptio complanata* or *Chrysemys picta*'s vitellogenin antibody) were added to the wells. The plates were incubated again for 2 hours at 4°C followed by washing steps. Then 100 µL of secondary antibody (goat anti-rabbit IgG-alkaline phosphatase conjugate, Sigma) was added to each well and then

incubated for 2 hours at 4°C. Next, after another washing step, 100 µL of p-nitrophenyl phosphate solution was added to each well and incubated for 10 minutes at room temperature. After that, absorbance was read at 405 nm. A semi-log standard curve was generated and the vitellogenin concentration was read from this standard curve.

4.4.4 Data and Statistical Analysis

All data are statistically analyzed with Two-way ANOVA and Student-Newman Keuls tests by using the SigmaStat 2.0 program. The Spearman-Rank Order correlation were also used to determine correlation between hepatic metallothionein concentration and hepatic glutathione-S-transferase activities with hepatic cadmium concentration

4.5 Results and Discussion

Hepatic metallothionein levels of *Fejervarya limnocharis* caught from Mae Pa range from 1.496 mg/kg wet weight to 2.785 mg/kg wet weight. (Fig 4.1) For those caught from Mae Tao, the range is from 2.991 mg/kg wet weight to 3.907 mg/kg wet weight. The seasonal differences in hepatic metallothionein levels are not statistically different (P=0.759). However, the differences in hepatic metallothionein between the stations are statistically significant (P=0.048). The overall average hepatic metallothionein for frogs caught from Mae Pa is 2.363 mg/kg wet weight while the value is 3.578 mg/kg wet weight for frogs caught from Mae Tao.

Figure 4.2 showed the activities of glutathione-s-transferase in the livers of *Fejervarya limnocharis* caught from Mae Pa and Mae Sot. The glutathione-s-transferase activities of the frogs caught from Mae Pa range from 0.154 to 0.159 µmol/min/mg total protein. The differences of GST activity between the season is not statistically significant (P= 0.053). For Mae Tao, the range extends from 0.199 to 0.331 µmol/min/mg total protein. The differences of glutathione-s-transferase activities between the season is statistically significant (P=0.016). In Mae Tao, the highest GST activity is recorded from frogs caught during the early rainy season. The lowest is found in frogs caught during the early dry season. The difference of GST activity between the station is statistically significant (P<0.001). The overall average GST activity for frogs caught from Mae Pa is

0.157 µmol/min/mg total protein, while for Mae Tao; the overall average value is µmol/min/mg total protein.

For vitellogenin determination, no cross reaction was detected between the rice frog plasma samples with the readily available vitellogenin antibody of both *Elliptio complanata* and *Chrysemys picta*. This indicated that interspecific antibody-antigen cross-reaction could not be used to detect vitellogenin levels in *Fejervarya limnocharis*. Therefore, vitellogenin determination in the plasma from the rice frog was deemed unsuccessful. Consequently, the experiment was terminated.

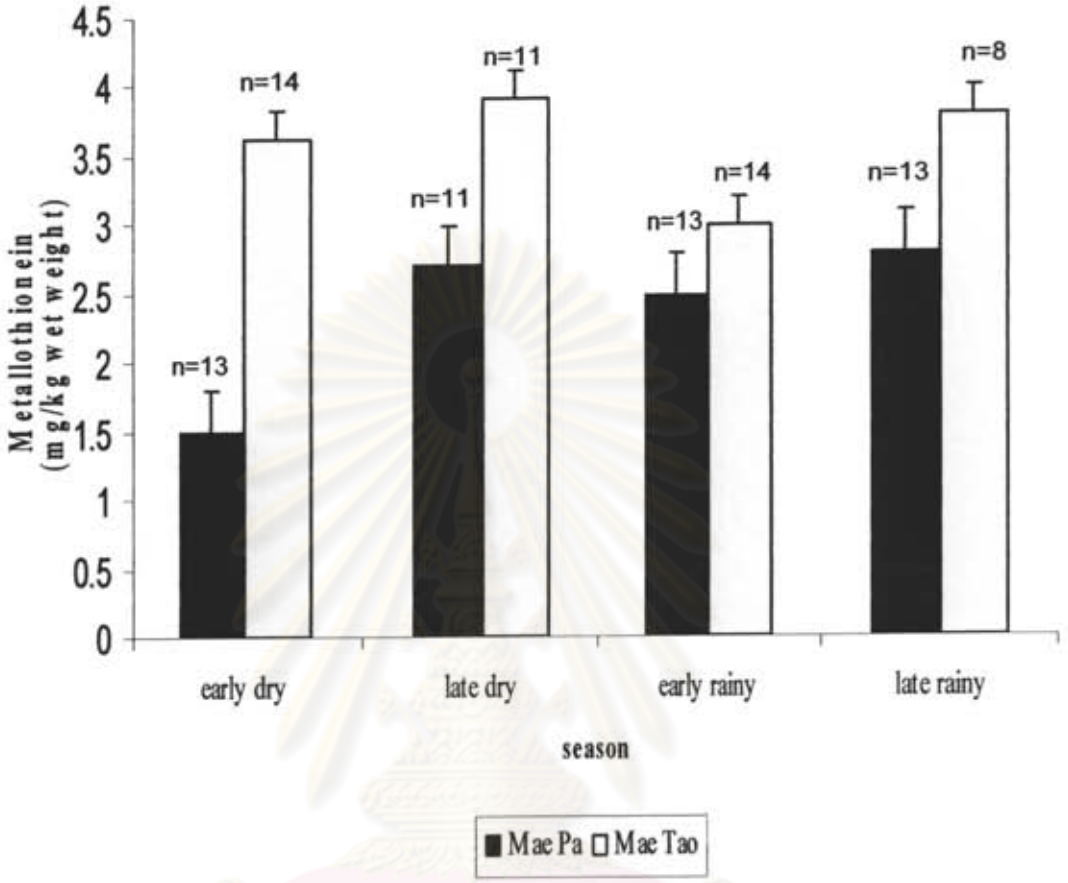

Figure 4.1: Quarterly average hepatic metallothionein in *Fejervarya limnocharis* caught from Mae Sot, Tak. Mean differences between stations are statistically significant (P=0.048)

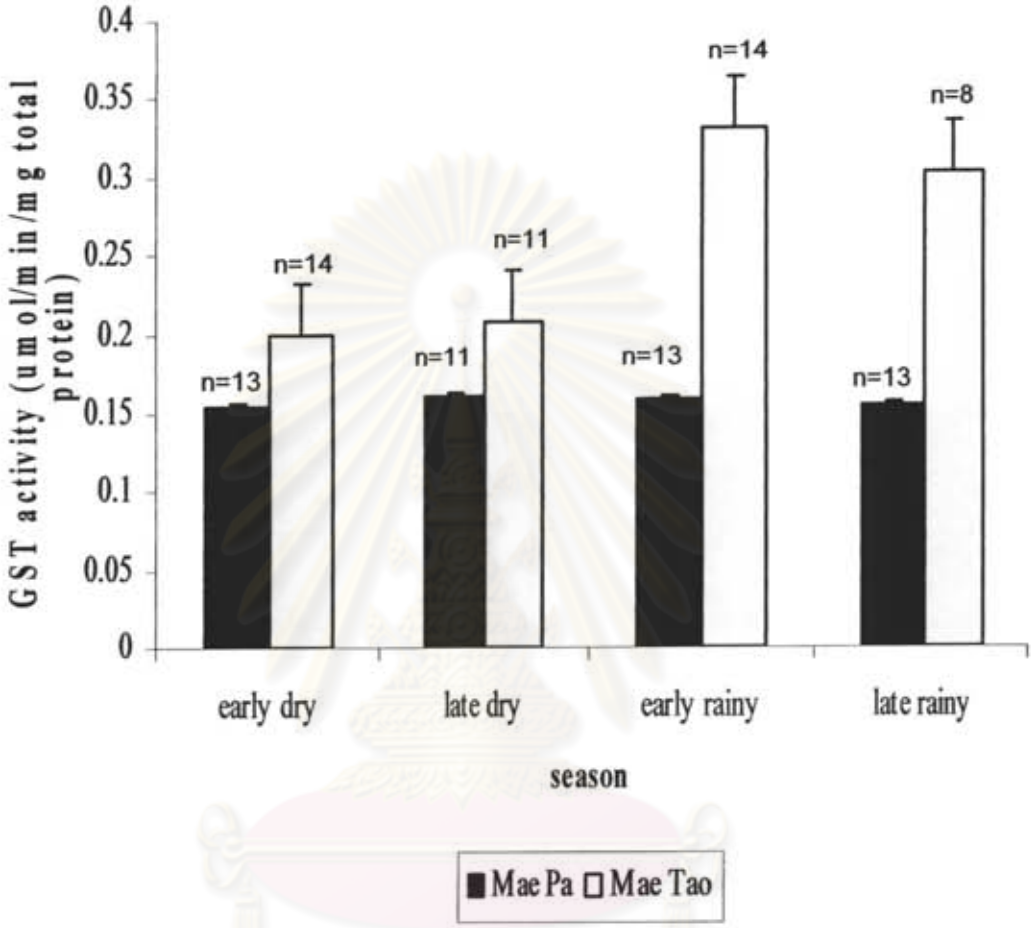

Figure 4.2: Quarterly average glutathione-S-transferase activity in *Fejervarya limnocharis* caught from Mae Sot, Tak. Mean differences between stations are statistically significant (P=0.048)

Table 4.1 Spearman-Rank Order Correlation showing the correlation coefficient between hepatic cadmium, hepatic metallothionein concentration and hepatic glutathione-S-transferase activity

Parameters	Correlation coefficient (r)	P
Hepatic cadmium – Hepatic metallothionein	0.548	0.139
Hepatic cadmium – Hepatic glutathione-S-transferase	0.802	0.0096
Hepatic metallothionein - Hepatic glutathione-S-transferase	0.731	0.029

Table 4.1 showed the correlation between hepatic cadmium with hepatic metallothionein concentration and hepatic glutathione-S-transferase activity. There was a strong positive correlation (r = 0.802) between hepatic cadmium and hepatic glutathione-S-transferase activity. This correlation was also statistically significant (P = 0.0096). For the hepatic cadmium-hepatic metallothionein pairing, there was a modest positive correlation (r = 0.548). However, this correlation was not statistically significant (P = 0.139). This may indicate that an increase in hepatic cadmium could result in an increase in hepatic glutathione-S-transferase activity. However, the same cannot be assumed from the hepatic cadmium-hepatic metallothionein interaction. Therefore, it is likely that an increase in hepatic cadmium will statistically increase hepatic glutathione-S-transferase activity, but not hepatic metallothionein. Another pairing showed significantly

positive correlation. The result showed that the coefficient correlation between hepatic metallothionein and hepatic glutathione-S-transferase was 0.731 with the P value of 0.029.

Hansen et al. (2006) reported that in *Salmo trutta*, metallothionein is an important mechanism in cadmium acclimatization. This was inferred from the research results that showed cadmium-acclimatized *Salmo trutta* had higher intercellular concentration of metallothionein-like proteins. A number of other studies also have found that organisms exposed to cadmium would be induced to produce metallothionein. The study organisms include *Xenopus laevis (*Mouchet et al., 2006; Herkovits et al., 1998), *Rana ridibunda* (Loumbourdis et al., 2007), *Oreochromis niloticus* (Baykan et al., 2007), *Platichthys flesus* (Povlsen, 1990), *Laternula elliptica* (Choi et al., 2006) and *Bufo arenarum* (Perez-Coll et al, 1997). Figure 5.1 of this study confirms the findings of the researches mentioned above because the result showed that *Fejervarya limnocharis* that lived in cadmium contaminated site (Mae Tao) had higher hepatic metallothionein concentration as opposed to those living in reference site (Mae Pa). This indicated that frogs exposed to high cadmium concentration in the environment would react by producing metallothionein as a line of defense from the toxic effect of cadmium. This is because induction of metallothionein would provide more binding sites for cadmium, hence would limit latent damage (Linde-Arias et al., 2008). Cadmium would bind to the thiol (-SH) group in the metallothionein molecule to form cadmium-thiolate clusters (Monserrat et al., 2007). This would limit the availability

of cationic cadmium from imparting its toxicity against the organ it was accumulated.

Isani et al. (2008) reported that cadmium that was absorbed from the environment would be transported to the liver. There it would induce the production of hepatic metallothionein. Therefore, it may be inferred that there is a positive correlation between the concentration of cadmium in the liver and the amount of metallothionein produced by the liver. According to Bird et al. (2008), in a study on *Anguilla anguilla*, there was a strong and significant relationship between hepatic metallothionein and hepatic cadmium concentration. The same conclusion was also obtained by Urena et al. (2007) where there was a strong positive association between metallothionein levels and cadmium concentration. However in this research, in spite of our assumption, the correlation between hepatic cadmium and hepatic metallothionein was not significant despite having positive correlation. This may be attributed to the insufficiency of metallothionein induction due to spillover effect (Mouchet et. al 2006). Spillover is defined as the metals still remaining due to overwhelmed defense capacity of the body to sequester metal. This means that initially, there would be a positive correlation between both parameters. However, after a certain threshold level, the liver is no longer able to produce enough metallothionein to bind with the ever increasing amount of cadmium. Therefore, in *Fejervarya limnocharis* caught from Mae Sot, the insignificant, but positive correlation between hepatic cadmium and hepatic metallothionein may be explained by the overwhelmed defense capacity that

leads to spillover effect. Due to the overwhelmed accumulation of cadmium, further increase in cadmium uptake may not result in the increase of cadmium being assimilated to metallothionein to form the cadmium-thiolate clusters in the metallothionein-cadmium complex. Instead, this would increase the concentration of free cadmium in the liver.

