Monitoring of heavy metal contamination in aquatic organism by applying Metallothionein biomarker and its situation in Thailand

Chutima Thanomsit

Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, Surin 32000, Thailand.

Corresponding author. E-mail address: chutima.tn@rmuti.ac.th

Abstract

Metallothionenein (MT) is produced in the liver after the organism exposed to heavy metals. MT is involved in homeostasis and detoxification process of heavy metals. Thus, MT has the potential to be applied as a bioindicator for heavy metal exposure of the organisms especially the aquatic ones. MT is characterized based on a high content of sulphur, cadmium and zinc. In present, the assessment on heavy metal contamination can be used to detect MT both directly and indirectly, i.e. (1) direct methods: the measurement of MT content by the difference pulse polarography (DPP) and spectrophotometry and (2) indirect methods: the measurement of MT content in each organ of aquatic organism by using antibody technique such as Enzyme-linked immunosorbent assay (ELISA) or western blot. The measurement on expression level of gene controlling MT production by many biomolecular techniques allowing to evaluate heavy metal exposure condition of the organism. In Thailand, the study on MT and its application have been inadequate. There were two cases of MT application as biomarker found: Ang sila (Chonburi province) and Map ta put (Rayong province). Thus it should be more studied for developing and extending its application.

Keywords: Metallothionein, Biomarker, Heavy metal, Aquatic organisms

Introduction

In General, an increasing of industrial development tends to worsen the aquatic environment by releasing harmful toxicants such as chemicals and heavy metals. After released to the aquatic environment heavy metals are assimilated in the aquatic organism, they can harm at the cellular level. Humans and animals can expose to those heavy metals by both direct and indirect contact by consuming the contaminated organisms (Castro-González and Méndez-Armenta, 2008). However, the assessment of heavy metal level in water of bottom sediment may not inform their true biological effects on organisms because the forms of heavy metals in the environment may not be in the active form which results in the negative effect (Adhikari et al., 2009). Castro-Gonzalez and Mendez-Armenta (2008) indicated that the ionic forms of cadmium tend to bind with anions to be cadmium peroxide (CdO_2), cadmium chloride ($CdCl_2$) or cadmium sulfate ($CdSO_4$). From all these forms, $CdCl_2$ form causes the most serious problem in fish or other seafood because of its high absorbable capability. Moreover, after heavy metals are assimilated in the organism body they can be accumulated and magnified along the food chain (Olsvik, Hindar, Zachariassen, & Andersen, 2001).

Biomarker is described as an alteration of biological indicator responding to toxicants exposure (Peakall, 1994). Walker, Hopkin, Sibly, and Peakall (2006) gave the meaning of biomarker that it was any biological response to an environmental chemical in the individual level or below differing from the normal status. Therefore, biochemical, physiological, histological, morphological, and behavioral changes should be considered to select the appropriate biomarker.

To select a suitable biomarker, Oost, Beyer, Vermeulen, and Nico (2003) suggested several criteria to consider. As described in the work of Stegeman et al. (1992) firstly, biomarker diagnosis should be reliable, cheap, and high performance. Secondly, it should detect xenobiotic exposure at quite low concentration for acting as an early warning parameter. Thirdly, the response profiles of biomarker after exposed to the contaminants should be easily understood. Fourthly, the expression of baseline value and biomarker response should be obviously dissimilar for well distinguishing the natural variability and pollution-induced stress. Next, the potential influencing factors such as age, gender, and reproduction state, on biological and physiological response should be limited and minimized. Finally, the responding mechanisms which is used as biomarker should be clearly studied and defined.

Metallothionein (MTs) can be used as an alternative method to monitor heavy metal contamination and its impact on the fish such as brown trout, *Salmo trutta* (Linde, Sanchez-Galan, Valles-Mota, and Garcia-Vazquez, 2001), Tilapia, *Oreochromis* sp. (Andrew, 2000) and dab, *Limanda limanda* (Lacorn, Lahrssen, Rotzoll, Simat, & Steinhart, 2001). Metallothioneins (MTs) are water soluble proteins generally found in both prokaryotic and eukaryotic cells. The molecular weight of MTs is low about 6-10 kDa comprising 30% cysteine and non aromatic amino acids, thus they can selectively bind with many metals such as Cd, Zn and Cu because of their high cysteine content (Knapen, Reynders, Bervoets, Verheyen, & Blust, 2007). However, the physiological role of MTs has not been fully understood, they primarily involve in homeostasis of some essential metals and toxic metal detoxification (Kim et al., 2008). Moreover, Montaser, Mahfouz, El-Shazly, Abdel-Rahman, and Bakry, (2010) reported that MT involved in controlling the alteration of cellular metal ion buffer by capturing essential metals such as Cu and Zn, thus, it could be summarized that MT level and measured heavy metal concentration are positively correlated.

