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Characterization of Metallothionein from Asian Sea Bass (*Lates calcarifer,* Bloch) and Application as a Biomarker for Heavy Metal Exposure in Thailand

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Abstract: In the present study, metallothionein (MT) proteins and cDNA were isolated from Asian sea bass (*Lates calcarifer*) livers. Immunochemical protocols (i.e. an enzyme-linked immunosorbent assay (ELISA) and Western blot) were developed for quantification of MT protein levels in Asian sea bass. These were applied to analyze MT protein levels in 15 different feral fish species from two different areas, one urban area Angsila and one industrial area Map Ta Phut in the Gulf of Thailand. An MT protein band was detected in Shrimp scad (*Alepes djadaba*) and Indian ilisha (*Ilisha melastoma*) from both areas. Higher MT protein expressions were seen in shrimp scad from cadmium-contaminated areas near shore as well as off shore. These results suggest that induction of hepatic MT immunoreactive proteins in the shrimp scad may be a good early warning signal for heavy metal exposure in environmental monitoring programmes in Thailand waters. Protocol for quantification of MT mRNA levels in Asian sea bass was developed using quantitative PCR. Asian sea bass were exposed in the lab to different doses of $CdNO_3$ and sampled at different times after injection. There appears to be a bi-phasic dose-response pattern with highest MT mRNA levels in fish injected with 4 mg $CdNO_3$/kg. In these fish, the highest expression was seen after one day and lowest induction after three days. These results suggest that induction of MT mRNA levels in Asian sea bass liver can be used as a sensitive early warning signal for cadmium exposure in tropical waters.

Key words: Metallothionein, *Lates calcarifer*, cadmium, fish, tropical waters.

Introduction

The industrial development affects the environment by increased chemical contaminations where most chemicals end up in the aquatic environment. This includes organic chemicals as well as metals. Exposure to metals can lead to harmful effects in fish and other aquatic organisms, either as a result of direct exposure or indirect exposure via the food chain. Human consumption of fish from areas exposed to metals can result in adverse health effects (Castro-González and Méndez-Armenta, 2008). Hence, it is important to monitor levels of metals in the aquatic environment. However, heavy metals detected in water and sediment samples may not reflect heavy metal contamination in fish as the composition of different metal ions may be

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different in the environment compared to that in fish (Adhikari et al., 2009). For example, cadmium can be biomagnified in the aquatic food chain (Ruangsomboon and Wangrat, 2006). Besides, the ionic form of Cd is usually present as oxygen (CdO_2), chloride ($CdCl_2$) or sulphur ($CdSO_4$) salts in the environment. In seafood, the $CdCl_2$ is the most dominant form, because of its higher bioavailability (Castro-González and Méndez-Armenta, 2008). Environmental exposures to heavy metals can be assessed by analyzing metallothionein (MT) levels in animals. There is generally a positive correlation between MT levels and heavy metal levels where Cd^{2+} is the strongest inducer of MT (Klaasssen et al., 1999) and causes most toxic effects in fish and mammals (Okocha and Adedeji, 2011). Consequently, induction of MT in fish has been widely used as a biomarker for metal exposures in temperate waters (e.g. Lacorn et al., 2001; Linde et al., 2001; Cheung et al., 2004).

The MT proteins are found in both prokaryotic and eukaryotic organisms and they are small water-soluble proteins of 6-10 kDalton, containing 30% cysteine. Due to the high sulphur content they are able to selectively bind to metal ions such as Cd, Zn and Cu (Klaasssen et al., 1999). Although the physiological functions of MTs have not yet been fully understood, MT proteins are supposed to be primarily involved in metal homeostasis and in metal detoxification in pufferfish, *Takifugu obscurrus* (Kim et al., 2008).

There are different methods to study MT proteins in fish. It has been suggested that the UV spectra and absorbance at 260 nm can be used to confirm that Cd^{2+} binds to MT proteins in rainbow trout (*Oncorhynchus mykiss*) and the Amazon fish (*Colossoma macroponum*) (Vergani et al., 2003; Honda et al., 2005). Levels of MT protein levels can be assessed using immunochemical techniques and specific antibodies to MT proteins. Since MT proteins are small, relatively high MT protein levels are needed as antigen for efficient antibody production. A one-step purification using affinity chromatography protocol was described for rapid isolation of MT proteins from the Amazon fish (Honda et al., 2005). An enzyme-linked immunosorbent assay (ELISA) was developed to analyze MT protein levels in hybrid tilapia exposed to Cd^{2+}, where elevated hepatic MT levels were observed 15 days after exposure (Wu et al., 2007). Furthermore, ELISA and Western blot protocols have been used to determine MT protein levels in *Lithognathus mormyrus* collected from two Cd^{2+} contaminated sites in the Mediterranean Sea. Fish from the two contaminated sites had 2-fold higher MT protein levels compared to fish collected from a clean site (Yodkovski et al., 2008).

Levels of MT mRNA can be characterized using various molecular techniques. Determination of MT transcription levels using molecular technique has been earlier used, because it is a specific and sensitive tool and can be used as an early warning signal for metal contaminations in the environment. Hence, a number of real-time PCR protocols have been developed to analyze MT mRNA levels in several fish species, e.g. striped sea bream (*Lithognathus mormyrus*), tilapia (*Orechromis mossambicus*), gudgeons (*Gobio gobio*) and sleek unicorn fish (*Naso hexacanthus*) (Tom et al., 2004; Cheung et al., 2005; Knapen et al., 2007; Monster et al., 2010).

The aim of the present study was to isolate hepatic MT proteins from Asian sea bass *(Lates calcarifer*) exposed to $CdNO_3$ in the lab and to develop specific protocols to detect MT mRNA and protein levels using quantitative reverse transcriptase polymerase chain reaction (qPCR) and ELISA. The immunochemical protocols were next applied to determine induction of MT in feral fish caught from two different locations: one urban aquacultural area (Angsila, Chonburi Province) and one industrial area (Map Ta Phut, Rayong Province) in Thailand.