From the Figure 5.2, it is apparent that *Fejervarya limnocharis* caught from the contaminated site (Mae Tao) had higher glutathione-S-transferase activity than those caught from the reference site (Mae Pa). To add, Table 5.1 also showed that there was a strong correlation between hepatic cadmium and the hepatic glutathione-S-transferase activity. This is in line with results from studies on *Rana ridibunda* (Loumbourdis et al., 1999; Loumbourdis et al., 2007), *S*almo trutta (Hansen et al., 2006), *Xenopus laevis* (Mouchet et al., 2006), *Sparus aurata* (Isani et al., 2008), *Sander vitreus* and *Perca flavescens* (Larose et al., 2008) that showed there was a higher level of antioxidant enzymes in organisms exposed to metals. The higher activity of glutathione-S-transferase in *Fejervarya limnocharis* caught from Mae Tao than those from Mae Pa and the strong correlation between hepatic cadmium and hepatic glutathione-S-transferase was anticipated because cadmium is known to induce oxidative stress (Hansen et al., 2006; Loumbourdis et al., 2007; Mouchet et al., 2006; Alvarez et al., 2004). This is because cadmium causes an increase in glutathione concentration (Kuroshima, 1995) and reactive oxygen species (Isani et al., 2008) and also promotes lipid peroxidation (Mouchet et al., 2006) and this would lead to the

increase in biotransformation enzymes production such as glutathione-S-transferase. According to Larose et al. (2008), researches have shown that high activity of glutathione-S-transferase can be induced by exposure to cadmium. Glutathione-S-transferase is one of the key enzymatic players in the defense mechanism against reactive oxygen species and oxidative stress (Hermes-Lima and Zenteno-Savin, 2002)

4.6 Conclusions

This research found that rice frog from the contaminated site had higher hepatic metallothionein concentration and hepatic glutathione-S-transferase activity when compared with rice frogs from the reference site. On the other hands, cross-reacting rice frog's vitellogenin with other species' anti-vitellogenin antibody was unsuccessful. Therefore no result was available for vitellogenin levels in the plasma of the rice frog

The results also showed that exposure to cadmium exposure leads to an increase in hepatic metallothionein concentration and hepatic glutathione-S-transferase activity. This is exhibited in the significant differences in the levels of both parameters between *Fejervarya limnocharis* caught from Mae Tao and Mae Pa. However, only glutathione-S-transferase activity showed significant strong positive correlation with hepatic cadmium while the correlation between hepatic cadmium and hepatic metallothionein was moderately positive but insignificant.

Therefore, in cases where a sentinel was exposed chronically to cadmium, the stressor-response correlation in the liver is better exhibited by the hepatic glutathione-S-transferase activities rather than hepatic metallothionein.

CHAPTER 5

MORPHOMETRIC AND GRAVIMETRIC STUDIES OF TWO POPULATIONS OF RICE FROG (*Fejervarya limnocharis*) NATURALLY EXPOSED TO DIFFERENT ENVIRONMENTAL CADMIUM LEVELS IN MAE SOT, TAK PROVINCE, THAILAND

5.1 Introduction

Morphometric and gravimetric data has always been a good indicator of the effect of pollution on an organism. Often, pollutant has a direct effect on growth of an organism. According to Monserrat et al. (2007), morphological and physiological alterations have been reported in individuals exposed either naturally or experimentally to different pollutants. Kitana et al. (2007) reported that morphometric analysis was used to compare site-related differences in body size. For instance, in a study by Norris et al. (2000), it was found that the hepatosomatic index of trout living in uncontaminated site was greater than those living in sites contaminated by cadmium and zinc. The same study also reported that there was an association between high renal cadmium with low hepatosomatic index in many fish species. Maes et al. (2005) reported that recent studies have established relationships between metal toxicity with

changes in gravimetric indices of European eel, especially hepatosomatic index. Therefore, the significance of assessing biometric response (weight, condition, growth) has been suggested as an important measure of pollution impact on an organism (Van Straalen and Timmermans, 2002)

Over the years, weight-length relationship and condition factor has been used to assess the well-being of fish species. The weight-length relationship can be used to reflect the impact of the environment on the growth of an organism. From this relationship, the value of Scaling coefficient is able to be determined. Eastwood and Couture (2002) stated that the Scaling coefficient is a descriptor of growth pattern of a specific population or organisms. The Scaling coefficient has been proposed to be used as a bioindicator for long term stress in populations subjected to environmental pollution. As for condition factor (CF), Bervoets and Blust (2003) stated that it is used to express the overall well being of an individual. Hence, CF is a useful tool to assess the effect of pollution on individuals. In addition, Urena et al. (2007) mentioned that condition factors are indicative of overall health and therefore, are good candidate to be considered when studying the effect of metal exposure. A study by Hansen et al. (2006) revealed that the condition factors are lower in metal-exposed population as compared to reference population which is an indicative of the effect of metal exposure on fitness of fish. Maes et al. (2005) also stated that there is a clear relationship between increased heavy metal content with lower condition factor.

The use of these parameters in *Fejervarya limnocharis* is novel because it has never been used in amphibians before

In this study, the morphometric and gravimetric indices studied includes hepatosomatic index, renosomatic index, gonadosomatic index, weight-length relationship and condition factor. Hepatosomatic, renosomatic and gonadosomatic indices are the ratio of organ mass to total tissue mass (Ji et al., 2006; Loumbourdis & Vogiatzis, 2002; Maitra et al., 2007). These indices are determined by these formulas

Hepatosomatic index (HSI) = (liver weight / body weight) x 100

Renosomatic index (RSI) = (kidney weight / body weight) x 100

Gonadosomatic index (GSI) = (gonad weight / body weight) x 100

Apart from that, Scaling coefficient and condition factor is also determined in this research. In order to do so, the weight-length relationship is determined by transforming the

$$W = a L^b$$

relationship (LeCren, 1951) into its logarithmic mode to form the

$$\log W = b \log L + \log a$$

relationship. From this, to Larose et al. (2008) reported that condition factor can be obtained from this formula

$$CF = (W / a L^b) \times 100$$

where W represents the weight and L refers to the snout-vent length of the individual.

5.2 Objective and Sub-objectives

The objective of this part of the research is to compare the contaminant analysis parameters of *Fejervarya limnocharis* from contaminated site with those from reference site. In fulfilling this objective, several sub-objectives have been identified. They are as follows

5.2.1 To compare the hepatosomatic index of *Fejervarya limnocharis* from contaminated site with those from reference site

5.2.2 To compare the renosomatic index of *Fejervarya limnocharis* from contaminated site with those from reference site

5.2.3 To compare the female and male gonadosomatic index of *Fejervarya limnocharis* from contaminated site with those from reference site

5.2.4 To compare the Scaling Coefficient and condition factors of *Fejervarya limnocharis* from contaminated site with those from reference site

5.3 Hypothesis

5.3.1 There are significant differences in the gravimetric indices between *Fejervarya limnocharis* from contaminated site with those from reference site.

5.3.2 There are significant differences in the Scaling Coefficients and condition factors between *Fejervarya limnocharis* from contaminated site with those from reference site

5.4 Methodology

5.4.1 Field Sampling and Laboratory Analysis.

Frogs were caught live at night time during visual encounter survey (Crump and Scott, 1994). The frogs were placed in plastic aquariums and then transported live to the lab. In the lab, the frogs were individually subjected to cold anaesthesia procedure before sacrificed by double-pith at the brain and spinal cord. Then the frogs were weighed and their snout-vent lengths (SVL) were weighed. Each frog was then autopsied and their livers (n=206), kidneys (n=206), ovaries (n=94) and testes (n=111) were removed and weighed. The organs were placed in plastic bags and then frozen until further analysis (Chapters 3 and 4).

5.4.2 Data and Statistical Analysis

The weight and the snout-vent length data (n=647) were used to determine Scaling coefficient and condition factor. Scaling coefficient was calculated from the extrapolation of the logarithmic transformed weight-length relationship by linear regression. The logarithmic-transformed weight-length relationship was also used to determine condition factor of each population.

Hepatosomatic index (Loumbourdis & Vogiatzis, 2002), renosomatic index and both male and female gonadosomatic indices (Goodwin et al., 1992; Tilton et al., 2003; Maitra et al., 2007) were calculated for each frog. These indices were based on the weight ratio of each tissue (liver, kidney, testis and ovary) to the body weight.

In order to compare the Scaling coefficient, condition factor, hepatosomatic index, renosomatic index and male and female gonadosomatic indices in *Fejervarya limnocharis* caught from Mae Sot, all data were statistically analyzed with two-way ANOVA and Student-Newman Keuls test using the SigmaStat 2.0 program. Linear regression analysis was also used for weight-length relationship.

5.5 Results and Discussion

Figure 5.1 showed the quarterly average Scaling coefficients of *Fejervarya limnocharis* caught from the sampling sites in Mae Sot. The Scaling coefficients of *Fejervarya limnocharis* caught from Mae Pa range from 2.783 to 3.223. For Mae Tao, the range extends from 2.762 to 2.978. The result also showed that in both sites, the highest Scaling coefficients are recorded from frogs caught during the late rainy season. The lowest is found in frogs caught during the late dry season. Figure 5.1 also showed that both Mae Pa and Mae Tao exhibit similar trend of Scaling coefficient seasonal fluctuation. The overall average Scaling Coefficient for frogs caught from Mae Pa is 2.975, while for Mae Tao, the overall average value is 2.856.

The quarterly average condition factors of *Fejervarya limnocharis* caught from Mae Pa and Mae Tao is shown in Figure 5.2. The result showed that condition factors of *Fejervarya limnocharis* caught from Mae Pa range from 9.781 to 11.093 while for those caught from Mae Tao, the range is from 9.511 to 9.931. In Mae Pa, the frogs have the highest condition factor during the late rainy season and the lowest condition factor during the early rainy season. However in Mae Tao, the highest condition factor is recorded during the late dry season while the lowest is recorded during the early rainy season. The overall average condition factor for frogs caught from Mae Pa is 10.296 while the value is 9.720 for frogs caught from Mae Tao.

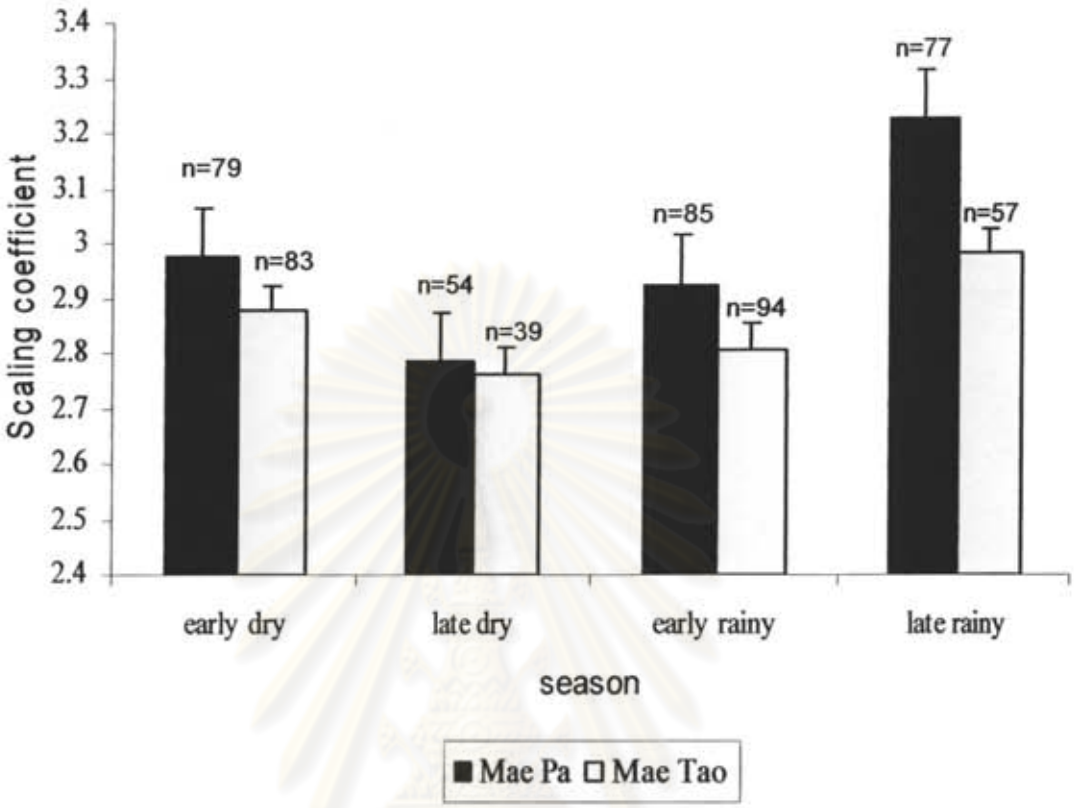

Figure 5.1: Quarterly average Scaling Coefficient of *Fejervarya limnocharis* caught from Mae Sot, Tak. There are no statistically significant differences in the means betweens seasons (P=0.053) and stations (P=0.082).

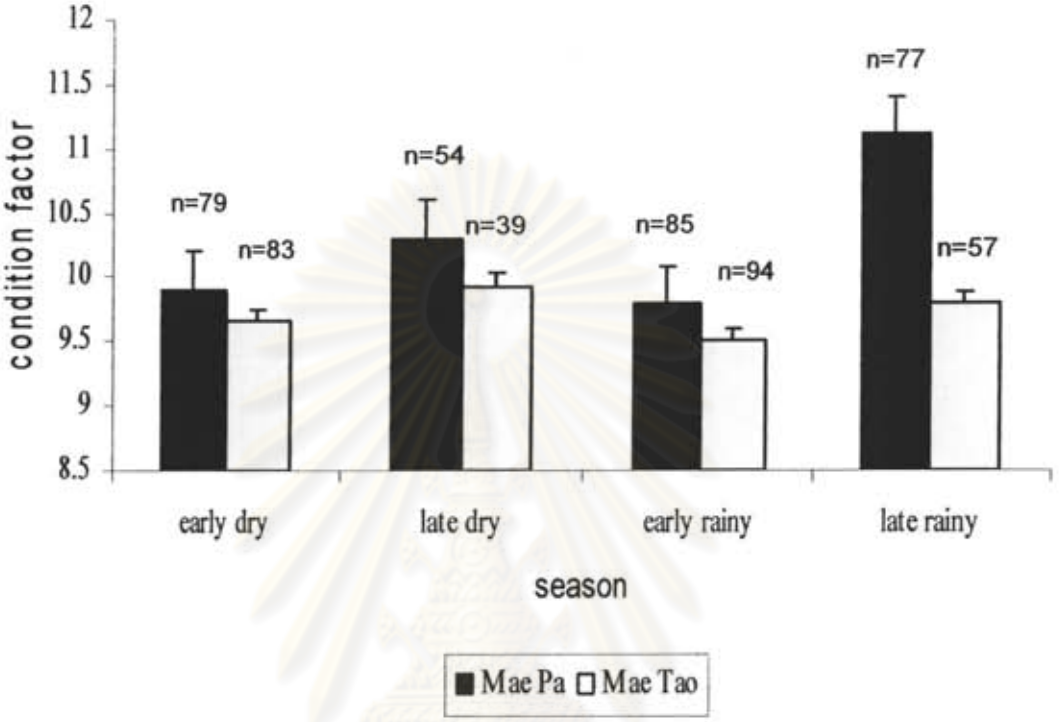

Figure 5.2: Quarterly average condition factor of *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences betweens season and stations are statistically significant (P<0.001)

The differences between the hepatosomatic indices of *Fejervarya limnocharis* caught from Mae Pa and Mae Tao is illustrated in Figure 5.3. The graph showed that hepatosomatic indices of the rice frog caught from Mae Pa range from 1.578 to 2.074. The rice frogs that were caught from Mae Tao showed hepatosomatic indices that ranged from 1.529 and 2.025. The figure also showed that in both sites, the highest hepatosomatic indices occur during the early rainy season, while the lowest occur during late dry season. It is also observable that both sites also show similar trend in the seasonal fluctuation of hepatosomatic indices. The overall average hepatosomatic index for rice frogs caught from Mae Pa is 1.913 while the value is 1.845 for rice frogs caught from Mae Tao.