Induction of Metallothionein

Metallothionein is in the conserved family closely relating with stress response protein which links to the cellular homeostasis. MT plays an important role in the management of essential heavy metal divalent cation, and serves to interfere the toxic effects of toxic heavy metals and free radicals. MT has been reported to regulate an activity of specific transcription factors. Induction of MT is responding mechanism to the initiators including divalent heavy metals, endotoxin, interferon, glucocorticoids, acute-phrase cytokines tumor necrosis factor-∝, interleukin-1 (IL-1), and interleukin 6 (IL- 6), reactive oxygen and nitrogen species, and toxic organic compounds (Roesijadi, 1992). Obviously, it is generally known that divalent metal is the major cause of MT induction in many organisms.

Campenhout et al. (2004) studied the induction of the MT level in carp (*Cyprinus carpio*) after exposed to cadmium in different concentrations (0, 0.1, 1, 2.5 and 10 μM) for 96 h. They found a significant increasing in MT induction only in the liver of the fish exposed to 10 μM of cadmium. For the kidney, a significant increase was found in the groups exposed to 2.5 and 10 μM of cadmium but not observed in the gill because of the low induction level and large inter individual variation. The highest MT levels found in the liver, kidney and gill were 29.48±2.58, 10.13±1.49 and 1.12±0.38, respectively. Moreover, Kovarova, Kizek, Harustiakova, Celechovska, and Svobodova (2005) investigated MT induction by cadmium chloride exposure in carp (*C. carpio*) at different concentrations (2.5, 5, 7.5, 10 and 12.5 mg/L). After exposed to $CdCl_2$, MT levels in liver

reached high level (above 130 ng/g) for the concentrations of 2.5, 5 and 7.5 mg/L and low level (50 ng/g) for the concentrations of 10 and 12.5 mg/L. This findings indicated that the MT induction and its binding capacity were restricted.

In General, MTs are mostly found in liver, kidney, gill and intestine of fish (Hogstrand and Haux, 1991; Filipovic and Raspor, 2003) but their content varies with species, reproductive stage, age and diet, thus these influencing factors must be considered in using MT as a biomarker (Livingstone, 1993; Filipovic and Raspor, 2003). In addition, Rotchell, Clarke, Newton, and Bird (2001) suggested that site and seasonal variations also significantly affected to MT level in the aquatic organisms.

The Structure and roles of Metallothionein

The study on cadmium binding-protein in equine kidney was firstly published in 1957 by Margoshes and Vallee and further was cited in Kagi and Kojima (1979) and Nordberg (1998). However this experiment was initially published in the form of abstract dealing with the fate of cadmium in human. MT structure has two parts consisting of one cluster with three and another one with four metal atoms as shown in Figure 1 MTs have high content of cysteinyl residues playing as the ligands for metal chelation (Filipovic and Raspor, 2003) thus primary MT structure cannot be stable. MT was characterized based on its high content of sulphur of 4.1% g/dry weight and 2.9% of Cd and 0.6% of Zn. In addition, many researchers reported its molecular weight as 10,000±260 Da. And, its specific absorption was 250 nm because MT was bound by cadmium mercaptide bonds. However, MT was hypothesized to lack of aromatic amino acids because it cannot be measured its absence at 280 nm. After the study on amino acid composition was performed, the results showed that MT has high concentration of sulphur at 8.5%, revealing the high content of cysteine in its molecules (Nordberg, 1998). In Figure 1, the two binding site in fish MT structure (ß cluster and α Cluster) is shown. Capasso et al. (2003) reported that the three-dimension MT structure in Black rockcod (*Notothenia coriiceps*) exposed to cadmium MT is identified by the presence of two domains linked; (1) Lys29-Lys30 comprising residues 31-60 and a four-metal cluster and (2) N-terminal ß domain comprising residues 1-28 and a three-metal cluster.

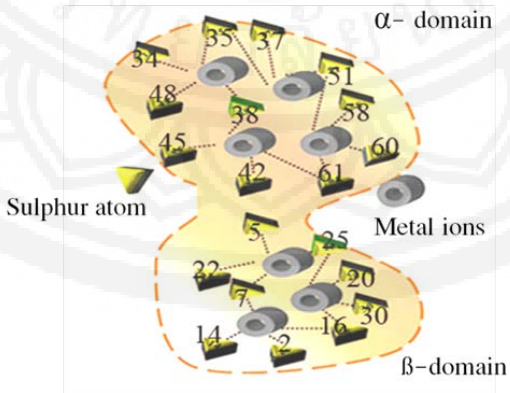

Figure 1 Model of two binding sites of MT in fish (ß cluster and α Cluster) (Adam et al., 2010)

The study on MT structure can be performed by using Nuclear magnetic resonance spectroscopy (NMR) such as MT structure in fish (*N. coriiceps*). Based on the result, it was found super position of the backbone heavy atom (N, C^{α}, and C') of residues 31-60 of the best energy-minimized structures (α domain). The terminal part are indicated by N and C labels (figure 2) (Capasso et al., 2003).