Materials and Methods

Chemicals

Cadmium nitrate was purchased from Unilab (Australia). The DNA gel extraction kit was purchased from VWR (Sweden), RT-PCR kit was from Applied Biosystems (Sweden) and DNA size marker was from Fermentas (Sweden). The RNAeasy plus mini kit was obtained from Qiagen (Sweden). The iScript cDNA synthesis kit and IQSYBR green supermix used for qPCR analysis were from BioRad (Sweden). Electrophoresis chemicals and affinity column chromatography chemicals were purchased from BioRad Inc. and Amersham Inc. (Thailand). All other chemicals used were obtained at the highest purity available from chemical suppliers in Thailand and Sweden.

Animals

Hatchery Reared Asian Sea Bass

Juvenile Asian sea bass were purchased from Angsila, Chonburi Province, Thailand and transported to the Burapha University. The animals were maintained in 2000 L tanks with aerated fresh water at a temperature of 28 °C and acclimated to these conditions for seven

days and were starved during the acclimation period. Five fish were used in each treatment group. For MT protein isolation, fish with an average body weight of 112 ±0.5 g and length of 15 ±1.1 cm were used. The big size of fish was needed to obtain enough amount of liver in order to yield MT protein. The fish were i.p. injected with $CdNO_3$ to reach a final dose of 4 mg/kg fish which was an optimal dose to induce MT protein comparing to 2 and 6 mg/kg fish. The $CdNO_3$ was dissolved (6.4 mg/ml) in 50 mM sodium phosphate buffer pH 7.4. For MT cDNA isolation, fish with an average body weight of 28 ±1.5 g and length of 8 ±1.1 cm were used. The fish were i.p. injected with $CdNO_3$ to reach final doses of 2, 4 and 6 mg/kg fish, respectively. Control fish were i.p. injected with the vehicle carrier alone (1 ml/kg fish). The fish were kept in 20 L aquaria with aerated fresh water at a temperature of 28 °C and were starved during the exposure experiment. The fish were sacrificed by cervical transections after two days for MT protein analysis and after 1, 2 and 3 days post injection for MT mRNA analyses. The livers were quickly dissected out, immediately placed in liquid nitrogen and stored for two days prior to further processing.

Feral Fish

Fish were caught in year 2011 with hook fishing at Angsila and Map Ta Phut in Chonburi and Rayong provinces in the Gulf of Thailand (Figure 1). In total, 15 different species (n = 2 to 90) were collected. The fish were immediately sacrificed by cervical transections and their livers were quickly dissected out and placed and stored in liquid nitrogen prior to analyses. Information about the fish (species name, dietary preferences, weight and length) is provided in Table 1.

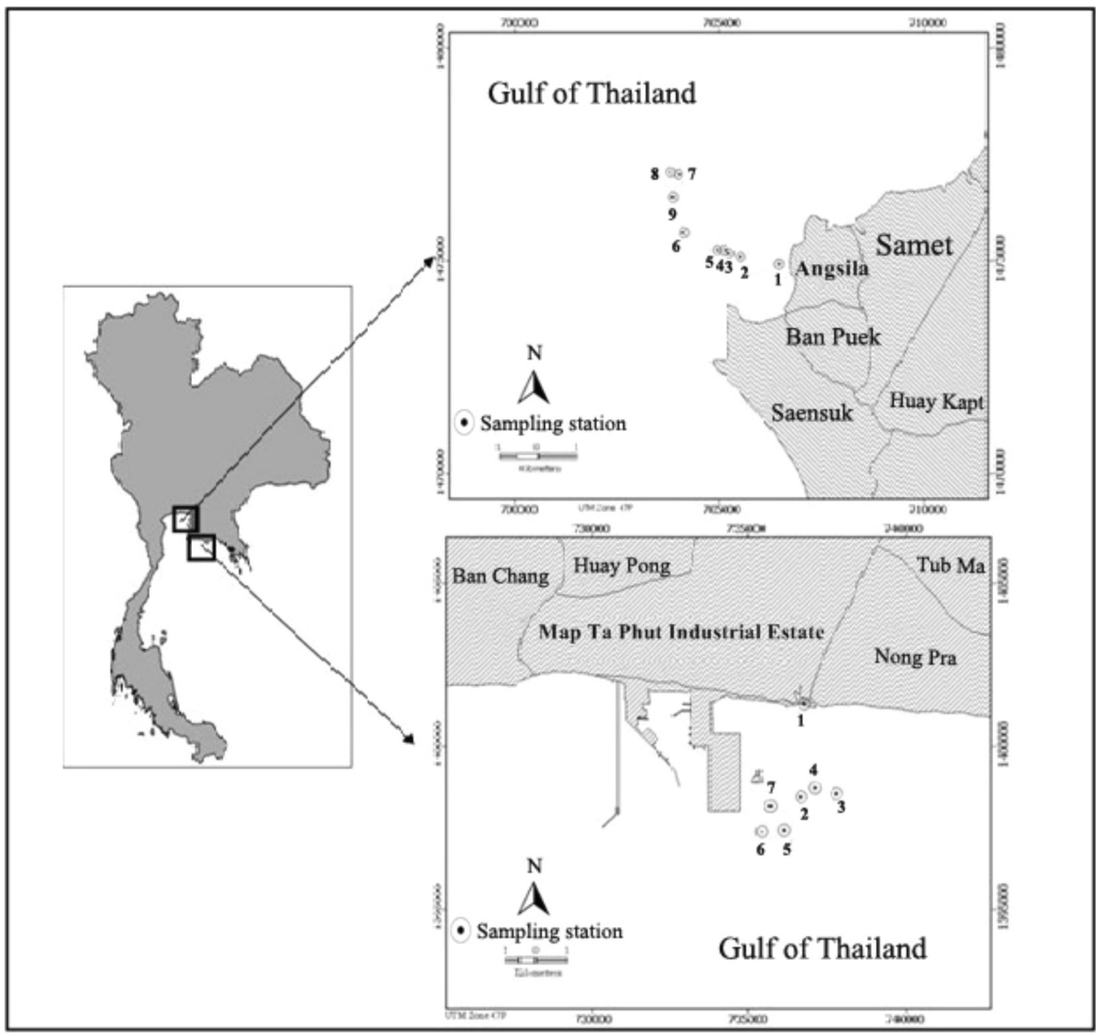

Figure 1: Map of the Chonburi province and Rayong province in the Gulf of Thailand. The four sampling sites are marked on the map. Site 1: Angsila (Near shore; 1-5), Site 2: Angsila (Off shore; 6-9), Site 3: Map Ta Phut (Near shore; 1-4) and Site 4: Map Ta Phut (Off shore; 5-7).