For renosomatic indices, the quarterly average is shown in Figure 5.4. Average renosomatic indices of *Fejervarya limnocharis* caught from Mae Pa ranged from 0.372 to 0.448. The highest renosomatic index of these frogs was recorded in during the early rainy season while the lowest renosomatic index occurred during the late rainy season. The overall average renosomatic index of *Fejervarya limnocharis* caught from Mae Pa was 0.413. For Mae Tao, the renosomatic indices of *Fejervarya limnocharis* caught ranged from 0.354 to 0.411. Just like Mae Pa, the highest renosomatic index of frogs caught from Mae Tao was recorded during the early rainy season. However, the lowest renosomatic index was recorded during the early rainy season. The overall average of renosomatic index for *Fejervarya limnocharis* caught from Mae Tao was 0.380.

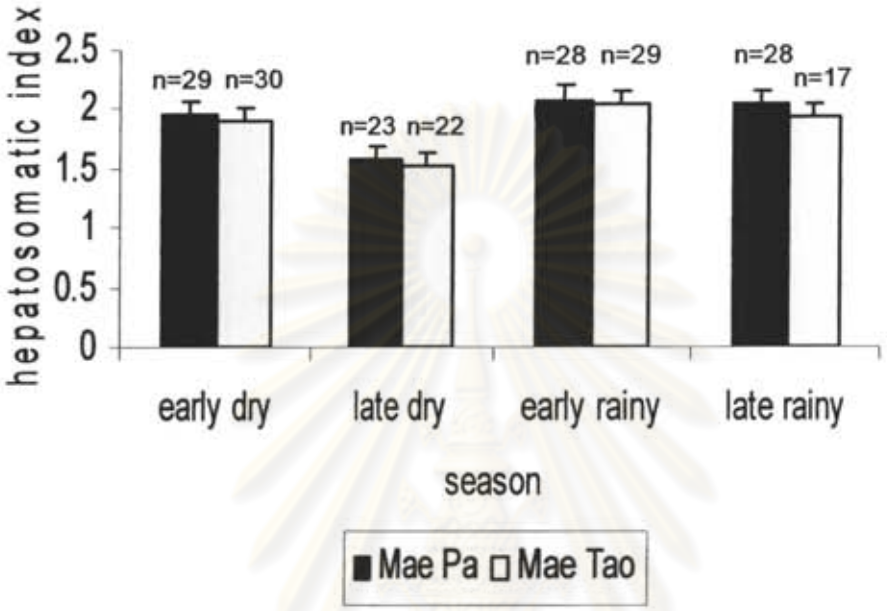

Figure 5.3: Quarterly average hepatosomatic index of *Fejervarya limnocharis* caught from Mae Sot, Tak. Mean differences betweens season are statistically significant (P<0.001) but the mean differences between stations are not statistically significant (P=0.359)

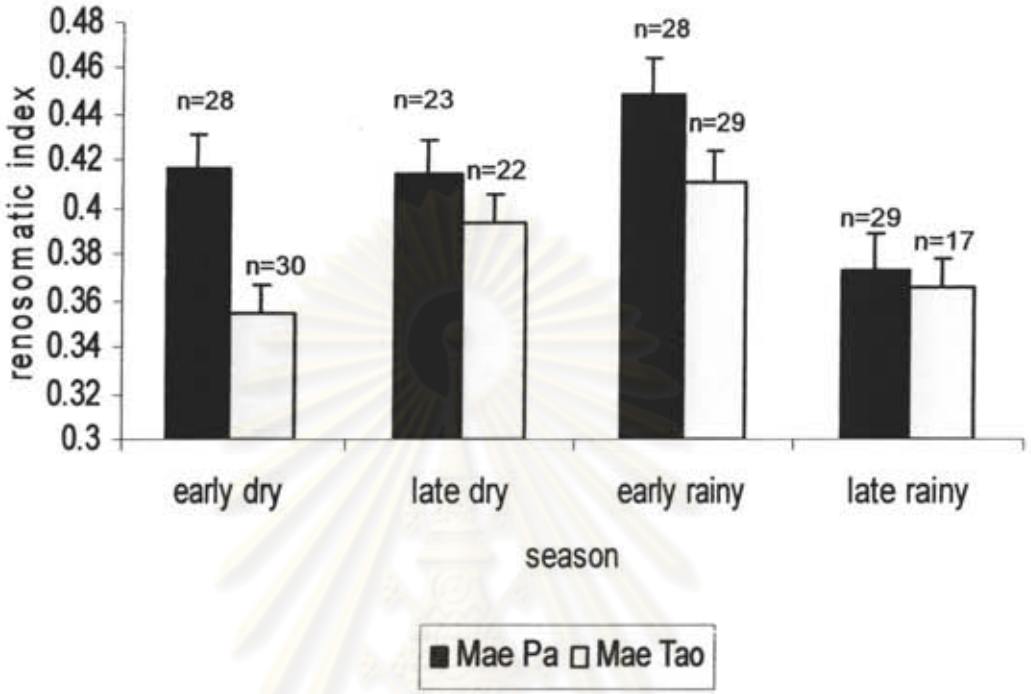

Figure 5.4: Quarterly average renosomatic index of *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences betweens season and stations are statistically significant ($P<0.001$)

Figure 5.5 exhibits the quarterly average female gonadosomatic indices of *Fejervarya limnocharis* caught from Mae Sot, Tak. The graph showed that female gonadosomatic indices of frogs from Mae Pa ranged from 3.591 to 12.082. On the other hand, female gonadosomatic indices of *Fejervarya limnocharis* caught from Mae Tao was recorded to be in the range of between 2.287 to 7.702. The overall average female gonadosomatic index for *Fejervarya limnocharis* caught from Mae Pa was 7.594 while for the frogs caught from Mae Tao, the average female gonadosomatic index was 4.919.

In Mae Pa, the highest average female gonadosomatic index was recorded in *Fejervarya limnocharis* caught during the late rainy season while for frogs from Mae Tao, the highest was recorded from those caught during the late dry season. For both stations, the lowest average female gonadosomatic index was recorded in frogs caught during the early dry season.

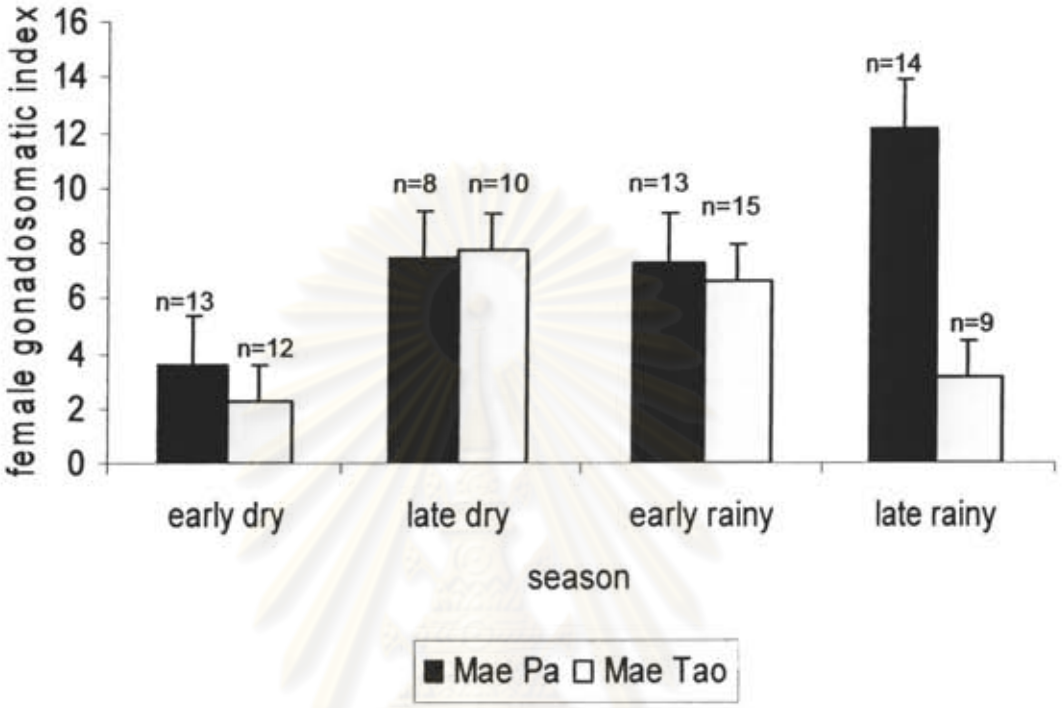

Figure 5.5: Quarterly average female gonadosomatic index of *Fejervarya limnocharis* caught from Mae Sot, Tak. There are statistically significant differences in the means betweens seasons (P=0.001) and stations (P=0.007)

For male gonadosomatic index, the data is illustrated in Figure 5.6. From the graph, it is shown that male gonadosomatic indices of *Fejervarya limnocharis* caught from Mae Pa ranged from 0.109 to 0.249. For those caught from Mae Tao, the range was recorded to in the range of 0.113 to 0.290. In Mae Pa, the highest male gonadosomatic index occurred in the frogs caught during the early rainy season while the lowest was recorded in *Fejervarya limnocharis* caught during early dry season. The highest male gonadosomatic index in Mae Tao frogs occurred in those caught during the late dry season while the lowest was those caught during the early dry season. The overall average male gonadosomatic index for frogs caught from Mae Pa was 0.192 while the value was 0.196 for frogs caught from Mae Tao.

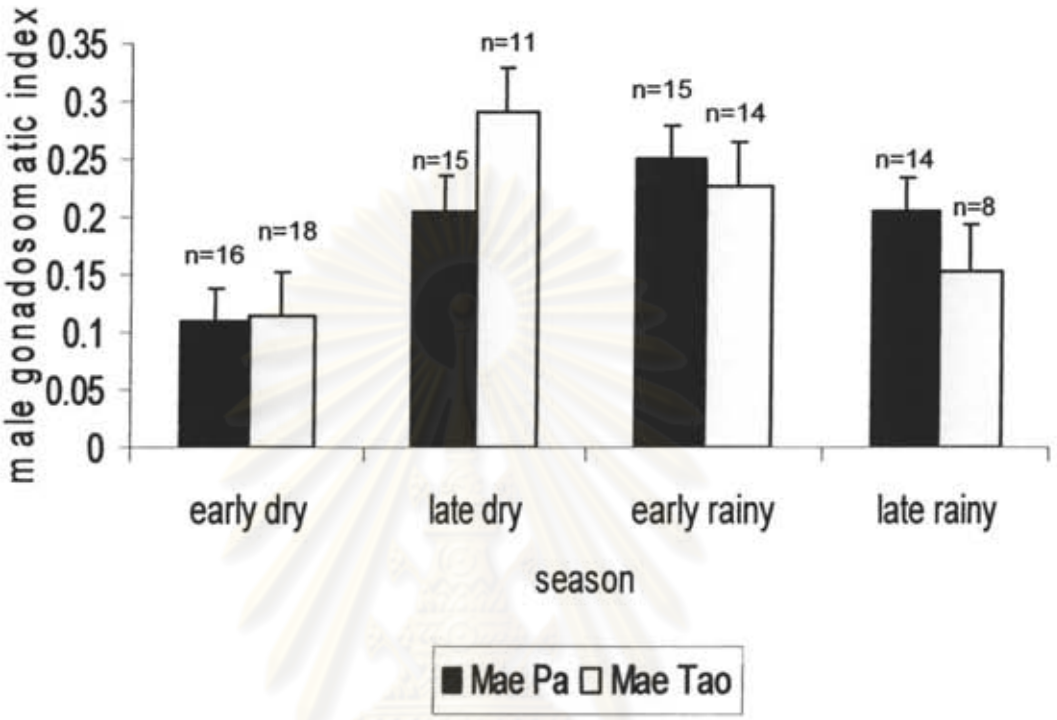

Figure 5.6: Quarterly average male gonadosomatic index of *Fejervarya limnocharis* caught from Mae Sot, Tak. Mean differences betweens season are statistically significant (P<0.001) but the mean differences between stations are not statistically significant (P=0.767)

In all the seasons, the differences in the mean values of Scaling coefficient of *Fejervarya limnocharis* caught from both Mae Pa and Mae Tao are not statistically significant. Scaling Coefficient reflects the ratio between logarithmic modes of weight and length. This means that for a certain snout-vent length, the difference in body weight of the rice frog may indicate whether the frog is in better condition or not. Therefore, it can be inferred that at this instance, cadmium accumulation is not severe enough to affect the ratio between weight and length of *Fejervarya limnocharis* caught from both reference and contaminated sites. Thus the Scaling coefficient is not significantly affected by the stress from living in a cadmium contaminated site. However, even though the differences are not statistically significant, there is an observable trend that shows frogs from reference site had higher Scaling coefficient than *Fejervarya limnocharis* caught from the contaminated site. Eastwood and Couture (2002) suggested that long term stress in population subjected to environmental pollution may lead to reduced weight to length ratio, hence reduced Scaling coefficient. Therefore, even though not statistically significant, the results showed there is a tendency that stress from cadmium accumulation in *Fejervarya limnocharis* may affect growth and development. This may eventually lead to reduced weight to length ratio. Perhaps, a longer duration of exposure may yield significant differences because Rosa et al., (2008) stated that age and exposure is an important factor in wildlife toxicology.

