Korean J. Limnol. 40 (4): 489~494 (2007)

Effects of Acute Metal Exposures on the Viability and mRNA Expression of Metallothionein in *Hemibarbus mylodon* Fry

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Transcriptional modulation of metallothionein (MT) during acute metal exposures (cadmium, copper or zinc) was examined in fry of *Hemibarbus mylodon*, a threatened fish species in Korean peninsula. Viability of *H. mylodon* fry was most affected by copper exposure (up to 79% of mortality at 1 ppm for 48 hours) and considerably by cadmium exposure (21 to 54% of mortality). On the other hand, Zn showed the least adverse effect on the viability (0 to 13% of mortality) of this species. Based on the semi-quantitative RT-PCR analysis, the stimulation of MT mRNA in response to metal exposures followed generally in a dose-dependent fashion where cadmium was the most potent inducer for the induction of MT transcripts in fry (up to more than 5-fold) while the lowest response was observed in zinc-exposed group (2-fold at maximum). From the exposure using environmentally realistic doses of cadmium (0 to 0.05 ppm for 24 hours), MT expression at mRNA level was also sensitively modulated toward upregulation up to more than 3-fold as relative to non-exposed control. Results from the present study would be a good basis for understanding the adaptive capacity and stress physiology of this endangered fish species during metal pollution.

Key words : *Hemibarbus mylodon*, metallothionein expression, heavy metal exposure, viability

INTRODUCTION

Hemibarbus mylodon, an endemic natural monument fish species in Korea, has been threatened by recent anthropogenic and/or industrial activities. Its population size has been gradually decreased in many local habitats with a high risk of extinction, and hence efforts for the conservation and protection of this species are urgently needed (Jang *et al.*, 2003; Kim *et al.*, 2007). Conservation or genetic restoration of the threatened fish species often requires the fine molecular bioindicator (s) to address the stress physiology occurred in the target species, which might possibly be related with environmental problems or pollutions.

Metallothionein (MT) is a cysteine-rich protein interacting with various essential and non-essential heavy metal ions. It is also involved in a number of cellular reactions including detoxification or sequestration of excess heavy metals and the reservation of essential metals for homeostatic regulation (Coyle *et al.*, 2002; Haq *et al.*, 2003). Due to its high inducibility by exogenously overloaded metal ions, MT has been considered as one of potential molecular biomarkers to evaluate aquatic quality especially with respect to the metal pollution (Cho *et al.*, 2005; Alvarado *et al.*, 2006). In addition, MT has also been recognized as one of general stress factors of which expression have been modulated by a wide spec-

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trum of stressors or stimuli (Coyle *et al.*, 2002). Generally speaking, the adoption of MT as a bioindicator for risk assessment of ecological environment would be more desirable in fish species with widespread geographic distribution in different ecosystems than in fish species having restricted habitats. However in spite of their restricted distributions in aquatic environment, the understanding the cellular response to pollutants at gene expression level would improve our knowledge on the adaptive capacity of the endangered fish species existing in a given environment, which consequently could contribute to the development of effective strategy for their protection and/or conservation. For this reason, this study was aimed to extend knowledge on the stress physiology arisen from the metal pollution in a threatened fish species, *H. mylodon* based on the examination of MT gene expression. We investigated effects of acute metal exposures on the expression of MT gene at mRNA levels in *H. mylodon* fry, and also examined any potential relationship between MT gene expression and mortality caused by heavy metal exposures.

MATERIALS AND METHODS

1. Nucleic acid preparation

Primary structure of *H. mylodon* metallothionein cDNA was determined previously and its full-length sequence is available in NCBI GenBank under the accession number, EF689139. For RT-PCR analysis, whole body total RNA from fry was extracted using TriPure Reagent (Roche, Germany). Total RNA was treated with DNase I (Roche, Germany) at 10 U μg^{-1} total RNA (37°C for 30 min) and then cleaned by Qiagen RNA Mini Clean-up Kit (Qiagen, Germany). All the procedures for preparing RNA template were carried out according to recommendations by manufacturers. RNA integrity (28S : 18S ratio) was checked using an MOPS-agarose electrophoresis and the concentration was determined using GeneQuant spectrophotometer (GE Healthcare, USA).