Table 1: Western blot and ELISA analyses using mouse PAb raised against Asian sea bass MT in liver of feral fish caught from four different sites that are shown in Figure 1

Sampling sites	*Species*	*Dietary preferences*	*Weight/Length*	*MT staining (Western blot)*	*Optical density of MT protein (ELISA)*
Site 1 Angsila (Near shore)	*J. carouna* (*n* = 8)	Carnivore	73.6-199.7g/ 18.0-25.5cm.	–	0.09±0.02^{A}
	A. djedaba (*n* = 20)	Carnivore	46.8-93.0 g/ 15.0-17.0cm.	++	0.39±0.26^{B}
	S. jello (*n* = 2)	Carnivore	618.5-639.6 g/ 51.0-51.2 cm	–	0.05±0.02^{A}
Site 2 Angsila (Off shore)	*A. djedaba* (*n* = 60)	Carnivore	53.8-97.8 g/ 15.3-18.3 cm.	++	0.16±0.11^{A}
Site 3 Map Ta Phut (Near shore)	*A. djedaba* (*n* = 60)	Carnivore	10.2-43.2 g/ 10.5-16.8cm.	++	0.41±0.05^{B}
	M. cordyla (*n* = 40)	Carnivore	22.6-40.0 g/ 13.0-16.8 cm	–	0.06±0.04^{A}
	I. melastoma (*n* = 60)	Carnivore	15.0-44.6 g/ 8.7-17.5 cm.	++	0.32±0.2^{B}
	L. fasciatus (*n* = 90)	Carnivore	10.6-29.2.6 g/ 7.5-13.0 cm.	–	0.07±0.02^{A}
	S. melanochi (*n* = 60)	Carnivore	33.2.0-79.4 g/ 16.0-21.7 cm.	–	0.07±0.03^{A}
	S. maculate (*n* = 40)	Carnivore	32.4-76.8 g/ 14.0-16.6 cm.	–	0.07±0.02^{A}
	R. brachysoma (*n* = 90)	Carnivore	18.2-44.4 g/ 12.5-15.4 cm	–	0.12±0.06^{A}
	S. commersoni (*n* = 4)	Carnivore	33.8-97.4 g/ 16.3-25.0 cm.	–	0.04±0.02^{A}
	A. dorab (*n* = 4)	Carnivore	180.0-350.0 g/ 38.0-48.0 cm	–	0.06±0.01^{A}
	A. chacunda (*n* = 10)	Scarvenger	46.6-60.8 g/ 14.8-16.0 cm	–	0.05±0.01^{A}
	T. lepturus (*n* = 10)	Carnivore	125.0-240.0 g/ 52.0-64.0 cm	–	0.06±0.01^{A}
Site 4 Map Ta Phut (Off shore)	*A. djedaba* (*n* = 30)	Carnivore	24.1-48.6g/ 11.5-14.5 cm	–	0.07±0.02^{A}
	S .jello (*n* = 2)	Carnivore	65.0-335.0g/ 21.0- 42.0 cm	–	0.09±0.01^{A}
	A. indica (*n* = 5)	Carnivore	45.5-145.0 g/ 11.5-22.0 cm	–	0.04±0.00^{A}
	L. blochii (*n* = 30)	Omnivore	14.4-26.4 g/ 9.0-11.5 cm	–	0.08±0.00^{A}

- No intensity; + Weak intensity; ++ Strong intensity: Mean ± SD, $p < 0.05$

Comparison of optic density at 490 nm using ELISA detection on feral fish from Angsila and Map Ta Phut areas were analyzed by one way ANOVA with SNK's comparison. Different letters (A and B) indicate a significant difference ($p < 0.05$). Each value represents mean optical density of each fish species ± SE.

Isolation of MT Protein from Asian Sea Bass Liver

Livers from individual fish were homogenized (35% w/v) in 50 mM Tris-HCl, pH 7.4; containing 0.1 mM PMSF, 0.5 mM DTT and 150 mM NaCl in a glass-teflon homogenizer. The homogenate was centrifuged at 15000 g for 90 min at 4 °C and the supernatant was immediately applied to a HiTrap™ Chelating HP column saturated with $NiSO_4.6H_20$. The MT fraction was eluted using a continuous gradient imidazol (0-50 mM) dissolved in 50 mM Tris-HCl pH, 7.4, at a flow rate of 1 ml/min. Fractions of 1.5 ml were collected and each fraction was next detected by separation using SDS-PAGE and 15% acrylamide according to standard protocols. The protein bands were stained with Coomassie Brilliant Blue R-250. The MT positive fractions were further analyzed using an electro-eluter and the MT proteins were stored at –20 °C. The protein concentrations in the MT containing fractions were determined using the Bradford reagent method using bovine serum albumin as a protein standard and analyzed according to the protocol provided by the manufacturers. The purified MT protein fraction was characterized by analyzing the absorbance at 260 nm after adding increasing concentrations of $CdNO_3$ from 8, 16, 24, 32, 40, 48, 56, 64, 72 to 80 µM. The A_{260} absorbances were plotted against $CdNO_3$ concentrations.

Production of Polyclonal Antibodies against Asian Sea Bass MT

Four ICR male mice of eight weeks old and 25 g of body weight were i.p. injected with an initial dose of 300 µg purified MT protein from Asian sea bass liver mixed with complete Freund's adjuvant. Next, booster doses each with 300 µg MT protein mixed with incomplete Freund's adjuvant were i.p. injected after two and four weeks, respectively. Seven days after the final antigen boost, the mice were bled from the tail vein to obtain polyclonal antibodies (PAb). Serum were collected and stored at –20 °C. The specificity of the PAb to MT proteins was determined using Western blot and ELISA analyses (data not shown).

Isolation of an MT cDNA from Asian Sea Bass Liver

Pieces of liver tissues of about 30 mg were quickly dissected out and immediately placed in RNA *later* solution (Sigma, code No. R0901, purchased in Thailand) and transported to the University of Gothenburg in Sweden. Preserved liver tissue, carried by the air plane which was spending around 15 hours at room temperature, samples were immediate placed in –20 °C upon arrival to Sweden. The total RNA fraction was isolated using the RNeasy plus mini kit with DNA elimination. The concentration and quality of the RNA samples were analyzed using the Experion system and RNA StdSens analysis kit from BioRad. The RNA samples with acceptable concentrations and quality for PCR analysis were selected and stored at –80 °C. Total RNA (1 µg) was reversed transcribed using the PCR kit from applied Biosystems and universal primers.