While Scaling coefficient values do not show statistically significant differences between both Mae's Pa and Mae Tao's frogs, condition factor shows a clearer picture. There are two important premises that can be derived from the result of the research. Firstly, it is shown that the difference in season plays a statistically significant role in the change in the condition factors of the frogs. This means that in different seasons, the frogs live in different conditions which eventually affect their overall well being. This is because condition factor is a provider of information on the health status (Linde-Arias et al., 2008), overall health (Urena et al., 2007) and general well-being (Bervoets and Blust, 2003) of an organism. However, a more important point is revealed in the second premise derived from the result of this research. The result shows that there is a statistically significant difference in the condition factor between *Fejervarya limnocharis* caught from the reference site than those caught from the contaminated site. Mae Pa's frogs had higher condition factors than Mae Tao's frogs. This is in line with the results obtained by other researchers where metal-exposed *Salmo trutta* was found to have lower condition factor as compared to reference populations (Hansen et al., 2006; Norris et al., 2000). Therefore, just to as many fish species, exposure to heavy metal especially cadmium leads to diminished quality of living condition which results in diminished condition factor.

Figure 5.3 showed the quarterly average hepatosomatic indices of *Fejervarya limnocharis* caught from both reference and contaminated sites. The differences in the average hepatosomatic indices between the frogs caught from the

contaminated site and those caught from reference site is not statistically significant. However, there is a common trend that can be observed despite the differences being statistically not significant. The data showed that in all the seasons, the frogs from Mae Pa have higher hepatosomatic indices than frogs from Mae Tao. According to Hansen et al. (2006), cadmium has the ability to induce the formation of reactive oxygen species. This will then result in the generation of oxidative stress (Loumbourdis et al., 2007) which will then lead to an increase in lipid peroxidation (Mouchet et al., 2006; Alvarez et al., 2004; Isani et al., 2008). Increased lipid peroxidation will eventually result in cell membrane damage and cell death, either via apoptosis or necrosis (Norris et al., 2000) what follows is the reduction of liver weight and consequently the reduction in hepatosomatic index. This may be a plausible explanation on why *Fejervarya limnocharis* caught from contaminated site tend to have lower hepatosomatic index than those caught from the reference site.

For renosomatic index, the difference in average values between rice frogs caught from Mae Pa and those caught from Mae Tao is statistically significant. The result shows that *Fejervarya limnocharis* living in cadmium contaminated site has lower renosomatic index than frogs from the reference site. There has been very limited explanation and information on how cadmium causes the reduction in kidney size, hence the reduction in renosomatic index of an organism. One possible cause is that the reduction could be attributed to the same mechanism that causes the reduction in liver weight. Exposure to cadmium leads to

production of reactive oxygen species which imposes oxidative stress to the kidney cells. Oxidative stress causes lipid peroxidation which results in cell membrane destruction and cell death. However more detailed and extensive studies need to be done in order to explain the mechanism how cadmium accumulation could lead to reduced kidney weight.

For reproductive tissues, the data are presented in Figure 5.5 and Figure 5.6. Differences in the female gonadosomatic index between *Fejervarya limnocharis* caught from cadmium contaminated site with those from reference site are statistically significant. The connection between cadmium accumulation with reduced ovarian weight could be attributed to the metal's effect on vitellogenesis. In a study on *Oncorhyncus mykiss*, Bon et al. (1997) found out that during the period of endogenous vitellogenesis and exogenous vitellogenesis, there is a great correlation between vitellogenin levels with female gonadosomatic indices. In another study, the presence of cadmium leads to the inhibition of vitellogenin production (vitellogenesis) in *Platichthys flesus* (Povlsen, 1990). This is because cadmium treatment and the resulting metallothionein synthesis compete with vitellogenesis in the liver cells. Apart from that, sub-lethal exposure to cadmium is often demonstrated by a marked hypocalcemic response (decrease in total blood plasma calcium) which may impair vitellogenesis (Haux et al., 1998). Therefore the disruption of vitellogenesis and the decline in vitellogenin level may be linked, although not exclusively, to cadmium exposure. Therefore, if cadmium can impair vitellogenesis and there is a great correlation between vitellogenin

levels with female gonadosomatic indices, it can be inferred that cadmium exposure may be able to result in the reduction of female gonadosomatic indices. This could explain why *Fejervarya limnocharis* caught from Mae Tao, the contaminated site, has lower female gonadosomatic index than those caught from Mae Pa, the reference site. However for male gonadosomatic index, the result offers no clear explanation. The result shows that there are no significant differences in male gonadosomatic indices of rice frog caught from both reference and contaminated site ($p=0.767$). A mixture of contradicting trend is also apparent in the result. During both early and late dry season, the quarterly average male gonadosomatic index for frogs from the contaminated site is higher than those from the reference site. However, during both early and late rainy season, the male gonadosomatic index showed a completely reverse trend where the quarterly average for frogs from the contaminated site is lower than those from the reference site. Most probably, longer period of study that involves two or more annual cycle is required to fully understand the fluctuation and changes in male gonadosomatic index.

5.6 Conclusions

This research found that there is a connection between cadmium contamination with morphometric and gravimetric indices of *Fejervarya limnocharis* living in contaminated and reference sites in Mae Sot, Tak. Albeit being statistically not significant, there is a trend that frogs from contaminated

site has lower Scaling coefficient and hepatosomatic index than frogs from reference site. This trend is even more apparent in the differences in condition factor, renosomatic index and female gonadosomatic index where the differences are statistically significant. For these parameters, *Fejervarya limnocharis* caught from Mae Pa had higher condition factor, renosomatic index and female gonadosomatic index than those caught from Mae Tao. On the other hand, result for male gonadosomatic index is rather mixed with contradicting trend according to season. However, these differences are not statistically significant.

In conclusion, the use of morphometric and gravimetric indices could give an idea on what would be the effect of cadmium contamination on the rice frog. Therefore, it is suggested that the use of morphometric and gravimetric can be used to determine whether *Fejervarya limnocharis* is a suitable candidate for sentinel species for cadmium contamination. However, it needs to be emphasized that morphometric and gravimetric data cannot be used alone in making the decision. These data need to be used in complementary with other parameters, especially contaminant analysis data.

CHAPTER 6

HISTOLOGICAL AND SKELETOCHRONOLOGIAL STUDIES OF TWO POPULATIONS OF RICE FROG (*Fejervarya limnocharis*) NATURALLY EXPOSED TO DIFFERENT ENVIRONMENTAL CADMIUM LEVELS IN MAE SOT, TAK PROVINCE, THAILAND

6.1 Introduction

Histological analysis is one of the important parameters often included in the study of sentinels. Hutchingson and Pickford (2002) stated that histology has been included as priority endpoints in the new OECD test guidelines for environmental monitoring. Often the initial effects of heavy metal pollution is evident only at the cellular or tissue level, thus causing histological changes before significant changes can be identified in a higher level (van Dyk et al., 2007). Some of the histological effects of cadmium exposure to a tissue include fibrosis, apoptosis and necrosis (Habeebu et al., 1998; Thijssen et al., 2007; Rosa et al., 2008), hyalinization, vacuolation, and cellular swelling (van Dyk et al., 2007) and cadmium deposition (Itokawa et al., 1978).

One of the most notorious effects of cadmium toxicity to a tissue is the formation of tumor cells. According to Alvarez et al. (2004) cadmium has been classified by

the International Agency for Research on Cancer as category I (human) carcinogen. This means that cadmium has the ability to induce abnormal and uncoordinated cell proliferation. A study on prostate epithelial cells by Martin et al. (2002) revealed that low level of cadmium is enough to mimic the effect of androgen and stimulate cell proliferation, thus propagating cancer cells.

Another common effect of cadmium in the tissue is the production of macro-melanophage centers. Macro-melanophage centers are produced in response to the presence of parasites and foreign materials in the tissue. Cadmium is reported to cause a depression in the immune system. (Loumbourdis and Vogiatzis, 2002). As a result of this depression, the tissue is prone to massive invasion of parasites which will then lead to the production of macro-melanophage centers. Apart from that, cadmium is reported to cause the rupture of small blood vessels in the tissue. This leads to the release of red blood cells in the tissue hence the activation of the aggregation of Kupfer cells to form macro-melanophage centers. Macro melanophage centers function to scavenge the parasites, red blood cells and other foreign materials in the tissue. In addition, since macro-melanophage centers are from Kupfer cells, its function also involves cadmium accumulation and scavenging (Loumbourdis and Vogiatzis, 2002). The presence of macro-melanophage centers has been reported in the liver, kidney and testis.

The effect of cadmium toxicity would result in different histopathological changes depending on what tissue it exerts its effect on. In the liver, cadmium was found to induced pathological changes including diffused hepatocyte and eosinophilic cytoplasm (Dudley and Klaasen, 1984); and hyalinization of hepatocytes, increased vacuolation associated with lipid accumulation, congestion of blood vessels and cellular swellings (van Dyk et al., 2007). In the kidney, cadmium was known to activate glomerular or interstitial infiltrated inflammatory cells, which would eventually lead to the over production of extracellular matrix component along with an increase in vacuolization and an increased amount of lysosomes (Thijssen et al., 2007). Interstitial fibrosis was also found in tissues suffering the effect of cadmium toxicity (Rosa et al., 2008).

The effect of cadmium on gonad histology has also been studied quite widely. According to Zorita et al. (2007), gonad histology was studied as a supporting parameter in order to establish the reproductive status of the animals and to detect possible pathological alterations. One histopathological observation that could be attributed to cadmium toxicity is testicular oogenesis that leads to the formation of testicular ovarian follicle. According to McDaniel et al. (2008), testicular ovarian follicles (TOFs) refer to the presence of ovarian follicles in the testes. Although no specific agent can be pointed as the cause of testicular ovarian follicle, it was suggested that they may be induced by exposure to pesticide or estrogenic endocrine disrupting compounds, and cadmium is one of the endocrine disrupting compounds. Apart from testicular ovarian follicle,

detectable eosinophilic leucocytes in the interstitial compartment, the presence of vacuolated areas within the tubules (Mosconi et al., 2005) and macro melanophage centers (Zorita et al., 2008) were also found in the testis. In the female individuals, cadmium was found to cause follicular atresia, inflammation, hemorrhage and necrosis in the ovary.

Skeletochronological study has been added in this study because it may be used to demonstrate the effect of cadmium on the population biology of the species. Skeletochronology usually provides data on individual age, speed of growth, age of sexual maturity and longevity. The use of data derived from skeletochronology has proven to be an important factor in the study of environmental effects on a species. In many cases that involve heavy metal pollution, the toxic effect of heavy metals manifested in an organ is mainly a function of concentration and exposure time, because many toxicants bioaccumulate. Thus in a study on *Balaena mysticetus*, Rosa et al. (2008) stated that age can be an important factor in wildlife toxicology and cadmium concentration was found to be age related. Therefore, Spear et al. (2009) recommended that skeletochronology is used along with morphometric data to estimate growth as an index of physiological health.

6.2 Objective and Sub-objectives

The objective of this part of the research is to compare the histological and skeletochronological parameters of *Fejervarya limnocharis* from contaminated site with those from reference site. In fulfilling this objective, several sub-objectives have been identified. They are as follow

6.2.1 To compare the histopathological features of *Fejervarya limnocharis* from contaminated site with those from reference site

6.2.2 To compare the skeletochronological parameters of *Fejervarya limnocharis* from contaminated site with those from reference site

6.3 Hypothesis

6.3.1 There are significant differences in the histopathological features between *Fejervarya limnocharis* from contaminated site with those from reference site

6.3.2 There are significant differences in the skeletochronology parameters between *Fejervarya limnocharis* from contaminated site with those from reference site

6.4 Methodology

6.4.1 Histology

Liver (n=30), kidney (n=30), testis (n=30) and ovary (n=20) tissues were fixed in 10% neutral buffered formalin before they were processed. Tissues were dehydrated and processed through graduated changes of 70% ethanol, 90% ethanol, 95% ethanol, n-butanol and xylene before they were embedded in paraffin wax. Each paraffin blocks were trimmed and then sections were cut from the block. The thickness of each section was 5 μm. The sections were mounted onto slides and then dried. The sections were then stained by Haemotoxylin and Eosin staining procedure involving graduated immersion into xylene, n-butanol, 95% ethanol, 90% ethanol, 70% ethanol, water, haematoxylin solution, differentiator and eosin solution. The slides were then finally cleaned with xylene before the sections were mounted with DPX mounting medium (distyrene and tricresyl phosphate in xylene, BDH Laboratory Supplies). The slides were dried and then observed under light microscope.

6.4.2 Skeletochronology

For skeletochronology, the third phalanges of the third digit of the upper limb of the frog were used. The phalanges were washed in running water for 24 hours before they were subjected to decalcification procedure in 5% nitric acid for

90 minutes. The decalcified phalanges were then washed under running water for another 24 hours. Then, the phalanges were sectioned by freezing microtome to 20-22 μm sections. The sections were transplanted onto the slides with a few drops of water. Then the sections were stained with Mayer's Haematoxylin (Mayer's acid hemalum). The sections were observed under a dissection microscope to select sections from the central part of the diaphysis only. The sections were rinsed in tap water before they were mounted onto a slide with glycerine. The slides were dried and then observed under light microscope.

6.4.3 Statistical Analysis

The difference in the average value of macro-melanophage center count was statistically analyzed by t-test. Prevalences of tumor-like aggregation, hemorrhage and testicular ovarian follicle were statistically analyzed by Fisher Exact Test for proportions. All statistical analyses were performed on the SigmaStat 2.0 program.

6.5 Result and Discussion

Figure 6.1 showed the liver sections of *Fejervarya limnocharis* caught from Mae Sot, Tak. The sections showed that there was an observable difference in the number of macro-melanophage centers (MMC) or Kupfer cells in liver

sections of frog caught from both sites. This observation is further confirmed in Figure 6.2 that shows average macro-melanophage center count in liver section from *Fejervarya limnocharis* caught from Mae Sot, Thailand. It was found that frogs caught from Mae Pa had an average hepatic MMC count of 0.672 ± 0.299 cells/1000μm^2 (n=15). For Mae Pa, the average hepatic MMC count was 0.949 ± 0.267 cells/1000μm^2 (n=15). The mean differences between station is statistically significant at P=0.049. Hyalinization and vacuolation were not observed in the liver sections. However, the hepatocytes of frogs from the contaminated site showed polygonal hepatocyte shape and an increase in the cytoplasmic area. Figure 6.1(d) also showed some possible necrotic cells/necrotic area similar to those found by Dudley and Klaasen (1984). The liver section also revealed chromatin margination which is a morphologic sign of apoptosis.