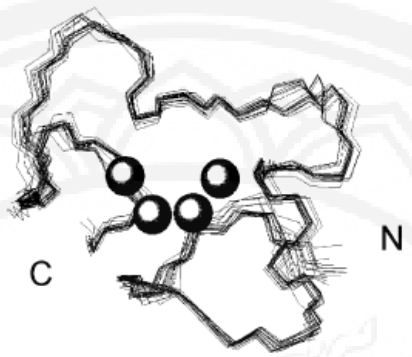

Figure 2 Nuclear magnetic resonance spectroscopy (NMR) structure of MT in fish (*N. coriiceps*) (Capasso et al., 2003)

Many studies reported that MT was found in both intracellular and extracellular compartments. The intracellular pool of Cysteine-rich protein plays the important role as a reservoir of essential nitrogen species i.e. an antagonist of toxic metals and organic molecules, and as a regulator of transcription process. In addition, MT can also be found outside the cells due to the influence of stressors suggesting that this protein may importantly contribute as a danger signal inducing the response of cellular damage management, while conventional knowledge has believed that extracellular MT is the consequence of cell death or leakage from stress response proteins. This finding suggested that MT is selectively released, and additionally the pool of extracellular MT acts as an important regulator for various cellular functions (Klaassen, Lui, & Choudhuri, 1999).

Multiple isoforms of MT have been studied and identified. And, polymorphism shows particularly important roles in invertebrate compared to mammal. The variety of MT molecular mass has also been observed, and also found their monomeric and dimeric forms (Amiard et al., 2006; Langston, Chesman, Burt, Pope, & McEvoy, 2002). Additionally, Honda et al. (2005); Vergani, Grattarola, Dondero, and Viarengo (2003) reported that the purified protein in fish liver could be characterized as MT by the UV spectra at 260 and 254 nm, respectively.

Because of variety of chemical structure, it is essentially classified MT into three classes. For the first, class I MTs are defined to include all polypeptides relating in primary structure to equine renal MT. Metallothionein primary structure has 38 classes including the amino acid sequence of MTs isolated from specific fish (*Pleuronectes platessa*), crab (*Scylla serrata*), lobster (*Homarus americanus*) and oyster (*Crassostrea virginica*). Next, class II MTs have none or only a very distant evolutional corresponding to the mammalian forms such as the MTs from the sea urchin (*Strongylocentrolus purpuratus*) and yeast (*Saccharomyces cerevisiae*). Besides class III MTs comprise γ-glutamyl-cysteinl oligopeptides isolated from plants, the microorganisms (*Euglena gracilis*) and *Schizosaccharomyces pombe* (Huggett, Kimerle, Mehrle, & Bergman, 1992).

The MT behavior is influenced by the thiol (-SH) group. The metal-thiolate cluster within the MT structure results in rapid exchanges of metallic ions in and with other MT molecules. These specific binding and transference of metals is unique to MT and being fundamental to its biological role (Monserrat et al., 2007).

Biological functions of MT cover detoxification of both essential and non-essential metals, homeostasis of physiological important metal (Cu, Zn), and antioxidant defense process (Amiard et al., 2006; Monserrat et al., 2007; Roesijadi, 1992; Viarengo, Burlando, Cerratto, & Panfoli, 2000). In addition, many reports suggested that MT possibly acts as oxyradical scavenger, which can be observed by the high sulfhydryl content in this protein. MT could protect the cells from oxidative stress not only acting as oxyradical scavenger but also binding with toxic metals and excreting (Monserrat et al., 2007; Viarengo et al., 2000).

Many studies reported that MT plays the important role in detoxifying many metal ions such as $Zn^{2+}$and Cu^{2+}. Heavy metal can enter the nucleus through a membrane of cytoplasmic by special transporters and ionic channel. After entering into the cytoplasm, the ion will intereact with the complex of Metal-regulatory Transcription Factor-1 (MTF-1) and Metal-synthesis inhibitor (MTI). The ion further will be bound to MTI. Because of this phenomenon, MTF is then released and bind to a regulatory sequence of DNA that is called metal-responsibility elements (MREs) resulting in MTs synthesis which is transcribed and translated from mRNA precursor. Finally, MT will bind to the heavy metal. However, the metal-MT complex can be transported to the kidney and then be excreted by MT degradation or pH alteration as shown in figure 3. In summary, MTs in both liver and kidney were correlated to detoxification process.