The PCR was next carried out using primers designed against conserved regions of MT genes obtained from GenBank from four different fish species (i.e. killifish, Japanese medaka, zebrafish and rainbow trout). The forward primer was 5′-ATG GA(C/T) CC(C/T) TG(C/T) GA(A/C/G) TGC-3′ and the reverse primer was 5′-CAC (G/A)CA GCC (T/A)GA (G/T/A)GC (G/A)CA-3′. Three different annealing temperatures were tested, 45 °C, 50 °C and 55 °C, respectively. The samples were first denaturated at 94 °C for 5 min followed by 30 cycles [denaturation at 95 °C for 30 s, annealing at 45 °C, 50 °C or 55 °C for 30 s, polymerization at 72 °C for 45 s]. The PCR products were analyzed on 2% agarose gels stained with ethidium bromide and visualized in UV light. The PCR bands were excised from the agarose gel and the DNA were purified using a Gel extraction kit from Omega Biotek. The DNA samples were sequenced by Eurofins MWG Gmbt (Ebersberg, Germany). A 150 base pair long cDNA sequence was obtained and positive identification to MT was confirmed using a BLAST search against NCBI GenBank database.

Phylogenetic Analysis

The deduced amino acid sequence of the partial MT cDNA sequence from Asian sea bass was aligned using Clustal X2 with MT sequences from 13 fish species, i.e. Java medaka (*Oryzias javanicus*), Indian medaka (*Oryzias melastigma*) MT, Japanese medaka (*Oryzias latipes*) MT, gold fish (*Carassius auratus*) MT, chum salmon (*Oncorhynchus keta*) MT A, rainbow trout (*Oncorhynchus mykiss*) MT A, pink salmon (*Oncorhynchus gorbuscha*) MT A, coho salmon (*Oncorhynchus kisutch*) MT A, arctic char (*Salvelinus alpinus*) MT A, European grayling (*Thymallus thymallus*) MT A, chinook salmon (*Oncorhynchus tshawytscha*) MT A, Atlantic salmon (*Salmo salar*) MT and MT A. A phylogenetic tree was next constructed on the fish MT amino acid sequences using neighbour-joining analysis with MEGA 5.1 model. The robustness of the tree topology was determined using bootstrap analysis with 1000 pseudo-replicates.

Quantitative Reverse Transcriptase PCR (qPCR) Analysis of MT mRNA Levels

Gene specific primers targeted to the Asian sea bass MT cDNA sequence were designed for qPCR analyses and synthesized by Eurofins MWG. The qPCR primers were: forward 5′-CAC CTG CAC AAC TGC TCC TG -3′ and 5′-ACG CAG CCT GAG GCA CAC T-3′. The qPCR was done with IQ SYBR green super mix and instrument iCycler/MyIQ from BioRad, and 25 ng of transcribed total RNA template was used in each reaction in a total volume of 25 µl with 0.5 µM of each primer. The following PCR profile in 40 cycles was used [94 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s].

Statistical Analysis

Statistical analysis of MT protein levels from feral fish were performed by a one way analysis of variance (ANOVA) procedure, significant differences was considered at $p < 0.05$. Statistical analysis of MT mRNA expression levels from $CdNO_3$ Asian sea bass was evaluated by a two-way analysis of variance (ANOVA) with SNK's comparison and significant differences was considered at $p < 0.05$. The data are presented as mean ($n = 5$) ± standard error (SE).

Results

Isolation of MT Protein from Asian Bass and Development of Antibodies

A 10 kDalton protein from juvenile Asian sea bass liver was isolated and purified using metal affinity column chromatography followed by electro-elution. The isolated protein was characterized by analyzing absorbance at 260 nm upon addition of increasing concentrations of $CdNO_3$. There was a positive correlation between A_{260} and $CdNO_3$ concentrations (Figure 2). This positive correlation supports that the isolated protein is an MT protein. This protein was next used for immunization of mice for production of PAb. These PAb recognized a 10 kDalton protein band in a Western blot loaded with 40-70 µg MT proteins from $CdNO_3$-treated Asian sea bass (Figure 3).

Isolation of MT cDNA Fragment from Asian Sea Bass

A 150 base pair cDNA sequence was isolated using a reverse transcriptase approach and primers against conserved regions in fish MT genes. This PCR product was obtained using the highest annealing temperature tested (55 °C) but not at the lower annealing temperatures (i.e. 45° and 50 °C) tested (Figure 4). The

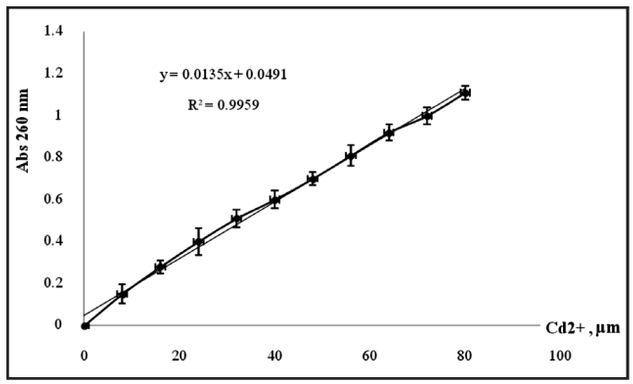

Figure 2: Absorbance at 260 nm was analyzed in the purified proteins after addition of 8-80 µM of $CdNO_3$ for MT characterization.