For kidney, the section is presented in Figure 6.3. The sections showed the presence of tumor-like cell aggregation in the kidney of *Fejervarya limnocharis* caught from Mae Tao. The cells within the aggregation had a denser nucleus with much reduced cytoplasm. The prevalence of this tumor-like aggregation in the kidneys of *Fejervarya limnocharis* caught from both Mae Pa and Mae Tao is presented in Figure 6.4. The result of this study revealed that the prevalence of tumor cell in the kidney of Mae Tao's frog was 15% (n=20), while the prevalence in Mae Pa's population was 0% (n=20). Apart from that, the result also showed that the prevalence of hemorrhage in the kidney of *Fejervarya limnocharis* caught from the contaminated site was 5% (n=20), while none were observed in the

kidney sections of the frogs caught from Mae Pa (n=20). For both observations, Fisher Exact Test revealed that the proportion of tumor-like cell aggregation and hemorrhage were not significantly different than were expected from random occurrences (P=0.112 and P=1.00 respectively)

Figure 6.5 showed sections from the testis of *Fejervarya limnocharis* caught from Mae Pa and Mae Tao. The photomicrographs showed that testicular ovarian follicle was apparent in the testis of rice frog caught from the contaminated site, as opposed to the testis of rice frog caught from the reference site. Further study also revealed that the prevalence of testicular ovarian follicle (Figure 6.6) in the frogs caught from Mae Tao was 21.4% (n=15) while the prevalence for frogs caught from Mae Pa is 13.3% (n=15). However the differences in the proportions of testicular ovarian follicles were not significantly different than were expected from random occurrences (P=0.651). For ovarian section, there were no discerning differences in the ovarian sections of frogs in the contaminated site with those caught from reference site. Therefore no distinction on the effect of cadmium to the ovary can be made. Perhaps, this observation can be cross-referred with the ovarian cadmium accumulation and bioconcentration data. The data showed that cadmium showed very little accumulation, hence have very low bioconcentration data.

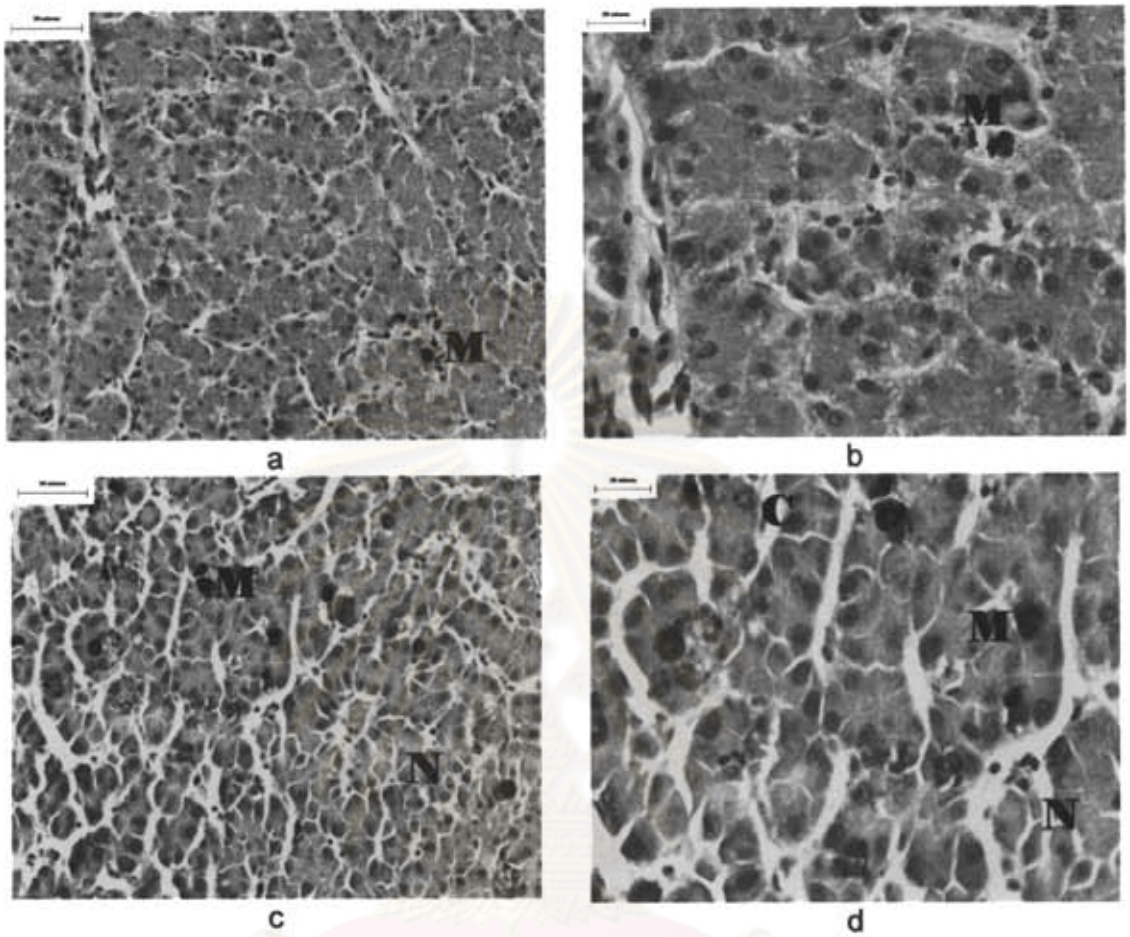

Figure 6.1: Liver sections of *Fejervarya limnocharis* caught from Mae Pa (a & b) and Mae Tao (c & d) at 20x (left; bar = 50μm) and 40x (right; bar = 20μm) magnifications (H&E staining). The sections showed macro-melanophage centers (M), necrotic cell area (N) and chromatin margination (C).

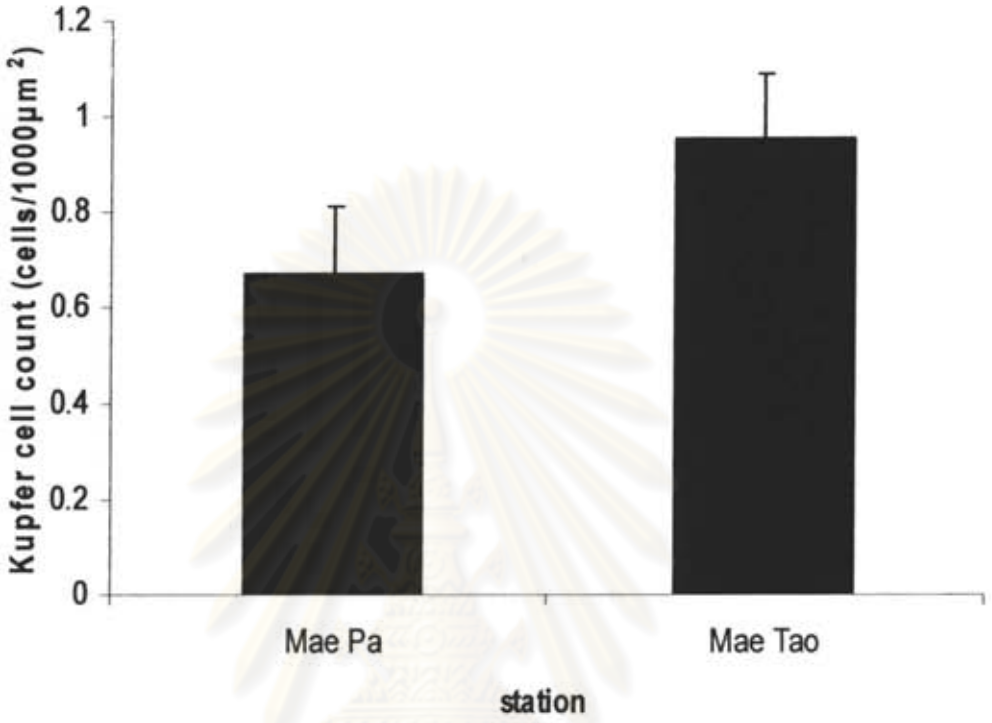

Figure 6.2: Mean macro-melanophage center count in liver section from *Fejervarya limnocharis* caught from Mae Pa (n=15) and Mae Tao (n=15). Mean differences between station is statistically significant (P= 0.049)

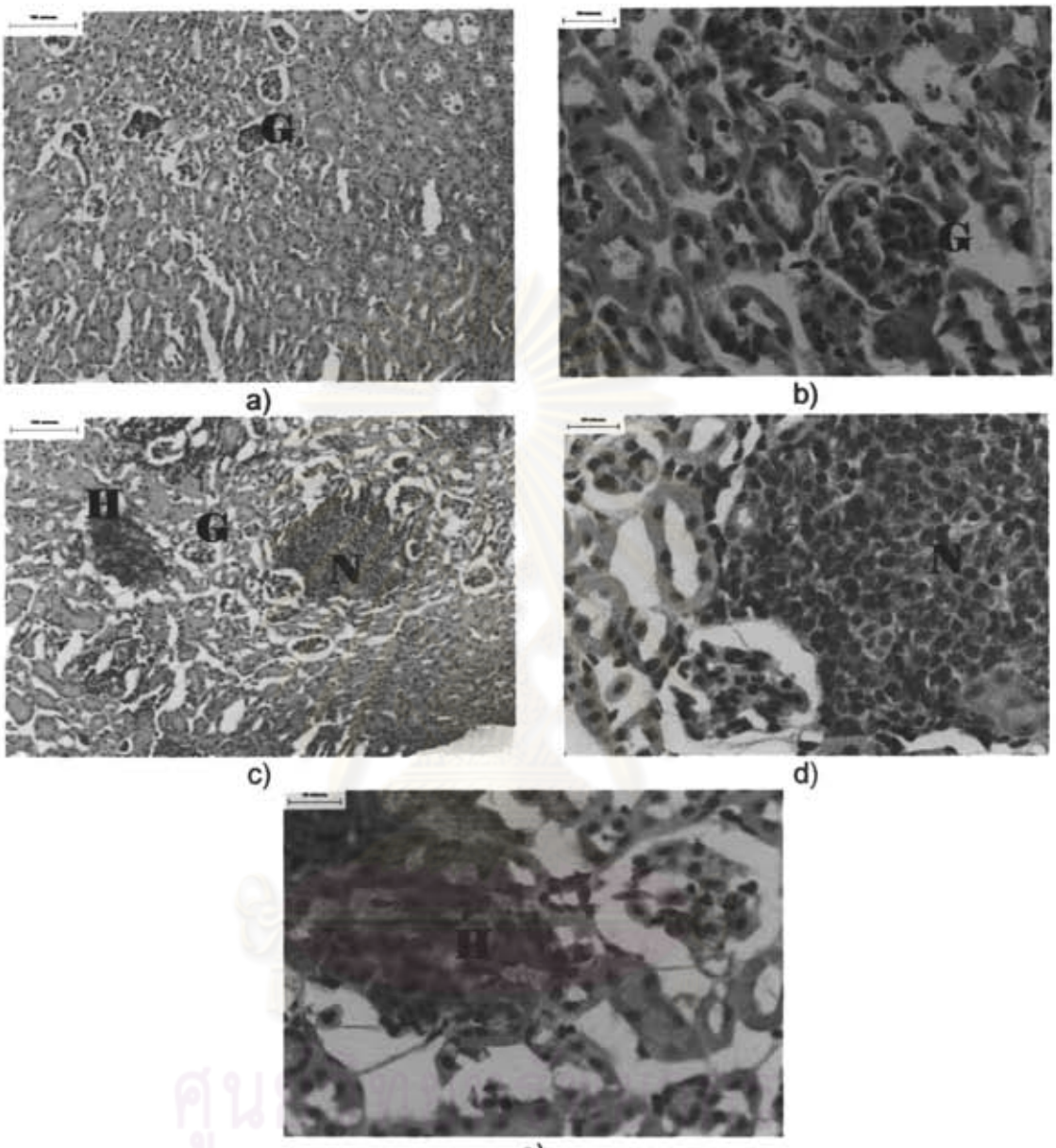

Figure 6.3: Kidney sections of *Fejervarya limnocharis* caught from Mae Pa (a & b) and Mae Tao (c, d and e) at 10x (bar = 100μm) and 40x (bar = 20μm) magnifications (H&E staining). The sections showed glomerulus (G), tumor-like cell aggregation (N) and hemorrhage (H).