2. Fish specimen and *in vivo* metal exposures

In order to examine the effects of overloaded waterborne heavy metals on the viability of *H. mylodon* fry, fish were exposed to 0, 0.2, 0.5 and 1.0 ppm of copper, cadmium or zinc. Fry (average body weights=0.9±0.2 g) used in this study were the laboratory stock (Marine Molecular Genetic Breeding Lab, Soonchunhyang University) that had been artificially propagated in spring of 2005. Twelve individuals were allocated into 30 L tank containing 10 μm-filtered tap water (pH 7.7; conductivity 325 μS cm^{-1}; hardness 268 ppm; temperature 20°C in average), and the desired concentrations of each heavy metal was adjusted by adding stock solution (100 ppm). Exposure was carried out for 2 days. Non-exposed control was also prepared identically except metal treatment. Two replicate tanks per treatment were prepared. Half of water was exchanged 1 day after exposure, and metals were renewed at that time. Viability of fish belonging to each replicate group was checked with 12 hours of intervals, and cumulative mortality was estimated at the end of exposure experiment. When exposures were finished, 4 randomly chosen individuals per treatment were subjected to RT-PCR analysis. Based on the results from the first experiment, the second exposure was performed in order to confirm the potency of each heavy metal for the induction of MT gene expression in fry. Sixteen fry of the same sized was exposed to a given concentration (0.5 ppm) of each heavy metal (Cu, Cd or Zn) for 48 hours again. Treatment conditions were the same as described above. After treatment, five to six randomly chosen fish from each treatment were used for RT-PCR analysis. In the third exposure, fry (n=12) were exposed to low doses of cadmium (0.01 to 0.05 ppm), the most potent metal proven in the previous experiment. Treatment duration was 24 hours. MT transcripts were analyzed with pooled total RNA prepared from six randomly chosen fry per treatment.

3. Semi-quantitative RT-PCR analysis

Prior to semi-quantitative RT-PCR assays, RT-PCR conditions were optimized especially regarding the input amount of total RNA and the number of PCR cycles for both MT and 18S rRNA genes (data not shown). Based on preliminary results, complementary DNA strands were synthesized from 2.5 μg of DNase-treated total RNA using Omni-transcript RTase (Qiagen, Germany) and oligo $d(T)_{20}$ primer according to recommendations by manufacturer. The 18S rRNA primer of the species *Hemibarbus mylodon* (reverse primer; 5′-CAAGAATTTCACCTCTAGCGGC-3′)

was also included in order to prepare an internal control standard. PCR amplification of MT fragments (298 bp) was carried out using AccuPower PCR Premix (Bioneer, Korea) including 30 pmoles of HM MT 2F (5′-TCAAGGGACTTTCGGACTCT-3′) and 2R (5′-GCGATGCAGAACGAT GACTA-3′) primers and 1 μL of RT product (cDNA). Reaction was cycled 22 times with the following conditions: 94°C for 30s, 58°C for 30 s and 72°C for 30s with an initial denaturation step at 94°C for 2 min. The 569 bp of 18S rRNA fragment was also amplified as an internal control using the same thermal cycling condition with 18S rRNA 1F (5′-TAACGGGGAATCAGGGTTCGAT-3′) and 1R primers (for sequence see above). PCR products (6 μL) were separated onto a 1.5% agarose gel by electrophoresis and visualized by GelRed™, a nucleic acid gel stain (Biotium, USA). Stained bands were analyzed Quantity-One image analysis software implemented into VersaDoc 4000 (Bio-Rad, USA). Triplicate amplifications per cDNA were made in an independent manner, and relative MT mRNA levels were normalized against 18S rRNA controls.