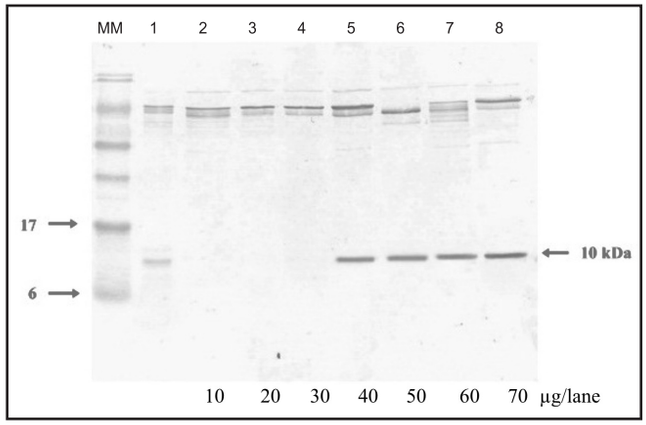

Figure 3: The specificity to MT of PAb (diluted 1:200) was analyzed in Western blot in serial dilutions of MT protein from $CdNO_3$-treated Asian sea bass. The gel was loaded as followed: molecular mass marker (MM); MT protein from $CdNO_3$-treated Asian sea bass 10-70 µg protein/lane (lanes 2-8).

PCR product was sequenced and the deduced amino acid sequence aligned with other fish and mammalian MT sequences (Figure 5A-B). The Asian sea bass MT showed 56-65% amino acid sequence identity with other fish MT genes (Table 2). Phylogenetic analyses further shows that Asian sea bass MT forms a cluster with the medaka MT genes (Figure 5C).

MT mRNA Analysis Using qPCR

A qPCR protocol was developed for mRNA analyses in Asian sea bass exposed to $CdNO_3$ (2, 4 or 6 mg/kg) in a dose-response and time course study (1, 2 and 3 days). There appears to be a bi-phasic dose-response pattern with highest MT mRNA levels in fish injected with 4 mg $CdNO_3$/kg. These fish had 7-fold higher MT

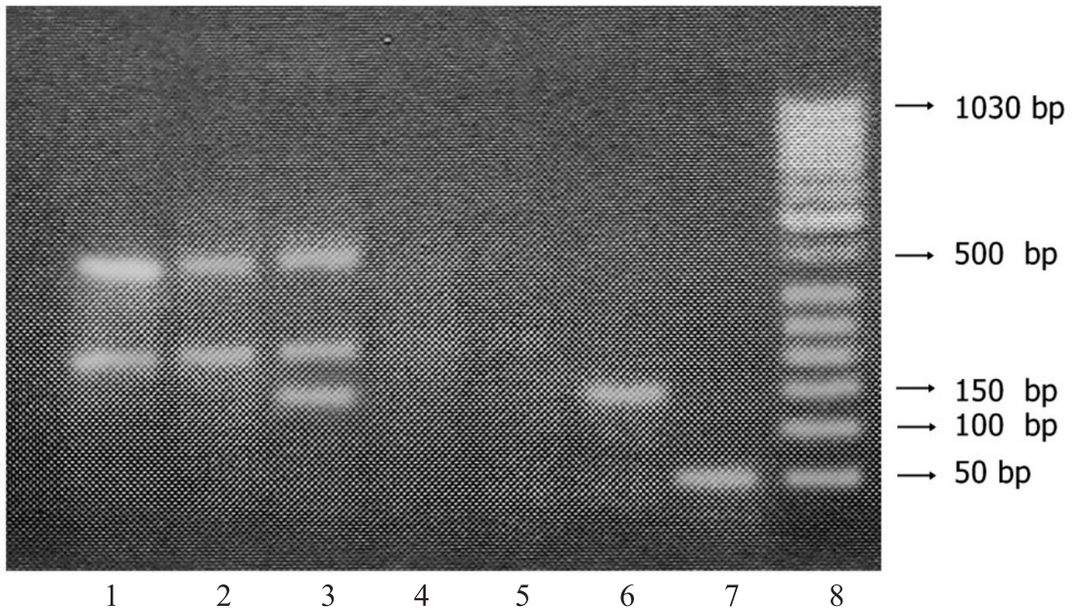

Figure 4: Optimization of reverse transcriptase PCR protocol for isolation of a 150 base pair MT cDNA fragment from the liver from Asian sea bass, using three different annealing temperatures 45 °C (lanes 1 and 4), 50 °C (lanes 2 and 5) and 55 °C (lanes 3 and 6), respectively. The PCR products were analyzed on 2% agarose gel stained with ethidium bromide and visualized by UV-light. Lanes 1-3: fish treated with the vehicle carrier (phosphate buffer); Lanes 4-6: fish treated with $CdNO_3$ (6 mg/kg fish); Lane 7: Negative control and Lane 8: DNA size marker.

ATGGATCCTTGTGAGTGCCAAGAGTGGAACCTGCAACT GCGGGGGATCCTGCACCTGCACAACTGCTCCTGT

M D P C E C Q E W N L Q L R G I L H L H N C S C

ACCACCTGCAAGAAGAGCTGCTGCGCATGCTGCCCGTCCGGCTGCAGCAAGTGTGCCTCAGGCTGCGTA

T T C K K S C C A C C P S G C S K C A S G C V—

Figure 5(A): Deduced amino acid sequence of Asian sea bass partial cDNA MT sequence.