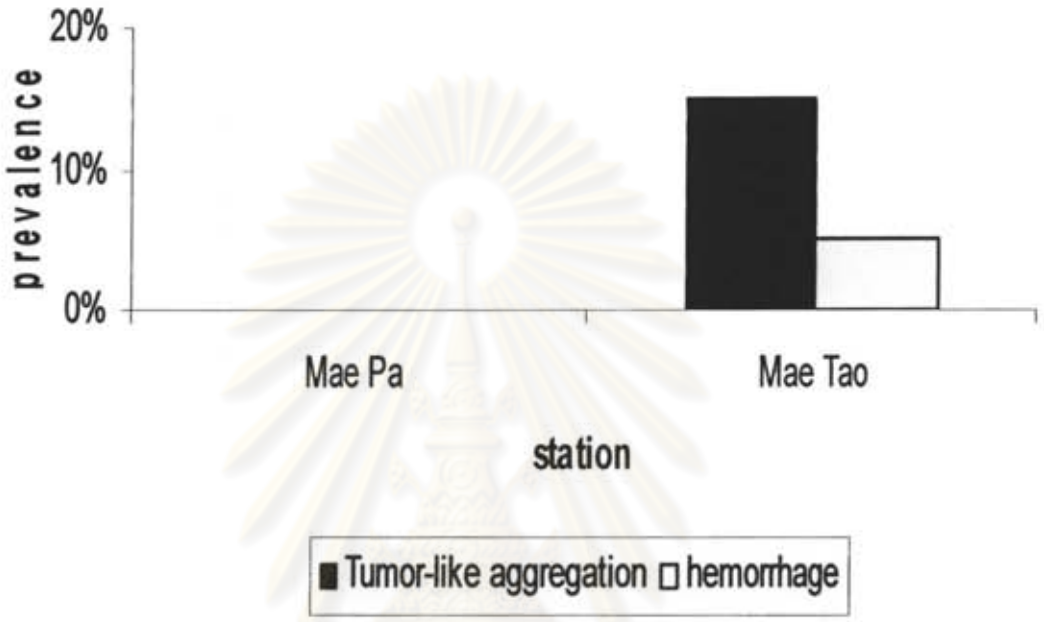

Figure 6.4: Prevalence of tumor-like cell aggregation and hemorrhage in the kidney sections from *Fejervarya limnocharis* caught from Mae Pa (n=15) and Mae Tao (n=15)

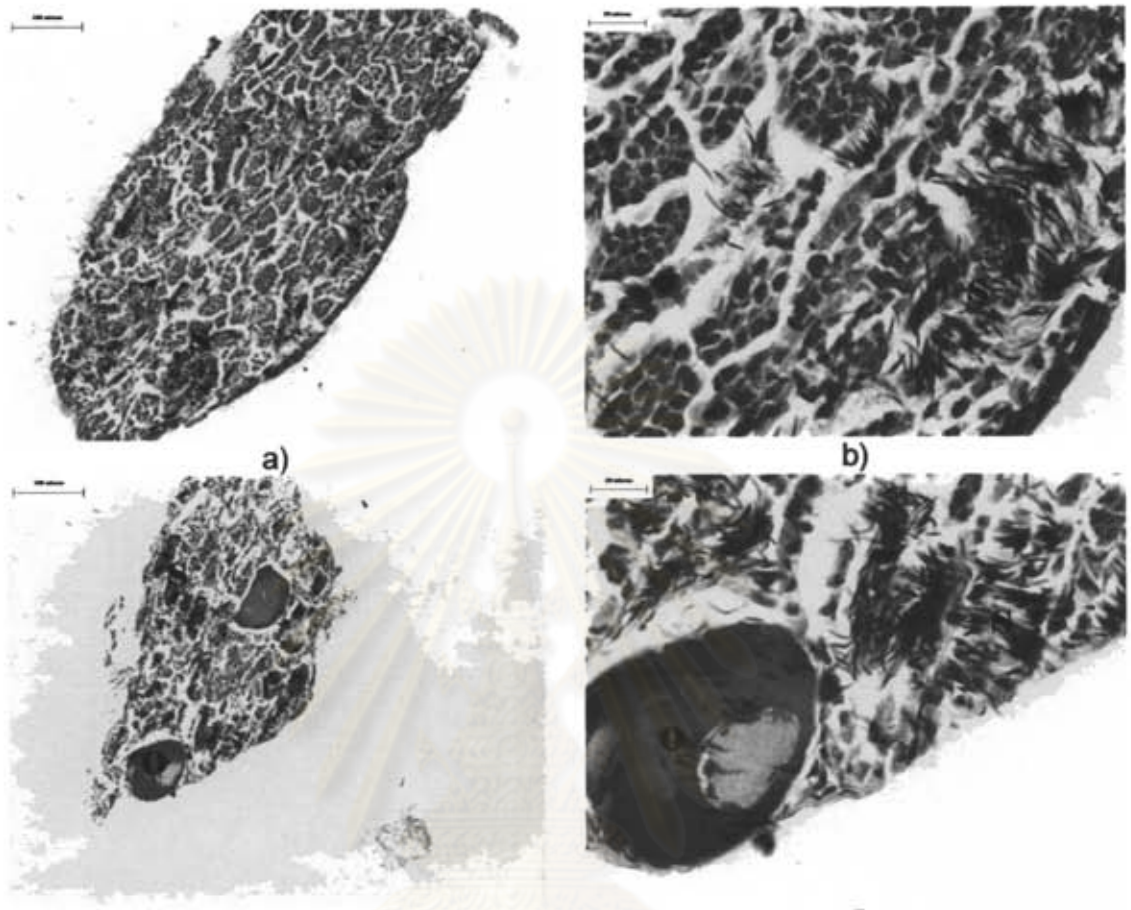

Figure 6.5: Testis sections of *Fejervarya limnocharis* caught from Mae Pa (a & b) and Mae Tao (c & d) at 10x (left; bar = 100µm) and 40x (right; bar = 20µm) magnifications (H&E staining). The sections showed mature sperms (S) in all sections and a developing oocyte (O) in the testis section from Mae Tao

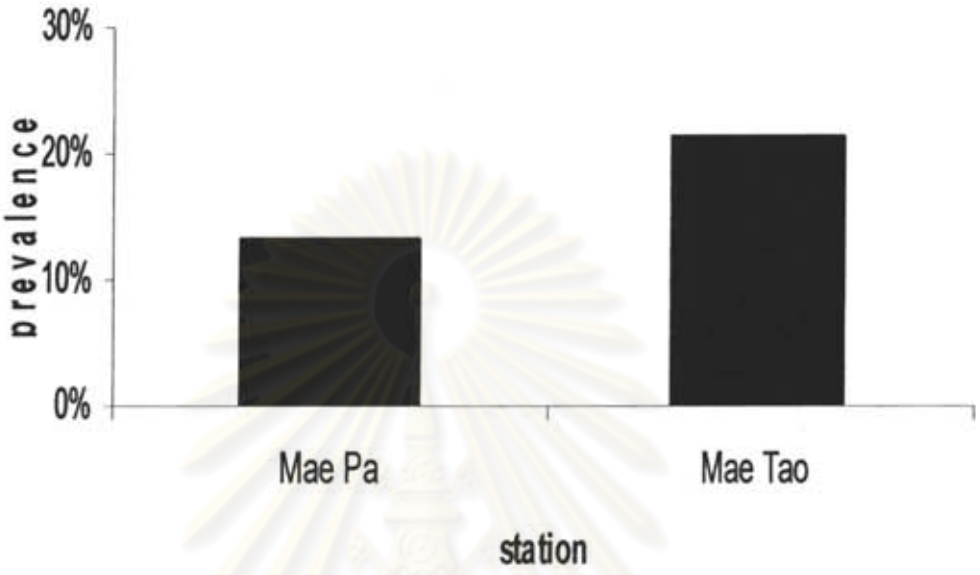

Figure 6.6: Prevalence of testicular ovarian follicle in the testis sections from *Fejervarya limnocharis* caught from Mae Pa (n=15) and Mae Tao (n=15)

The presence of macro-melanophage centers in the livers of the frogs in both reference and contaminated site was expected. This is because frogs living in the wild are often exposed to parasitic infections. However, this study found that the macro-melanophage center count of *Fejervarya limnocharis* caught from the contaminated site was statistically significantly higher than the macro-melanophage count of those caught from reference site. To explain, there is an association between cadmium accumulations with the production of macro-melanophage centers. Cadmium was known to cause a depression in the immune system (Loumbourdis and Vogiatzis, 2002). When this occurs, the body lacks the defense against parasitic infection, which would cause a massive invasion of parasites in the liver. As a response to this parasitic invasion, the liver would produce Kupfer cells to scour the parasites. The presence of Kupfer cells that has engulfed the parasites and foreign bodies is also often termed the macro-melanophage centers. Apart from that, Loumbourdis and Vogiatzis (2002) also reported that cadmium may cause the rupture of small vessels in the tissue. This would cause red blood cells to be discharged into the surrounding tissue. The presence of red blood cells in the tissue would also trigger the aggregation of Kupfer cells that would act as scavenger of the foreign bodies, forming the macro-melanophage centers.

A study by van Dyk et al. (2007) reported that some of the common manifestations of cadmium toxicity to the microstructure of the liver were hyalinization, vacuolation and cellular swelling. In this study, however,

hyalinization and vacuolation were not observed in the liver sections. Yet, moderate cellular swelling can be observed in the section from frog from Mae Tao, signified by polygonal-shaped hepatocytes and an increase in the cytoplasmic area in each hepatocyte (Dudley and Klaasen, 1984). This is because, van Dyk et al. (2007) stated that cadmium contamination could cause water influx into the cell, thus causing hydropic degeneration or cellular swelling. Possible necrotic cell/necrotic area similar to those found by Dudley and Klaasen (1984) were also found in the sections in this study. The liver section also revealed chromatin margination which is a morphologic sign of apoptosis (Habebu et al., 1998).

In the kidney, the result showed that tumor-like cell aggregation and inflammation were found only in the sections from frogs caught from the contaminated site. A study by Martin et al. (2002) showed that cadmium was shown to stimulate the proliferation of epithelial cells of the prostate. In another study, Stoica et al. (2000) found that cadmium has the potential to increase the risk of breast cancer via the activation of estrogen receptors while Alvarez et al. (2004) reported that cadmium has been classified by the International Agency for Research on Cancer as carcinogen. Therefore, cadmium may be able to activate the proliferation of the kidney cells, hence explaining the presence of tumor-like cell aggregation in the kidney of contaminated frogs. However, great care should be practiced before making the relationship between cadmium and cancer of the kidney. Since the diagnosis of cancer cells requires more extensive tests,

therefore this study would only label the finding as tumor-like cell aggregation, distinguishing them from the actual tumor cells or neoplasm. Also, the presence of renal hemorrhage can also be attributed, albeit indirectly, to cadmium. As stated before, cadmium has the ability to cause the rupture of blood vessels, hence causing hemorrhage in the tissue.

The presence of testicular ovarian follicle in the testis of *Fejervarya limnocharis* may also be attributed to cadmium contamination and accumulation in the tissue. According to Garcia-Morales et al. (1994), cadmium is the new environmental estrogen that is able to activate estrogen receptors in the cell. Since the oogenesis process is regulated by estrogen, most probably cadmium may play a role in the presence of testicular ovarian follicle in the testis. McDaniel et al. (2008) reported that the frequencies of the presence testicular ovarian follicles of less than 2% may represent natural background levels and there are some suggestions that they may be induced by exposure to pesticide or estrogenic endocrine disrupting compounds. Since cadmium is considered as an estrogenic endocrine disrupting chemical, there might be some correlation between cadmium accumulations with the high prevalence of testicular ovarian follicle in the male *Fejervarya limnocharis.* However, further studies into the origin of testicular ovarian follicle are necessary to evaluate the consequences of this condition in wild frog populations. Furthermore, in a field study, because of the large variation in environmental and chemical parameters it is difficult to pinpoint the cause with the effect.

For skeletochronological analysis, data obtained from this research showed a very inconsistent trend. Some individuals would show clear lines of arrested growth and resorption lines, while most other would show very faint lines or no line at all. Therefore, it was clear that the inconsistency would prevent the skeletochronological data to be used in this study. Hence, further use of skeletochronological data was terminated.

6.6 Conclusions

This research found that frogs from the contaminated sites had higher hepatic macro-melanophage centers, higher prevalence of tumor-like cell aggregation and inflammation in the kidney and higher prevalence of testicular ovarian follicle in the male individuals. However, only hepatic macro-melanophage count showed statistically significant difference between the two sites. Cellular swelling, possible necrotic area and possible apoptotic cell could be observed in the liver sections. No discerning histological differences were found in ovarian sections of frogs from the contaminated site with those from reference site. While the histological data are not considered as specific parameter, they might be able to support the notion that cadmium may have an effect on the histological and histopathological features of a tissue. However, any conclusion should be approached with great caution because any abnormalities that occur in the tissue most definitely cannot be traced back to cadmium contamination alone.

Skeletochronology analysis was considered unsuccessful because the data did not show consistent presence of line of arrested growth and resorption line. Therefore the data from this analysis is not used for this research.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

This research attempted to determine whether the rice frog, *Fejervarya limnocharis*, is a suitable candidate as a sentinel species for cadmium contamination. The district Mae Sot was chosen as a study site because of its history of cadmium contamination. Therefore, the cadmium contamination history offered a great opportunity for this study to proceed, using one of its native species. Our preliminary study confirmed the cadmium contamination in the contaminated sited, Mae Tao. While the level of cadmium in water remains low, the concentration of cadmium sorbed to the sediment was high in Mae Tao, as opposed to the reference site, Mae Pa.

Both Mae Pa and Mae Tao are located in the district of Mae Sot, in Tak province, northwestern part of Thailand. Mae Pa, located about 8 kilometers north of Mae Pa was chosen as a reference site because it shared several features. Sites with similar features helped reduce bias associated with land use, habitat and climate. Both sites are open plain areas used for rice fields and are irrigated by small creeks. Therefore, similar aquatic habitat feature could be expected between both sites. Since both of the sampling sites are located within the same district,

Mae Sot, both also share the same weather fluctuation and climate. This further helped reduce bias associated with climate and weather.

The amphibian species in concern was the rice frog, *Fejervarya limnocharis*. Amphibian species was chosen because previous researches have shown that amphibians would make an excellent candidate for cadmium contamination. Amphibians are able to uptake and accumulate cadmium via various modes. Cadmium can get into the amphibian body through oral and dermal exposure, as well as through the food chain. Furthermore, amphibian species are exposed to cadmium starting from the egg phase right up to its adult life. Therefore, it is more vulnerable to cadmium accumulation throughout its life cycle as compared to other group of animals with external protections such as eggshell and scales.

The rice frog was chosen based on several factors. The main determining factor was the fact that this species is not threatened by extinction. Being categorized as LC (Least Concerned), the utilization of this species would not put it under undue extinction pressure. In addition, this species has a wide range of distribution, covering South Asia, East Asia and Southeast Asia. Therefore its use as sentinel species could be utilized by the whole region. This species is also of the right size, hence making sampling and sample management easier. In certain parts of its distribution range, the rice frog is also considered as a food source, including at the sampling sites. Thus this species could provide the

important link between environmental cadmium and the bioavailable cadmium to human.

In order to determine whether *Fejervarya limnocharis* is a suitable candidate as a sentinel species for cadmium contamination, this research had been divided into four parts, encompassing the various levels or organization, from molecular/chemical level to the individual/population level. A battery of parameters was chosen because one single parameter may not be able to explain the extent of the effect of cadmium on the organism. To add, this research was not designed around standardized laboratory conditions. Instead, this study looked at what are the changes that are already occurring in the natural population of a sentinel species. In this case, the sentinel species under scrutiny was the rice frog, *Fejervarya limnocharis*. This is because Henry (2000) suggested that it may be productive that toxicological studies evaluate the contaminants' effects on the organisms and their adaptation within the environment. The overall parameters used and their summarized results are exhibited in Table 7.1.