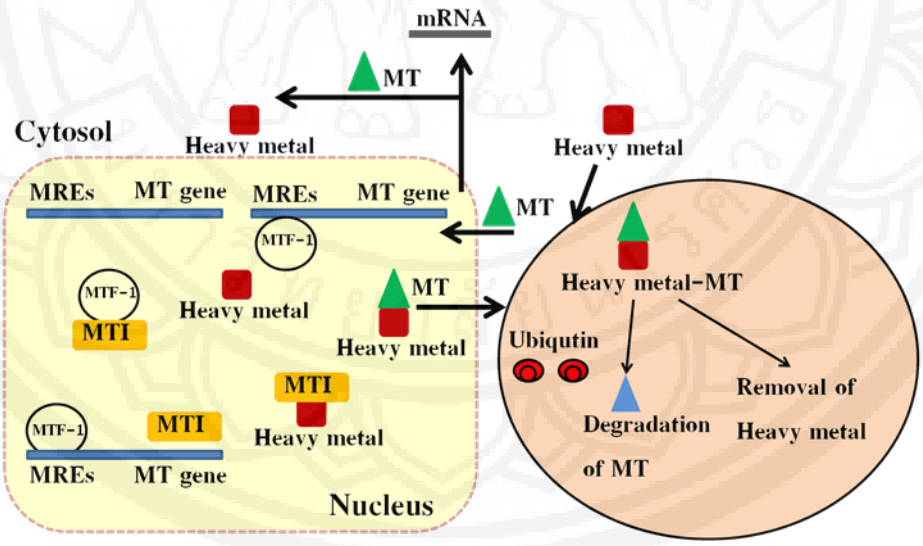

Figure 3 Detoxification process of heavy metal ions by MT (Adam et al., 2010)

In addition, some studies reported that MT played an important role in genotoxicity, carcinologenicity and immune response. Thus, many researchers try to describe the cellular functions of MT for obtaining the accurate analysis of MT isoforms.

Detection and quantification of Metallothionein

Metallothioneins are a potential biomarker for evaluating and monitoring environmental quality. Although, the primary MTs structures are extremely variable and their low molecular mass making MT level measurement not an easy task, many methods of detection and quantification were developed. Adam et al. (2010) suggested that the analytical methods for MTs should be based on detection of bound metal ions, determination of free-SH groups, evaluation of protein mobility in electric field and investigation of the interaction with different types of sorbent. The examples of methods applying in detection and quantification of MT are shown in the Table 1.

For detecting MTs, differential pulse voltammetry with a modification called Brdicka reaction after the founder. It is the most common used electrochemical method to detect MT in various fish tissues (Adam et al., 2010). In addition, there are many ways to detect MT such as antibodies specification (immunoblotting and ELISA) because it is very precise and can be applied to many samples. However, there is a main obstacles in using ELISA and other immunological methods; degradation of the target molecules, cross reactivity with polyclonal antibodies and possible interferences of metal molecule. Thus, these methods are appropriate for qualitative detection of MTs.

Table 1 The examples of methods applying in detection and quantification of MT

Sample assay	Method	Detection limit	References
Crustacean, *Artemia sp.* and *Procanbarus clarkitt*	Silver saturation method	0.03 ppm Ag	Ramo, Torreblanca, Martinez, Pastor, and Diaz-Mayans (1995)
Lake trout (*Salvoclinus namaycus*)	Hg saturation	-	Klaverkamp, Wautier, and Baron, (2000)
Fish liver	IEC-ETAAS (Ion-exchange chromatography-Electrothermal atomic absorption spectrometry)	2 ng/mg protein	Lacorn et al. (2001)
Mussel (*Mytilus galboprovincialis*)	Spectrophotometric	-	Viarengo, Burlando, vangelisti, Mozzone, and Dondero (2001)
Turbot (*Scophthalmus maximus*)	Difference pulse polarography (DPP)	-	Alvarado et al. (2006)
Tilapia (*Oreochromis mossambicus*)	ELISA	-	Wu, Shih, and Ho (2007)
Perch (Perca fluviatilis)	Electrochemical	-	Krizkova et al. (2007)
Lithognathus mormyrus	ELISA	-	Yudkovski et al. (2008)
Fish liver	Difference pulse polarography (DPP)	-	Siscar, Koenig, Torreblanca, and Solé (2013)
Fish (liver, gill and kidney)	Differential pulse voltammetry Brdicka reaction	-	Sevcikova et al. (2013)