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                        10         20         30         40         50         60
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....
Asian sea bass  MDP--CECQE WNLQLRG-IL HLHNCSCTTC KK-SCCACCP SGCSKCASGC V--------- ---- 47
AAB32777        MDP--CECAK TGACNCGATC KCTNCQCTTC KK-SCCFCCP SGCSKCASGC VCN-GNSCGS SCCQ 60
ABA03252        MDP--CECSK TGSCNCGGSC KCSNCACTSC KKASCCDCCP SGCSKCASGC VCK-GXTCDT SC-- 59
ABA03254        MDP--CECSK TGSCNCGGSC KCSNCACTSC KKASCCDCCP SGCSKCASGC VCK-GRTCDT SCC- 60
ABA03251        MDP--CECSK TGTCNCGGSC KCSNCACTSC KKASCCDCCP SGCSKCASXC VCK-GHTCDT IC-- 59
ABA03255        MXP--CECSK TGSCNCGGSC KCSNCACTSC KKASCCDCCP SGCSKCASGC VCK-GKTCDT SC-- 59
NP001117149     MDP--CECSK TGSCNCGGSC KCSNCACTSC KKASCCDCCP SGCSKCASGC VCK-GKTCDT SCCQ 61
ACN09889        MDP--CECSK TGSCNCGGSC KCSNCACTSC KKASCCDCCP SGCSKCASGC VCK-GKTCDT SCCQ 61
AAP31403        MDP--CECSK TGSCNSGGSC KCSNCACTSC KKASCCDCCP SGCSKCASGC VCK-GKTCDT SCCQ 61
ABA03250        -DP--CECSK TGSCKCGGSC KCSNCACTSC KKPSCCDCCP SGCSKCASGC VCK-GKTCDT SCCQ 60
AEZ55097        MDP--CDCSK TGKCNCGGSC TCANCSCTSC KK-SCCACCP SGCTKCASGC VCK-GKTRDK SCCQ 60
AAW83513        MDP--CDCSK TGKCNCGGSC TCANCSCTSC KK-SCCACCP SGCTKCASGC VCK-GKTCDK SCCQ 60
NP001098255     MDP--CDCSK TGKCNCGGSC TCTNCSCTSC KK-SCCACCP SGCTKCASGC VCK-GKTCDT TCCQ 60
NP001134810     MDP--CDCSK TGRCSCGGLC KCTNCGCA-- -TKSCCSCCP TGCSKCASGC VCKEGKTCDT SCCQ 59
AAP97267        MDPN-CSCTT GVSCACTGSC KCKECKCTSC KK-SCCSCCP VGCAKCAHGC VCK-GTLENC SCCA 61
AAA20233        MDPGECTCMS GGICICGDNC KCTTCSCKTC RK-SCCPCCP PGCAKCARGC ICK-GGSDKC SCCP 62
Clustal Consensus   *  * *                 * *     . *** ***  **:***  * :
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Figure 5(B): Alignment of Asian sea bass MT amino acid sequence with the corresponding region of other fish and mammalian MT genes obtained from the GenBank database. These MT genes are *C. auratus* (AAB3277), *O. kisutch* (ABA03252), *O. keta* (ABA03254), *O. gorbuscha* (ABA03251), *O. tshawytscha* (ABA03255), *S. salar* (ACN09889), *O. mykissgairdneri* (NP001117149), *S. alpinus* (AAP31403), *T. thymallus* (ABA03250), *O. melastigma* (AEZ55097), *O. javanicus* (AAW83513), *O. latipes* (NP001098255), *S. salar* (NP001134810), *H. sapiens* (AAP97267) and *M. musculus* (AAA20233).

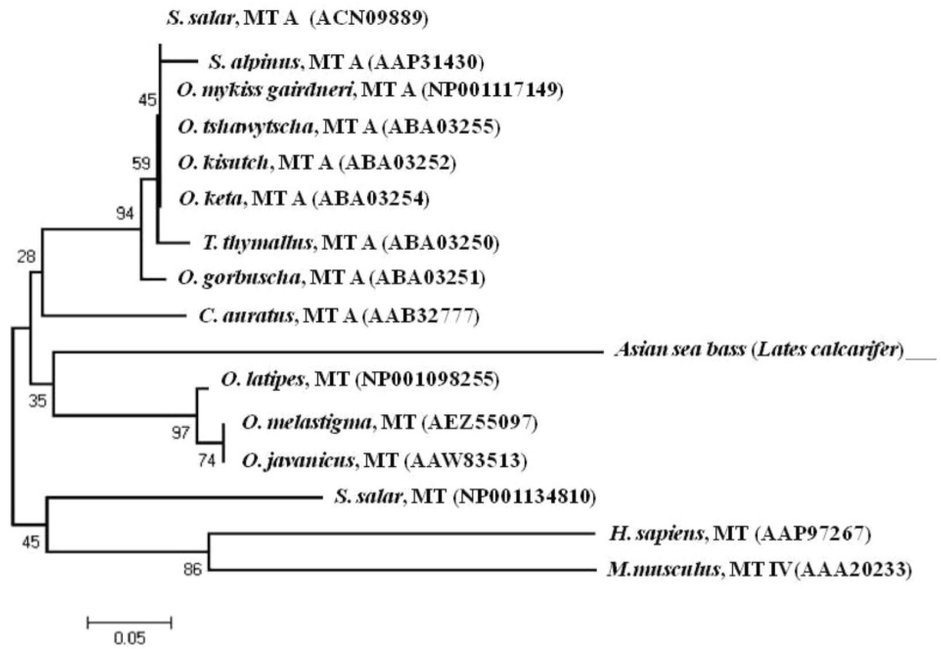

Figure 5(C): Phylogenetic tree of MT amino acid sequences from 13 different fish species was constructed using Neighbour-Joining and the MEGA 5.1 model. The tree was rooted with the MT protein sequences from human (AAP97267) and mouse (AAA20233). Robustness of tree topologies was evaluated by bootstrap analysis with 1000 pseudo-replicates.

Table 2: Amino acid sequence identity between Asian sea bass MT and MT deduced amino acid sequences from other fish species according to NCBI BLAST

Fish species	*GenBank Accession number*	*Sequence identity (%)*
Java medaka *Oryzias javanicus* (MT)	AAW83513.2	65
Indian medaka *Oryzias melastigma* (MT)	AEZ55097.1	65
Japanese medaka *Oryzias latipes* (MT)	NP001098255.1	65
Gold fish *Carassius auratus* (MT)	AAB32777.1	65
Chum salmon *Oncorhynchus keta* (MT A)	ABA03254.1	63
Rainbow trout *Oncorhynchus mykiss* (MT A)	NP001117149.1	63
Pink salmon or Humpback salmon *Oncorhynchu sgorbuscha* (MT A)	ABA0352.1	61
Coho salmon *Oncorhynchu skisutch* (MT A)	ABA03252.1	63
Arctic char *Salvelinus alpinus* (MT A)	AAP31403.1	63
European grayling *Thymallus thymallus* (MT A)	ABA03250.1	63
Oncorhynchus tshawytscha (MT A)	ABA03255.1	61
Atlantic salmon *Salmo salar* (MT A)	NP 001117149.1	59
Atlantic salmon *Salmo salar* (MT)	NP 001134810.1	56

mRNA levels compared to non-treated fish (Figure 6). In fish exposed to 4 mg/kg there was a time difference in MT induction with highest expression after one day and lowest induction after three days. However, in fish treated with the lower dose 2 mg/kg and the highest dose 6 mg/kg there were no apparent effect of duration of exposure (Figure 6). Although, in fish injected with the highest dose (6 mg/kg) had hemorrhage and 30% mortality was evident after day 2 in this treatment group, which suggests that this dose of $CdNO_3$ was lethal for Asian sea bass.