The first part of this study was the contaminant analysis. In this part, cadmium concentrations were compared between the livers, kidneys, testis and ovaries of rice frogs caught from Mae Pa with those caught from Mae Tao. Whole organismal cadmium was also compared between there two sites. Further analysis was done by comparing the bioconcentration factors between

Fejervarya limnocharis caught from the reference site with those from the contaminated sites. This research found out that rice frog from the Mae Tao had higher hepatic, renal and testicular cadmium when compared with rice frogs from the reference site. The same trend was also seen in the ovarian cadmium levels. However, the differences were not statistically significant. The results also showed that kidney is the greatest cadmium accumulating organ while the ovary showed the least accumulation. This was shown in the result where kidney was the organ with the highest concentration of cadmium and the highest bioconcentration factor. We also found out that rice frogs caught during the late dry and early rainy seasons tend to have higher tissue and organismal cadmium than those caught during late rainy and early dry seasons. Therefore, if and when *Fejervarya limnocharis* is used in the biomonitoring of cadmium accumulation, types of tissues used and the season when sampling is performed should be taken into consideration. In this study, it was found out that the use of liver, kidney, testis and whole organism was appropriate in determining whether *Fejervarya limnocharis* can be used as sentinel species for cadmium contamination.

The next part of this research is the biomarker study. In this part, three biomarkers were used to determine whether the rice frog is a suitable sentinel species for cadmium contamination. The first biomarker used was hepatic metallothionein. The use of metallothionein was considered as biomarker of exposure, and a specific biomarker. This is because metallothionein are

produced in response to exposure to heavy metal contamination. In addition, it is considered as a specific biomarker because in this study, the level of metallothionein measured is the metallothionein-cadmium complex, hence rendered it as specific to cadmium contamination. This research found that frogs caught from contaminated site had higher hepatic metallothionein than those from the reference site. This shows that the use of hepatic metallothionein as a sentinel parameter for cadmium contamination is justified. The next biomarker was glutathione-S-transferase, which is a biomarker of effect and a non-specific biomarker. The findings of this research revealed that frogs from Mae Tao had higher hepatic glutathione-S-transferase than those from Mae Pa. Even though glutathione-S-transferase is not a specific biomarker, this research also found that it showed significant strong positive correlation with hepatic cadmium, hence exhibit a strong stressor-response correlation in the liver. The third biomarker was vitellogenin where the use of this biomarker would explain the effect of cadmium on the reproductive axis of the species. However, our attempt to cross-react rice frog's vitellogenin with other species' anti-vitellogenin antibody was unsuccessful. Therefore no result was available for vitellogenin levels in the plasma of the rice frog. In conclusion, the use of hepatic metallothionein and hepatic glutathione-S-transferase as parameters indicating the rice frog's suitability as a sentinel species is justified because of their statistically significant differences in their levels in the frogs from both sites. However, if the researcher needs to go one step further, the use of hepatic glutathione-S-transferase would

exhibit a better stressor-stress response in the frogs that were exposed to cadmium chronically, over a long period of time.

The third part of this research was the morphometric and gravimetric study. Morphometric and gravimetric data has always been a good indicator of the effect of pollution on an organism, while weight-length relationship, Scaling coefficient and condition factor has been used to assess the well-being of the species and can be used to reflect the impact of the environment on the growth of an organism. The use of Scaling coefficient and condition factor in *Fejervarya limnocharis* is novel because it has never been used in amphibians before. This research found that there is a connection between cadmium contamination with morphometric and gravimetric indices of *Fejervarya limnocharis* living in contaminated and reference sites in Mae Sot, Tak. *Fejervarya limnocharis* caught from Mae Pa had higher condition factor, renosomatic index and female gonadosomatic index than those caught from Mae Tao. This shows that, if this species is to be used as sentinel species for cadmium contamination, and if morphometric and gravimetric data are to be used, condition factor, renosomatic index and female gonadosomatic index are the justified parameters to choose. However the use of hepatosomatic index and Scaling coefficient should not be overlooked. This is because, albeit being not statistically significant, they showed a trend where frogs from contaminated site had lower values than those from reference site.

Table 7.1: Summary of the results from this study

Parameter	Unit	Range (low-high)		Significant
		Mae Pa	Mae Tao	difference
Contaminant analysis				
Environmental cadmium				
Cd in water	mg/L	0.0015 – 0.0020	0.0019 – 0.0023	No
Cd in sediment	mg/kg	0.1013 – 0.2206	2.9260 – 3.2888	Yes
Tissue cadmium				
Hepatic Cd	mg/kg	0.044 – 0.592	0.199 – 3.543	Yes
Renal Cd	mg/kg	0.239 – 1.715	1.890 – 12.175	Yes
Ovarian Cd	mg/kg	0.006 – 0.041	0.009 – 0.053	No
Testicular Cd	mg/kg	0.044 – 1.089	0.266 – 4.626	Yes
Whole organismal Cd	mg/kg	0.024 – 0.045	0.180 – 0.549	Yes
Bioconcentration factor (BCF)				
BCF_{liver}	-	22.025 – 379.18	84.55 – 1819.80	Yes
BCF_{kidney}	-	119.53 – 458.32	804.56 – 6229.75	Yes
BCF_{ovary}	-	3.35 – 22.26	4.47 – 26.95	No
BCF_{testis}	-	21.99 – 585.83	113.38 – 2367.47	Yes
BCF_{whole}	-	11.93 – 25.83	76.71 – 281.30	yes

Note: BCF = Bioconcentration factor

Parameter	Unit	Range (low-high)		
		Mae Pa	Mae Tao	
Biomarker study				
Metallothionein	mg/kg wet weight	1.496 – 2.785	2.991 – 3.907	Yes
Glutathione-S-transferase	µmol/min/mg total protein	0.154 – 0.159	0.199 – 0.331	Yes
Vitellogenin	µmol/min/mg total protein	No result	No result	-
Morphometric & gravimetric analysis				
Scaling coefficient	-	2.783 – 3.223	2.762 – 2.978	No
Condition factor	-	9.781 – 11.093	9.511 – 9.931	Yes
Hepatosomatic index	-	1.578 – 2.074	1.529 – 2.205	No
Renosomatic index	-	0.372 – 0.448	0.354 – 0.411	Yes
GSI_{female}	-	3.591 – 12.082	2.287 – 7.702	Yes
GSI_{male}	-	0.109 – 0.249	0.113 – 0.29	No
Histological analysis				
MMC count	cells /1000µm^2	0.177 – 1.146	0.601 – 1.304	Yes
Renal TLA prevalence	%	0	15	No
Renal hemorrhage prevalence	%	0	5	No
TOFs prevalence	%	13.3	21.4	No
Skeletochronology	-	No result	No result	-

Note: GSI = gonadosomatic index; TLA = Tumor-like aggregation; TOFs = Testicular ovarian follicles;
MMC = macro-melanophage center

The final part of the research was the biological and ecological study. In this part, histology analysis and skeletochronology were employed. The result of the histological study revealed that *Fejervarya limnocharis* caught from Mae Tao had higher hepatic macro-melanophage count and higher prevalences of renal tumor-like aggregation, renal hemorrhage and testicular ovarian follicle than the rice frogs caught from Mae Pa. Cellular swelling, possible necrotic area and possible apoptotic cell could also be observed in the liver of contaminated frogs. The presence of ovarian follicle in the testes of the frogs caught from the contaminated site may also help explain the higher male gonadosomatic index in these frogs as compared to those caught from reference site. The findings showed that the use of histological data can support the notion that *Fejervarya limnocharis* is a suitable candidate as a sentinel species for cadmium contamination.

The skeletochronology data obtained from this research showed very inconsistent findings. Some individuals would show clear line of arrested growths while the majority of others would not. Therefore, further use of skeletochronological data was terminated. This maybe partly attributed to the fact that this is a tropical species where a significant period of hibernation and reduced growth was not observed.

Overall, the results showed that *Fejervarya limnocharis* shows a great potential to be used as a sentinel species for cadmium contamination. However, it needs to be emphasized that non-specific indicators (glutathione-S-transferase, all morphometric and gravimetric data, condition factor and histological observations) should not be used alone in making the decision. These data need to be used in complementary with other contaminant-specific parameters namely contaminant analysis data and cadmium-metallothionein level.

As mentioned above, *Fejervarya limnocharis* has a great potential to be used as a sentinel species for cadmium contamination. However, it is also justified that more research should be done on this species in order to verify the suitability of this species. A list of recommended future researches involving *Fejervarya limnocharis* is shown below.

- Controlled-laboratory experiments for verification of field observation: This study should include all research parameters studied in this project (contaminant analysis, biomarker study, morphometric and gravimetric analysis, and histology and skeletochronology) but in the laboratory setting.
- Developmental studies of *Fejervarya limnocharis* exposed to baseline and environmentally relevant cadmium concentration: This study should include embryology, tissue development and gonad development and maturation.

- Detailed histological and histopathological studies of tissues of the rice frog: This study should also include quantification of several histological features highlighted in the report.
- Development and purification of specific vitellogenin antibody for *Fejervarya limnocharis* followed by determination of vitellogenin level in the plasma of rice frog caught from Mae Sot.
- Cadmium biomagnification study associated with other organisms in the rice frog's line of food chain.
- Study on the effect of zinc and lead and their co-exposure with cadmium on the rice frog since these two metals are also found in high concentration in Mae Tao.
- Study on risk assessment associated with eating rice frog: This study should also include risk communication to the locals.

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Cadmium Accumulation in Two Populations of Rice Frogs (*Fejervarya limnocharis*) Naturally Exposed to Different Environmental Cadmium Levels

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Abstract Contaminant accumulation analysis is important in the study of sentinels. This research determined cadmium accumulation and bioconcentration factors of whole organism, liver, kidney, ovary and testis of *Fejervarya limnocharis* exposed to different environmental cadmium levels. Frogs from contaminated sites had significantly higher hepatic (1.939 mg/kg), renal (7.253 mg/kg) and testicular (1.462 mg/kg) cadmium than those from the reference sites (0.205, 0.783 and 0.379 mg/kg, respectively). Cadmium accumulation was the highest during the late dry and early rainy seasons. If this species is used as a sentinel for cadmium accumulation, the utilization of its whole organism, liver, kidney and testis is appropriate.

Keywords Cadmium · Environment · *Fejervarya limnocharis* · Sentinel species

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Cadmium in the environment has the ability to accumulate in the body. Bervoets et al. (2001) reported that levels of accumulated metals in tissue were related to metal levels in sediment, water and food. This is further supported by Bervoets and Blust (2003) saying that it is more likely that tissue levels reflect environmental levels because metal concentrations in tissue follow concentrations in the environment. Francis et al. (1984) reported that *Carrasius auratus*, *Rana pipiens* and *Micropterus salmoides* showed strong correlations between cadmium concentrations in water and tissue, and sediment and tissue. Different tissues have the ability to accumulate metals differently (Loumbourdis and Wray 1998; Bervoets et al. 2001; Loumbourdis and Vogiatzis 2002; Bervoets and Blust 2003 and Burger et al. 2007). Loumbourdis and Vogiatzis (2002) reported that the liver is one of the main target organs of cadmium accumulation in *Rana ridibunda*. In another study, Loumbourdis and Wray (1998) found that *Rana ridibunda* liver has higher cadmium concentrations than carcass. A study by Loumbourdis et al. (2007) found that upon exposure, cadmium started to deposit in the liver, kidney and the gut of *Rana* sp. However, the kidney is found to be the main site of accumulation. Flament et al. (2003) reported that cadmium is also readily incorporated in the kidneys and reproductive tissues. Lee (1983) supported this notion by saying that cadmium directly targets testis. In addition, adult *Chrysemys picta*

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from impacted sites has higher cadmium concentration in liver, kidneys and gonads (Rie 2000).

In this study, the rice frog, *Fejervarya limnocharis* has been chosen to be a representative sentinel species for cadmium accumulation. This species is chosen based on a few criteria. The first and the most important reason for choosing the species is because it is categorized under the "Least Concern" or LC category by the IUCN (IUCN, Conservation International and NatureServe 2006; AmphibiaWeb 2008). The status is given to the species because of its wide distribution, its tolerance of a broad range of habitats and its large and stable population. Therefore, using this species will not put it under undue extinction pressure. The rice frog is also of a suitable size. It is not too large that it may cause logistic problem and yet it is also not too small that sampling and analysis are greatly hampered. This species also has a wide range of distribution, covering Southeast Asia, South Asia and parts of East Asia. Hence it is a suitable candidate as the region's sentinel species. *F. limnocharis* is also utilized as human food in some countries (IUCN, Conservation International and NatureServe 2006), including Thailand. Therefore, the species plays an important role as a link between environmental cadmium and bioavailable cadmium through the food chain. However, data on the use of *F. limnocharis*, either from wild populations or lab raised populations, for cadmium monitoring is non-existent. Therefore, this research aimed to determine the accumulation of cadmium in wild *F. limnocharis* that are naturally exposed to different cadmium levels. Accumulation is investigated in selected tissues (liver, kidney, ovary and testis) as well as in whole organisms.

Materials and Methods

Frog samples were collected on monthly basis during November 2007 and October 2008 from several rice fields in Mae Tao and Mae Pa in Mae Sot District, Tak Province. The contaminated site, Mae Tao, was located at 16°45′13″N; 98°35′25″E. This area is irrigated by the Mae Tao Creek. Simmons et al. (2005) reported that there were elevated cadmium levels in the paddy soils and rice grain in the vicinity of Mae Tao Creek downstream of a zinc mining area. The reference site, Mae Pa, was located 8.4 km north of the contaminated site at 16°40′43″N; 98°35′36″E. The area is irrigated by Huay Luek Creek and not on the path of the potential contaminated plume. Preliminary analysis showed that the cadmium concentrations from the contaminated site ranged from 0.0019 to 0.0021 mg/L in water samples, and 2.9260 to 3.2888 mg/kg in sediment samples. The concentration ranges at the reference site were 0.0018 to 0.0020 mg/L (water) and 0.1013 to 0.2206 mg/kg (sediment).

Frogs were individually subjected to cold anesthesia before killed by double-pith at brain and spinal cord. The liver, kidney and gonad were removed and weighed. Tissue and whole organism samples were dried in an oven at 80°C to a constant weight. The samples were then subjected to a microwave digestion procedure with concentrated nitric acid followed by cadmium determination using Graphite Furnace Atomic Absorption Spectrometer (AAS ZEEnit 700 by Analytik Jena). The standard curve range used was 0–10 μg/L and the detection limit of this instrument is 0.02 μg/L.

All data were statistically analyzed with two-way ANOVA and Student-Newman Keuls test using the SigmaStat 2.0 program.