Recently, many studies suggested that biomarkers based on mRNA expression levels can only reflect to the initial step of a cascade of the phenomenons linking to ecological factor alterations (Kessabi et al., 2010). Therefore, the molecular technique based on Polymerase Chain Reaction (PCR) had a potential to quantify MT-mRNA expression levels. Cho et al. (2009) indicated that MT could be isolated from mud loach (*Misgurnus mizolepis*). The MTs isolated from fish liver have 2 isoforms: MLMT-IA and MLMT-IB. From the PCR analysis, both MLMT-IA and MLMT-IB isoforms had a tripartite structure. The sequence identity at the genomic levels showed that there were 88% similarity between MLMT-IA and MTMT-IB. The sequence characteristic of the mud loach (*M. mizolepis*) is shown in Figure 4.

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                          * *      * *   * *   * * *     ** **    *  *   * *     *   **
Misgurnus mizolepis MT-IA MDPCDCSKTG TCNCGATCKC TNCKCTTCKK -SCCSCCPSG CSKCASGCVC KGNSCDSSCC Q (FJ015060)
Misgurnus mizolepis MT-IB .......... .......... ...Q...... -......... .......... ...T...... . (FJ015059)
Carassius cuvieri MT-A    ......A... P......... ...Q...... -......... .......... .....G.... . (AY165047)
Carassius cuvieri MT-B    ......A... A......... ...Q.K.... -...P..... .......... .D...G.... . (AY165048)
Cyprinus carpio MT-1      ......A... .......... ...Q.K.... -...P..... .......... .....G.... . (AY789469)
Cyprinus carpio MT-2      ......A... .......... ...Q...... -......... .......... .....G.... . (AY789468)
Gobio gobio MT-1          ......A... .......... ...Q...... -...P..... .......... .....G.... . (AY953544)
Gobio gobio MT-2          ......A... .......... ...Q...... -...P..... .......... .D...G.... . (AY953546)
Danio rerio MT-IIA        ....E.A... .......... ...Q...... -......... .......... .....G.... . (EU877072)
Danio rerio MT-IIB        ....E.A... A......... ...Q...G.. -......... .......... .....GT... . (EU877073)
Oncorhynchus mykiss MT-A  ....E..... S....GS... S..A..S... AS..D..... .......... ..K...T... . (M81800)
Oncorhynchus mykiss MT-B  ....E..... S....GS... S..A..S... -...P....D .......... ..K...T... . (M22487)
Salvelinus alpinus MT-A   ....E..... S....GS... S..A..S... AS..D..... .......... ..K...T... . (AF013800)
Salvelinus alpinus MT-B   ....E..... S....GS... S..A..S... -...P....D .......... ..K...T... . (AF013801)
Chionodraco hamatus MT-I  ........S. .....GS.T. ...S.KS... -...P..... .T........ ..K...T... . (Y12580)
Chionodraco hamatus MT-II ........S. .....GS.T. ...S..S... -...P..... .T........ ..K...T... . (Y12581)
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Figure 4 Sequence characteristic of the mud loach (*M. mizolepis*) (Cho et al., 2009)

Additionally, Kessabi et al. (2010) investigated MT mRNA expression in liver of killfish (*Aphanius fasciatus*) after exposed to cadmium by using quantitative RNA biomarker and found that the MT mRNA expression level increased approximately 200 folds after 24 h of exposure, and then the expression level deceased 80% after 48 h. Moreover, the study on the expression of MT mRNA can be applied to assess in field study. Montaser et al. (2010) reported that MT mRNA expression level could be also applied in the field study on Sleek unicorn fish (*Naso hexacantus*) at Jeddah Coast. In reference area, MT mRNA expression level was only 0.95±0.05 compared to 2.4±1.02 in the contaminated area, in conclusion, contaminated area was 3 folds higher than that in reference area.

As mentioned above, MT mRNA expression level can be used as biomarker for evaluating metal contamination in aquatic environment, however, Kessabi et al. (2010) recommended that the obvious disadvantages on the use of mRNA marker in environmental monitoring is require reference gene of the impacted species. Thus, gene expression observed should be firmly supported by the data from biochemical, immunochemical, morphological and pathological studies.

Application Metallothionein (MT) as biomarker to evaluate heavy metal contamination in aquatic environment and its current status in Thailand

At first, the aquatic ecosystem was chosen to study on environmental monitoring of metals contamination and released MT levels in organisms. Roesijadi et al. (1992) reported that at least 80 species of aquatic organisms not only fish but also vertebrates, had MT protein though MT could be induced by various factors. In present, heavy metal especially divalent metals is mainly research focus on induced MT structure and functions. The various

forms of MTs found in a single species indicate that each MT form regulates and functions responding to an individual form of xenobiotic (Roesijadi, 1992).