Detection of MT Proteins in Feral Fish from the Gulf of Thailand

The mouse PAb raised against Asian sea bass MT was applied to detect MT protein levels in 15 different fish collected in the Gulf of Thailand. Four different sampling sites (Figure 1) were selected: Klong Bangprong Angsila (Site 1, near shore), Klong Bangprong Angsila (Site 2, off shore), Map Ta Phut (Site 3, near shore) and Map Ta Phut (Site 4, off shore). Western blot and ELISA techniques were used to detect MT immunoreactive proteins in livers from these fish. Western blot analysis detected MT proteins in three of these four sampling sites. Besides, of all 15 species tested only two species had detectable MT protein bands. Thus, at sites 1 and 2, an MT protein band was detected in shrimp scad (*Alepes djadaba*). In site 3, an MT protein band was also observed in shrimp scad as well as in Indian ilisha (*Ilisha melastoma*). In site 4, no MT protein band was observed in any of the four fish species analyzed. The Western blot results were confirmed using ELISA showing increased MT protein levels in shrimp scad from site 1, 2 and 3 and in Indian ilisha from site 3 (Table 1).

Discussion

Biomarkers

The most compelling reason for using biomarkers in environmental risk assessment is that they can give information about biological effects in wildlife. Besides, biomarkers also provide information on type of exposure in the environment. There are classical biomarkers such as induction of detoxification enzyme cytochrome P450 1A (CYP1A), which is a good biomarker for aromatic hydrocarbon exposure (Mihailovic et al., 2010); induction of the egg-yolk

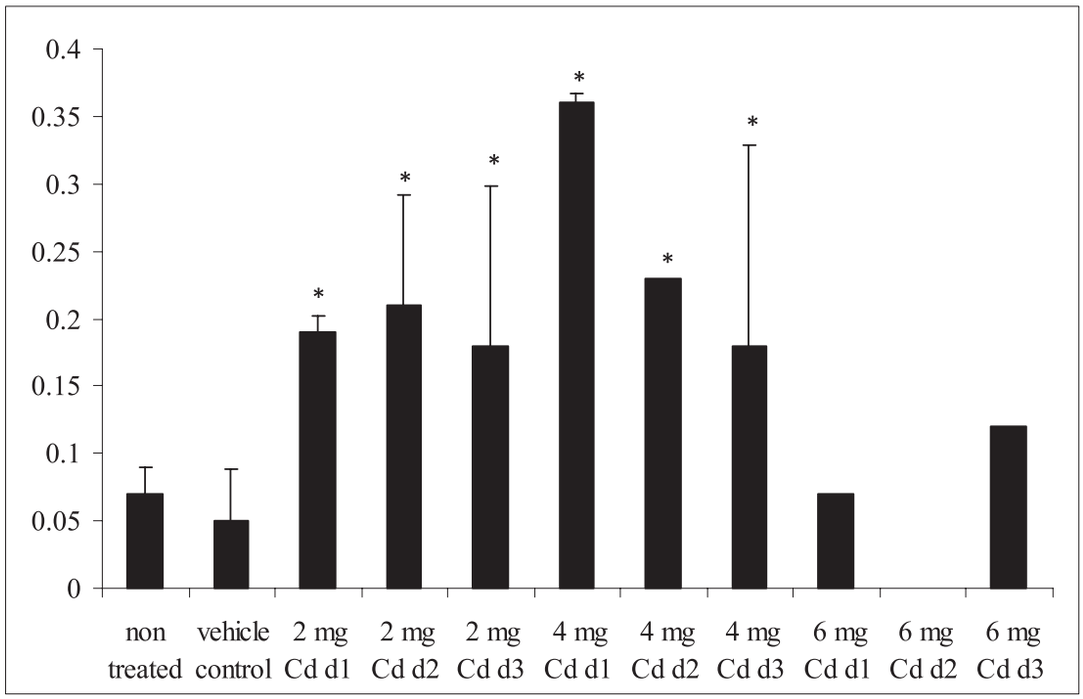

Figure 6: Expressions of MT mRNA levels were analyzed using quantitative PCR (qPCR) in livers from juvenile Asian sea bass. The fish were i.p. injected with $CdNO_3$ at 2, 4 or 6 mg/kg fish and the fish were sacrificed 1, 2 or 3 days post injection compared with untreated group and vehicle control group by two-way ANOVA. The values are presented as mean (n = 3) ± SE. * mean significant difference from untreated group and vehicle control group (p < 0.05).

precursor vitellogenin is a biomarker for estrogenic exposure (Hennies et al., 2003); and induction of MT is a biomarker for heavy metal exposure (Langston et al., 2001). Theses biomarkers have been commonly used in various biomarker programmes in temperate waters and have aided to decrease certain anthropogenic chemicals into the environment in Europe and North America. In tropical waters, less has been studied and there is a need to develop biomarkers that can be used to assess chemical exposures in these areas. In a previous study, we isolated and characterized CYP1A from Asian sea bass and applied it as a biomarker for exposure to aromatic hydrocarbons such as petroleum hydrocarbons in the Gulf of Thailand (Kanchanopas-Barnette et al., 2010). In addition to aromatic hydrocarbons, this area is also exposed to heavy metals. Therefore, our research aimed to study MT in fish in tropical areas and to develop a biomarker as an early warning signal for heavy metal pollution in tropical fish species.

Isolation of MT from Fish

In the present study, an MT protein was isolated from Asian sea bass and used to immunize mice to develop immunochemical protocols (i.e. Western blot and ELISA) to quantify MT proteins in Asian sea bass liver with a calculated molecular mass of 10 kDalton. Moreover, MT protein in $CdNO_3$ injected group was higher than in control fish. A study in the Amazon fish (*Colossoma macropomum*) showed induced MT protein levels 48 h post injection with $CdNO_3$ and 8-9 kDalton MT protein was isolated using a Hitrap column and a G-25 column (Honda et al., 2005). It is possible that there are multiple MT isoforms in fish. In fact, different MT isoforms of 37.7, 16.5 and 16.0 kDalton were isolated from hepatopancreas in white shrimp (*Litopenaeus vannamei*) exposed to $CdCl_2$ and $ZnSO_4$, using gel filtration chromatography of Wu and Chen (2005). In addition, two MT isoforms, between 6 and 10 kDalton, were isolated from crab (*Portunus pelagicus*) (Ang and Chong, 1998). However, the methods used to isolate MT proteins also need to be considered and that exposure conditions needs to be carefully considered. For example, tissue differences as well as possible differences in metal ion binding between different MT isoforms was suggested in brown trout (*Salmo trutta*) sampled from two different rivers in Norway, one contaminated with Cu ions and the other contaminated with Cd and Zn ions (Olsvik et al., 2001). At this stage, we do not know if there is more than one MT form in Asian sea bass. In the present study, we isolated a 150 base pair cDNA sequence from Asian sea bass using a PCR based approach. This sequence showed up to 65% amino acid sequence identity with other fish MT sequences obtained from the GenBank database. The phylogenetic analyses suggested that the Asian sea bass sequence clusters with the medaka MT genes.