Results and Discussion

Figure 1a shows the quarterly average hepatic cadmium in *F. limnocharis* caught from both sites. Hepatic cadmium in *F. limnocharis* caught from Mae Pa ranges from 0.044 to 0.592 mg/kg. On the other hand, in *F. limnocharis* caught from Mae Tao, the range is from 0.199 to 3.543 mg/kg. Further analysis showed that hepatic cadmium concentration in Mae Tao frogs are between 4.5 and 32.2 times higher than Mae Pa frogs. Frogs in both sites show similar fluctuation of hepatic cadmium when compared seasonally. In both sites, hepatic cadmium concentration is the highest during the early rainy season (April, May and June).

For cadmium in kidney, the results are shown in Fig. 1b. It is found that renal cadmium in *F. limnocharis* caught from Mae Pa ranges from 0.239 to 1.715 mg/kg. In *F. limnocharis* caught from Mae Tao, the range is from 1.890 to 12.175 mg/kg. The result showed that Mae Tao frogs had renal cadmium concentration of between 5.5 to 16.2 times higher than Mae Pa frogs. Seasonal fluctuation-wise, renal cadmium concentration is the highest during the early rainy season for Mae Pa frogs and during the late dry season for Mae Tao frog.

Site-wise comparison of ovarian cadmium revealed that the differences in ovarian cadmium are not statistically significant. When season-wise comparisons are made, ovarian cadmium concentrations in both sites are the highest during the late dry season.

Figure 1c shows the results for quarterly testicular cadmium in *F. limnocharis* caught from Mae Pa and Mae Tao. The graph showed that testicular cadmium in *F. limnocharis* caught from Mae Pa ranges from 0.044 to 1.089 mg/kg. Meanwhile in *F. limnocharis* caught from Mae Tao, the range is from 0.266 to 4.626 mg/kg. Site-related comparison shows that testicular cadmium

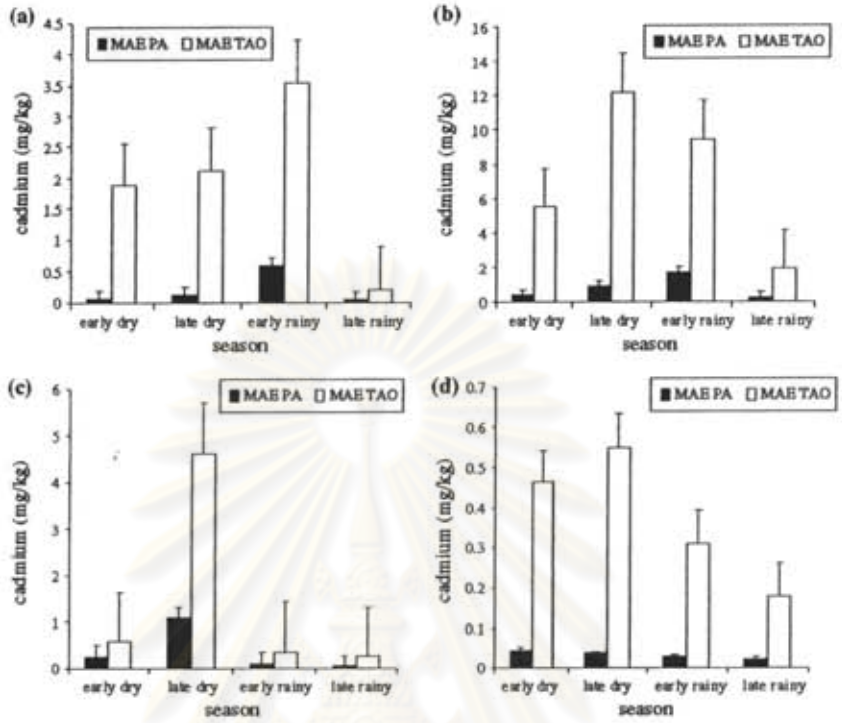

Fig. 1 Quaterly average **a** hepatic, **b** renal, **c** testicular and **d** whole organismal cadmium concentration in *F. limnocharis* caught from Mae Sot, Tak. All mean differences betweens stations are statistically significant ($P < 0.05$)

concentration in Mae Tao frogs are between 2.2 and 6.1 times higher than Mae Pa frogs. For frogs from both sites, the testicular cadmium concentrations are the highest during the late dry season.

The results for whole organismal cadmium are shown in Fig. 1d. Whole organismal cadmium concentration in *F. limnocharis* caught from Mae Pa ranges from 0.024 to 0.045 mg/kg. On the other hand, in *F. limnocharis* caught from Mae Tao, the range is from 0.180 to 0.549 mg/kg. To compare, whole organismal cadmium concentration in Mae Tao frogs are between 7.5 and 14.5 times higher than Mae Pa frogs. Seasonally, Mae Pa frogs caught during the early dry season showed the highest whole organismal cadmium concentration. For Mae Tao, the highest whole organismal cadmium concentration is shown in frogs caught during the late dry season.

Overall comparisons also show that in both sites, renal cadmium concentrations are higher than hepatic cadmium. Renal cadmium concentration in Mae Pa frogs are between 2.9 and 6.8 times higher than hepatic cadmium concentration. In Mae Tao frogs, the renal cadmium concentrations are between 2.7 and 9.5 times higher than hepatic cadmium concentration. Tissue-by-tissue comparison showed that in both sites, renal cadmium concentration is the highest while ovarian cadmium concentration is the lowest (Fig. 2a). The result is in line with bioconcentration factor analysis where for both sites, kidney showed the highest cadmium bioconcentration factor (Fig. 2b). The average cadmium bioconcentration factor in kidney is 467.75 for Mae Pa frogs and 3,672.32 for Mae Tao frogs.

Loumbourdis and Vogiatzis (2002) reported that liver is one of the main target organs of cadmium accumulation in *Rana ridibunda*. In another study, Loumbourdis and Wray (1998) found that *Rana ridibunda* liver has higher cadmium concentration than carcass. Pérez-Coll et al. (1997) found out that 26% of the cadmium uptake is deposited into the liver. Foran et al. (2002) stated that cadmium can accumulate and be retained in the liver. However, our sets of results showed a different trend. While accumulation of cadmium in the liver is quite high, it is clear that renal cadmium accumulation is even more apparent. This may be because the liver is the primary accumulation site while the kidney is the final accumulation site. Loumbourdis et al. (2007) reported that cadmium may gain entry into hepatocytes via endocytosis mediated by Fe binding protein such as ferritin. In the liver, cadmium will bind with

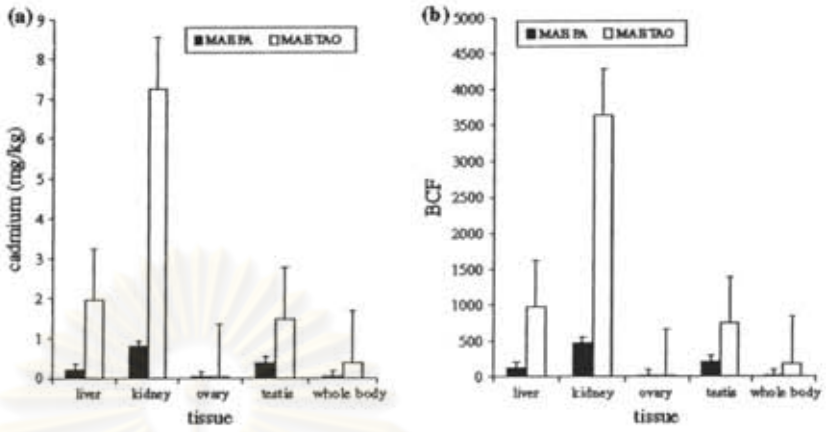

Fig. 2 Comparison of **a** average cadmium level and **b** cadmium bioconcentration factor in *F. limnocharis* caught from Mae Sot, Tak

metallothionein or stay as free cadmium in the hepatocytes. Free cadmium from liver is then released into the gastrointestinal lumen by the secretion of bile contents in the bile duct. Some of the cadmium is removed by feces while most will enter the blood stream through the enterohepatic circulation. Cadmium will then be transported to the various target organs, especially kidney. Therefore, throughout the life span of the frog, cadmium will be continually accumulated in the liver, and then transported to the kidney. So far, there has been no account on whether cadmium in the kidney is excreted or not. Hence, it is assumed that all cadmium that accumulates in the kidney will be retained there. High cadmium accumulation in the kidney is also reported by various other studies. The highest renal cadmium accumulations were found in *Gobio gobio* (Bervoets and Blust 2003), *Salmo trutta* (Olsvik et al. 2000), *Rana ridibunda* (Loumbourdis et al. 2007), *Pleurodeles waltl* (Flament et al. 2003) and *Gasterosteus aculeatus* (Bervoets et al. 2001).

While kidney showed higher cadmium accumulation than liver, we also found that both are actually suitable indicators for biomonitoring of cadmium accumulation. This is because both hepatic and renal cadmium levels are significantly higher in *F. limnocharis* caught from the contaminated site as compared to those from the reference site.

Among reproductive tissues, ovarian cadmium showed very little accumulation. To add, the differences in ovarian cadmium accumulation between reference and contaminated sites were also not significant. This shows that the ovary probably is not a suitable organ to be used in biomonitoring of cadmium accumulation. However, this study found out that high cadmium accumulation is found in the testis. This is shown by the high testicular cadmium and high testicular cadmium bioconcentration factor in *F. limnocharis* from the impacted site as compared to those from the reference site. This is expected because cadmium is a known toxicant directly targets testis (Lee 1983).

In this study, all the frogs used for the analysis of whole organismal cadmium weighed less than two grams. In these frogs, the use of organs, especially kidney and testis was rather difficult. Therefore, for these small frogs, whole body cadmium analyses were performed. We have included the use of small frogs in this research because we anticipate that in the future, not all field sampling activities will be able to have a yield of large frogs. In this case, instead of determining cadmium accumulation in organs and tissues, whole organismal cadmium accumulation may be a better choice of analysis. In our study, we found out that there were significant differences in whole organismal cadmium level and whole organismal cadmium bioaccumulation factor between *F. limnocharis* caught from contaminated site with those from reference site. Frogs from contaminated site had higher whole organismal cadmium level and whole organismal cadmium bioaccumulation factor. Therefore, in cases when organ cadmium accumulation determination in large frogs is not available, the use of whole organismal cadmium in small frogs is also considered as suitable indicator for biomonitoring of cadmium accumulation.

When we compared cadmium accumulation according to season, we found that the highest cadmium accumulation occurred either during the late dry season or during the early rainy season. This is because these two seasons are the active season when reproductive tissues are developing and when reproduction actually takes place. Zug et al. (2001) stated that rainfall is one of the major determinants of timing of reproduction. AmphibiaWeb (2008) confirmed this by stating that the breeding of the *F. limnocharis* is triggered by rain and it is usually the first species to come to the calling sites. In order for the rice frog to be the first species to come to the calling sites during the early rainy

season, their ovaries and testes will have to start developing and maturing during the late dry season. Reproductive tissues development and maturation requires energy, hence the frogs would have to increase food and water intake during the late dry season for these purposes. During the early rainy season, the actual breeding occurs, and again these efforts require a lot of energy. Zug et al. (2001) stated that egg development would constitute a large portion of their overall energy budget. Therefore, it is during these two seasons, more food and water were consumed. And with increased food and water consumption, there was also a chance of increased uptake of cadmium along with it. This would explain the high cadmium concentration in the liver, kidney and testes of the frogs during the late dry and early rainy seasons.

In conclusion, this research found that frogs from the contaminated site had higher hepatic, renal, testicular and whole organismal cadmium when compared to frogs from the reference site. The results also showed that the kidney is the greatest cadmium-accumulating organ. We also found that frogs caught during the late dry and early rainy seasons tend to have higher tissue and organismal cadmium than those caught during late rainy and early dry seasons. Therefore, when using *F. limnocharis* in biomonitoring cadmium accumulation, types of tissues used and seasonal sampling period should be taken into consideration.

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Abstract of Poster Presentation

The 16th International Congress of Comparative Endocrinology

June 22-26, 2009

Hong Kong SAR, China

Poster Presentation (P)

P-69

HEPATIC BIOMARKER RESPONSES IN THE FROG, *FEJERVARYA LIMNOCHARIS*, NATURALLY EXPOSED TO ENVIRONMENTAL STRESS FROM CADMIUM CONTAMINATION

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Glutathione-S-Transferase (GST) and metallothioncin are important biomarker endpoints in studying the effect of cadmium exposure. This research studied correlation between hepatic cadmium with GST and metallothioncin in the wild frog, *Fejervarya limnocharis*, exposed to different levels of environmental stress from cadmium contamination. The results showed that frogs from contaminated site had significantly higher hepatic cadmium (1.939 mg/kg), metallothioncin (3.578 mg/kg wet weight) and GST activity (0.259 mol/mL/min/mg protein) than those from the reference site (0.205 mg/kg, 2.363 mg/kg wet weight and 0.157 mol/mL/min/mg protein, respectively). There was a significantly positive correlation between hepatic cadmium and GST activity ($r = 0.802$, $P = 0.0096$). However, the correlation between hepatic cadmium and metallothioncin was not significant ($r = 0.548$, $P = 0.139$). The results concluded that while frogs from the contaminated site had higher GST and metallothioncin, only GST showed significant positive correlation with hepatic cadmium levels. Therefore, in the case that the sentinel species used was chronically exposed to environmental cadmium, hepatic GST activity may be used as a better biomarker endpoint than hepatic metallothioncin.

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BIOGRAPHY

Mohd Sham Bin Othman was born on the 2nd of November 1969 in Kuala Lumpur, Malaysia. He graduated his first degree in 1996 by obtaining an upper second class Bachelor of Applied Science (Applied Biology) majoring in Aquatic Biology and minoring in English Language Studies from Universiti Sains Malaysia, Penang. His final year research project was entitled "A Study on the Primary Productivity of Pedu and Muda Dams in Kedah". After that, he worked as a Biology teacher at the Matriculation Center of Universiti Kebangsaan Malaysia, Selangor. He then continued his graduate study in Universiti Kebangsaan Malaysia and obtained a Master's Degree of Science in Conservation Biology in 2002. His thesis title was "A study on the Ecology and Diversity of Stream Fishes in Sungkai Wildlife Reserve, Perak". Upon graduation, he became a lecturer in the Environmental Health Program in the Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia. In 2006, he took a study leave to continue his study in the Doctor of Philosophy Program in Environmental Management in the National Center of Excellence for Environmental and Hazardous Waste Management, Chulalongkorn University, Thailand.