In Thailand, the study on MT application is inadequate which may be limited by the essential devices and MT structure is very small and complex. Moreover, MT is very difficult to being extracted and the study requires multidisciplinary. In Thailand, the application of MT in aquatic organism as biomarker can be used to monitor heavy metal contamination, for examples, molecular technique and immunological technique. Chinda (2003) applied MT as biomarker to test mercury contamination in shellfish. The study particularly performed in the digestive tract of the mussels which plays an important role in MT distribution. The results of reverse transcription polymerase chain reaction analysis indicated that there was a significant difference between treatments and control in digestive tract MT mRNA after exposed to mercury within 2 to 4 weeks. Mercury chloride ($HgCl_2$) at the concentration of 1 mg/L could significantly induce MT mRNA production compared to the control. In addition, this study also investigated the differentiation of MT forms in mercury-exposed mussels. Six isoforms of MT were identified by single strand conformation polymorphism (SSCP) method. This finding indicated that MT mRNA production in digestive gland of mussel was induced by mercury exposure, thus this responding mechanism could be used in the assessment of mercury contamination in marine environment. Besides, Barnette et al. (2013) suggested that MT could be used as bioindicator for heavy metal contamination in Ang Sila subdistrict, Chon buri province and Map ta put industrial estate, Rayong province. It was found that MT expression (size 10 kDa) was 10 out of 60 samples (16.6%) in the mussel collected from Ang Sila compared with 34 out of 60 samples (56.7%) from Map ta put. For fish, they can be categorized by the consumption behavior in the trophic levels of carnivore, omnivore and herbivore, however there was no significantly influence on the expression of CYP1A and MT. In addition, Thanomsit et al. (2013) reported that MT and cDNA were also isolated from Asian sea bass (*Lates calcarifer*) liver. Immunochemical assessments such as enzyme-linked immunosorbent assay (ELISA) and Western blot were developed to quantify MT level in Asian sea bass. These assessments could be applied to measure MT level in 15 different feral fish species in two different areas: urban area (Ang Sila) and industrial area (Map ta put), in the Gulf of Thailand. The MT protein band was founds in Shrimp scad (*Alepes djadaba*) and Indian ilisha (*Ilisha melastoma*) collected from both areas. The higher MT expressions observed in Shrimp scad (*A.djadaba*) from cadmium-contaminated areas in both near shore and off shore. These finding indicated that hepatic MT immunoreactive proteins induction expressed in the Shrimp scad (*A.djadaba*) may be an appropriate early warning signal for heavy metal exposure in environment in Thai waters.

Conclusion

Metallothioneins (MTs) are the protein induced after the organism exposed to heavy metal. Thus, it can be applied as bioindicator for heavy metal exposure especially for aquatic organism such as fishes. For the measurement of MT, it can be performed in both qualitative and quantitative such as immunological analysis, chemical analysis, and molecular technique. However, the study on MT which is depended on the objective is limited in Thailand by devices, samples, and time. These technique aiming to apply in monitoring heavy metal contamination should be developed for obtaining the appropriate method.

References

Adam, V., Fabrik, I., Eckchlager, T., Stiborova, M., Trnkova, L., & Kizek, R. (2010). Vertebrate metallthioneins as target molecules for analytical technique. *Trends in Analytical Chemistry, 29(5)*, 409-418.

Adhikari, S., Ghosh, L., Giri, B., & Ayyappan, S. (2009). Distributions of metals in the food web of fishponds of Kolleru Lake, India. *Ecotoxicology and Environmental Safety, 72*, 1242-1248.

Alvarado, N. E., Quesada, I., Hylland, K., Marigomez, I., & Sato, M. (2006). Quantitative changes in metallothionein in target cell-types in the gills of turbot (*Scopthalmus maximus*) exposed to Cd, Cu, Zn, and after a depuration treatment. *Aquatic toxicology*, 77, 64-77.

Amiard, J. C., Amiard-Triquet, C., Barka, S., Pellerin, J., & Rainbow, P. S. (2006). Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquatic toxicology, 76*, 160-170.

Andrew, G. K. (2000). Regulation of metallothionein gene expression by oxidative stress and metals ions. *Biochemistry and Pharmacology, 59*, 95-104.