Responsiveness to Cd^{2+} Exposure in Lab Studies

Acute and chronic effects of Cd ion exposures have been earlier described in different aquatic organisms (Castano et al., 1998). The present study was undertaken to explore dose response and duration of MT mRNA levels in Asian sea bass exposed to $CdNO_3$ using qPCR. A bi-phasic dose response pattern was seen with highest induction of MT mRNA in Asian sea bass injected with 4 mg/kg fish and a higher dose (6 mg/kg) resulted in toxic effects e.g. bleeding and death, which probably explains the lower inducibility of MT gene expression in these fish. In fish treated with 4 mg/kg, highest MT mRNA levels were seen 24 h post injection. Thus, a 7-fold induction was observed in these fish compared to control fish. Increased expression of MT mRNA levels have also been reported in *Tilapia* (*T. aurea* × *T. nilotica*) exposed to 5 mg Cd^{2+}/kg, where a 15-fold increase was seen after 24 h (Cheung et al., 2004). In addition, induced MT mRNA levels also have been reported in the obscure pufferfish (*Takifugu obscures*) after 24 h exposure to various doses of $CdCl_2$, ranging from 50 to 5000 ppb (Kim et al., 2008). Dose-dependent differences in MT mRNA have been proposed by others (Kim et al., 2008). In turbot (*Scophthalmus maximus*), exposure to a low dose of $CdCl_2$ (75 µg/kg) and the high dose at 500 µg/kg resulted in different responses on hepatic MT mRNA levels. Hence, a 4-fold increase in MT mRNA levels was seen in turbot exposed to low dose, whereas a 30-fold increase was seen in turbot exposed to the high dose. In addition to a dose-response relationship, there were time effects. In turbot exposed to the high dose, no chance in MT mRNA levels was observed after 21 days, but in fish exposed to a low dose lower MT mRNA levels were seen after 21 days (George et al., 1996). The decline in MT mRNA levels in Asian sea bass and Tilapia (Cheung et al., 2004) exposed to 6 and 10 mg/kg Cd^{2+}, respectively, is probably due to a general toxic effect. In fact, in the present study exposure to 6 mg/kg resulted in 30% mortality. Hence, gene expression of MT mRNA is dependent on dose and duration of exposure and it is likely that the route of exposure as well as other factors such as temperature, season and species affect MT inducibility. Nevertheless, data from our lab, and from others, strongly supports the usefulness of analyzing MT mRNA levels in fish

using qPCR for establishing sensitive and specific early warning biomarkers in order to assess environmental exposures to heavy metals.

Induction of MT Levels in Wildlife Fish

Cadmium in the liver of feral fish in this study was not measured body burden because of very costly, problem of low-limited detection of equipment and need to pool many liver samples. Thus, alternatively screening studied for heavy metal contaminant by using antigen-antibody based technique having the advantage of high specific, cheaper, rapidly, and can be applied to many samples. In the present study, we applied immunochemical protocols to investigate MT protein levels in feral fish from two different locations: the urban Angsila area and the industrial Map Ta Phut area in the Gulf of Thailand. Our results imply that feral fish have been exposed to heavy metals as indicated by the strong MT immunoreactive proteins in some of the fishes determined by Western blot analyses. Fish collected from Map Ta Phut (Site 3, near shore) had highest levels of MT proteins compared to the other three sites. Cadmium contamination has earlier been reported in this industrial area. Thus, in Map Ta Phut, 0.001-0.005 mg Cd^{2+}/L was detected in 2005 (Saengsupavanich et al., 2009). The safety limit for Cd ions is 0.005 mg/L according to the Thailand national standard.

In the present study, increased MT protein levels were seen in two fish species of totally 11 species analyzed near the shore, which suggests that these fish have been exposed to Cd ions. However, in fish collected off shore no MT proteins were detected. Increased levels of MT immune reactive proteins have also been reported in sentinel fish (*Lithognathus mormyrus*) collected from a polluted site in Israel along the Mediterranean coast, compared to fish from a cleaner site over a two-year sampling period (Yudkovski et al., 2008). Of all 15 different fish species analyzed, two species shrimp scad and Indian ilisha had statistically significantly higher MT protein levels compared to other fish from the Angsila and Map Ta Phut areas. Hence, both the aquaculture area in the urban Angsila and the industrial Map Ta Phut seem to be contaminated by heavy metals. Besides, in the aquaculture areas increased MT protein levels were also seen in fish sampled from off shore. The study implies that this aquaculture area is also exposed to heavy metals. It should be noted that dietary preferences may affect the responsiveness in the different fish species analyzed and this needs further investigations. However, shrimp scad seems to be a suitable fish for future biomonitoring programmes of heavy metal exposure in this area because it is the only sentinel species that can be found in both areas and it also had the highest MT protein levels.

Conclusions

Hepatic Asian sea bass MT protein and partial cDNA sequence were isolated and used to develop immunochemical protocols for MT mRNA and protein quantifications. These protocols were used to analyze MT mRNA and protein levels in Asian sea bass exposed to $CdNO_3$ in the laboratory. In addition, the immunochemical protocols were used to analyze MT protein levels in feral fish collected from two different areas in Thailand: one industrial area and one urban aquaculture area. The data suggested that fish are more exposed to heavy metals near shore compared to off shore in the urban aquaculture site. Furthermore, the results suggest that the shrimp scad may be a good candidate fish species for environmental monitoring programmes in Thailand waters.

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