Barnette, P., Panutrakul. S., Nanthanawat, P., Pattarabuddha, N., Maharachpong, N., Mokkongpai, P., & Celender, M. C. (2013). *Assessments of heavy metals and organic hydrocarbons exposure in selected marine animals from coastal industrial area at Map Ta Phut, Rayoung Province.* Burapha University: Chonburi.

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

Castro-González, M. I., & Méndez -Armenta, M. (2008). Heavy metals: Implications associated to fish consumption. *Ecotoxicology and Environmental Safety, 26*, 263-271.

Campenhout, K. V., Infante, H. G., Hoff, P. T., Moens, L., Goemans, G., Belpaire, C., ... Bervoets. (2004). Cytosolic distribution of Cd, Cu, and Zn and metallothionein levels in relation to physiological changes in gibel carp (*Carasius auratus gibelio*). *Ecotoxicology and Environmental Safety, 73*, 296-305.

Chinda, P. (2003). *Metallothionein as a biomarker for mercury contamination in mussel, Perna viridis.* Chulalongkorn University: Bangkok.

Cho, Y. S., Lee, S. Y., Kim, K. Y., & Nam, Y. K. (2009). Two metallothionein genes from mud loach *Misgurnus mizolepis* (Teleostei; Cypriniformes): gene structure, genomic organization, and mRNA expression analysis. *Comparative Biochemistry and Physiology: Biochemistry Molecular Biology, 153(4)*, 317-326.

Filipovic, V., & Raspor, B. (2003). Metallothionein and metal levels in cytosol of liver, kidney and brain in relation to growth parameters of *Mullus surmuletus* and *Liza aurata* from the Eastern Adriatic Sea. *Water Research, 37*, 3253-3262.

Hogstrand, C., & Haux, C. (1991). Binding and detoxification of heavy metals in Lower vertebrates with references to metallothionein. *Comparative Biochemical and Physiological, 113C(2)*, 109-115.

Honda, R. T., Araujo, R. M., Horta, B. B., Val, A. L., & Demasi, M. (2005). One-step purification of metallothionein extracted from two different sources. *Journal of Chromatography, 100C(1/2)*, 137-141.

Huggett, R. J., Kimerle, R. A., Mehrle, P. M., & Bergman, H. L. (1992). *Biomarkers Biochmical, physiological, and histological marker of anthropogenic stress*. Lewis: USA.

Kagi, J. H. R., & Kojima. (1979). Nomenclature of metallothionein: a proposal. *Experientia, Supplementum, 34*, 141-142.

Kessabi, K., Navarro, A., Casado, M., Said, K., Messaoudi, I., & Pina, B. (2010). Evaluation of environmental impact on natural population of the Mediterrenean killifish *Aphanius fasciatus* by quantitative RNA biomarker. *Marine Environmental Research, 70*, 327-333.

Kim, J., Wang, S., Kim, I., Ki, J., Raisuddin, S., Lee, J., & Han, K. (2008). Cloning of river pufferfish (*Takifugu obscurrus*) metallothionein cDNA and study of its induction profile in cadmium-exposed fish. *Chemosphere, 71*, 1251-1259

Klaassen, C. D., Lui, J., & Choudhuri, S. (1999). Metallothionein: An intracellular protein to protect against cadmium toxicity. *Annual Review in Phamacology, 39*, 267-294.

Klaverkamp, J. F., Wautier, K., & Baron, C. L. (2000). A modified mercury saturation assay for measuring metallothionein. *Aquaculture Toxicology, 50*, 13-25.

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

Kovarova, J., Kizek, A. V., Harustiakova, D., Celechovska, O., & Svobodova, Z. (2005). Effect of cadmium chloride on metallothionein levels in Carp. *Sensor, 9*, 4789-4803.

Krizkova, S., Zitka, O., Adam, V., Beklova, M., Horna, A., Svobodova, Z., ... Kizek, R . (2007). Possibilities of electrochemical techniques in metallothionein and lead detection in fish tissues. *Czech Journal of Animal Science, 52(5)*, 143-148.

Langston, W. J., Chesman, B. S., Burt, G. R., Pope, N. D., & McEvoy, J. (2002). Metallothionein in liver of eels *Anguilla Anguilla* from the Thames Estuary: an indicator of environmental quality? *Marine Environmental Research, 53*, 263-293.

Linde, A. R., Sanchez-Galan, S., Valles-Mota, P., & Garcia-Vazquez, E. (2001). Metallothionein as bioindicator of freshwater metal pollution: European eel and Brown trout. *Ecotoxicology and Environmental safety, 49*, 60-66.

Lacorn, M., Lahrssen, A., Rotzoll, N., Simat, T. J., & Steinhart, H. (2001). Quantification of metallothionein isoforms in fish liver and its implications for biomornitoring. *Environmental Toxicology and Chemistry, 20(1)*, 140-145.

Livingstone, D. R. (1993). Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology, 57*, 195-211.

Monserrat, J. M., Martinez, P. E., Geracitano, L. A., Amado, L. L., Martins, C. M. G., Pinho, G. L. L., ... Bianchini, A. (2007). Pollution biomarkers in estuarine animals: Critical review and new perspectives. *Comparative Biochemistry and Physiology Part C, 146*, 221-234.

Montaser, M., Mahfouz, M. E., El-Shazly, S. A. M., Abdel-Rahman, G. H., & Bakry, S. (2010). Toxicity of heavy metals on fish at Jeddah coast KSA: Metallothionein expression as a biomarker and histopathological study on liver and gills. *World Journal of fish and marine Science, 2(3)*, 174-185.

Nordberg, M. (1998). Metallothioneins: historical review and state of knowledge. *Talanta, 46*, 243-254.

Olsvik, P. A., Hindar, K., Zachariassen, K. E., & Andersen, R. A. (2001). Brown trout, *Salmo trutta*, metallothioneins as biomarkers for metal exposure in two Norwegian. *Biomarkers, 6(4)*, 274-288.

Oost, V. D., Beyer, R., Vermeulen, J., & Nico, P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology, 13*, 57-149.

Peakall, D. A. (1994). The role of biomarkers in environmental assessment. *Ecotoxicology, 3*, 157-160.
Ramo, J. D., Torreblanca, A., Martinez, M., Pastor, A., & Diaz-Mayans, J. (1995). Quantification of cadmium-induced metallothionein in crustaceans by the silver-saturation method. *Comparative Biochemistry and physiology, 141(C)*, 303-314.

Roesijadi, G. (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology, 22*, 81-114.

Rotchell, J. M., Clarke, K. R., Newton, L. C., & Bird, D. J. (2001). Hepatic metallothionein as a biomarker for metal contamination: age effects and seasonal variation in European flounders *(Pleuronectes flesus)* from the seven estuary and Bristol channel. *Marine Environmental Research, 52*, 151-171.

Sevcikova, M., Modra, H., Kruzikova, K., Zitka, O., Hynek, D., Adam, V., ... Svobodova, Z. (2013). Effect of Metals on Metallothionein Content in Fish from Skalka and Želivka Reservoirs. *International Journal of Electrochemical Science, 8*, 1650 – 1663.

Siscar, R., Koenig, S., Torreblanca, A., & Solé, M. (2013). The role of Metallothionein and selenium in metal detoxification in the liver of deep-sea fish from the NW Mediterranean Sea. *Science of the Total Environment, 1*(466-467), 898-905.

Stegeman, J. J., Brouwer, M., Digiulio, R. T., Forlin, L., Fowler, B. M., Sanders, B. M., & Van, P. A. (1992). *Molecular responses to environmental contamination: enzyme and protein systems as indicators of contaminant exposure and effect*. Lewis: USA.

Thanomsit, C., Nantanawat, P. Wassmur, B., Grans, J., Calander, M., & Barnette, P. (2013). Characterization of metallothionein from Asian sea bass (*Lates calcarifer*, Bloch) and application as a biomarker for heavy metal exposure in Thailand. *Asian journal of water, Environment and pollution, 10(4)*, 53-64.

Vergani, L., Grattarola, M., Dondero, F., & Viarengo, A. (2003). Expression, purification and characterization of metallothionein-A from rainbow trout. *Protein Expression and Purification, 27(2)*, 338-345.

Viarengo, A., Burlando, B., Cerratto, B., & Panfoli, I. (2000). Antioxidant role of metallothioneins: a comparative overview. *Cellular and Molecular Biology*, *46*, 407-417.

Viarengo, A., Burlando, B., Evangelisti, V., Mozzone, S., & Dondero, F. (2001). Sensitivity and specificity of metallothionein as a biomarker for aquatic environment biomornitoring. *Biomarker in Marine Organism: A Practical Approach*, 29-43.

Walker, C. H., Hopkin, S. P., Sibly, R. M., & Peakall, D. B. (2006). *Principle of Ecotoxicology*. Taylor & Francis: USA.

Wu, S. M., Shih, M., & Ho, Y. (2007). Toxicological stress response and cadmium distribution in hybrid tilapia (*Oreochromis sp.*) upon cadmium exposure. *Comparative Biochemistry and Physiology, Part C*, *145*, 218-226.

Yudkovski, Y., Rogowska-Wrzesinska, A., Yankelevich, I., Shefer, E., Herut, B., & Tom, M. (2008). Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium. *Marine Environmental Research*, *65*, 427-436.