4. Statistics

Difference in viability (expressed as percent cumulative mortality) and MT mRNA levels (relative levels normalized against 18S rRNA control based on semi-quantitative RT-PCR) among groups during exposures were assessed by ANOVA followed by Duncan's multiple range test. Difference was considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

1. Effects of metal exposure on viability of *H. mylodon* fry

No mortality was detected in non-exposed group. However, the metal exposure affected the viability of fry, and the amount of mortality was different depending upon metals treated. At the end of exposure (48 hours post exposure), the group exposed to Cu revealed the most severe mortality up to more than 79%. The treatment of Cd also exhibited significant depression of viability (mortality ranged from 21 to 54%), although the amount of loss was lower than that observed in Cu. On the other hand, zinc showed the least adverse effect on the viability of *H. mylodon* fry: the highest dose (1 ppm) resulted in 12.5% of cumulative mortality, however other lower doses caused no significant loss of fish (Fig. 1A). In the second exposure with a given concentration (0.5 ppm) of each metal, percent mortality was similar as observed with the first exposure experiment (68.8±8.8% for Cu, 34.4±4.4% for Cd and 3.1±4.4% for Zn; histograms not shown). In the third exposure with low doses of cadmium, non-exposed control and groups treated with 0.01 ppm Cd did not reveal any mortality. However,

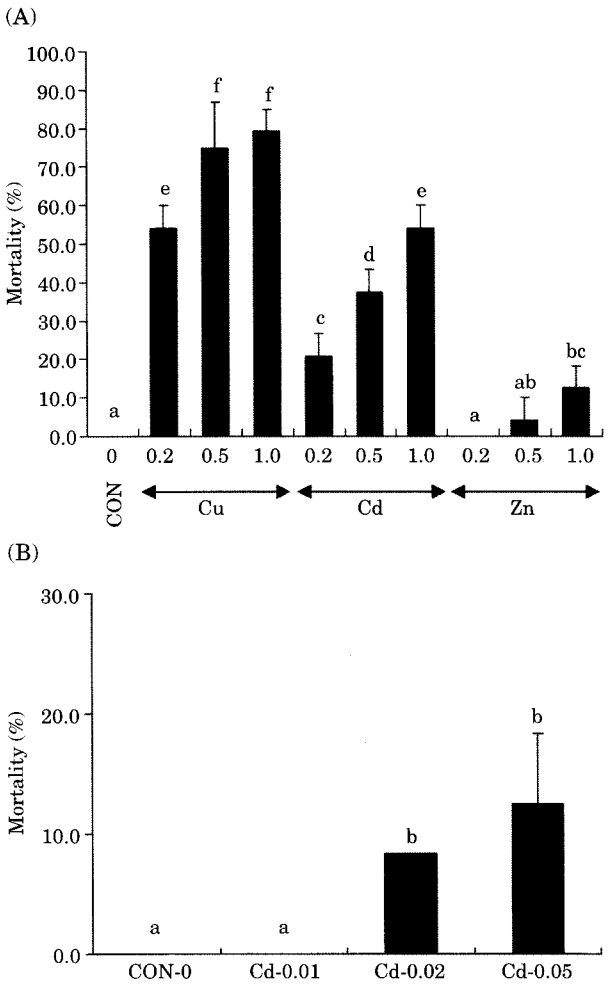

Fig. 1. Effects of metal exposures on the viability of *H. mylodon* fry with different doses (0 to 1 ppm) and/or heavy metal ions, copper (Cu), cadmium (Cd) or zinc (Zn). Cumulative mortalities of exposed groups were compared with non-exposed control (CON). For details, refer to Materials and methods. Means with same letters in each trial were not significantly different based on ANOVA ($p > 0.05$). T bars indicate standard deviations.

groups exposed to 0.02 and 0.05 ppm Cd displayed 8 and 12% of mortality in average, respectively (Fig. 1B).

It is widely accepted view that even essential heavy metals such as Cu and Zn, may act as toxicants to disrupt the body homeostasis in fish when its levels are unnecessarily excessive. The toxic effects of Cu have been particularly associated with the impaired ion regulation in gills, in which excess Cu would inhibit Na^+/K^+-ATPase activity, a vital enzymatic action for most animal species, and consequently hinder the proper ion uptake/excretion in gills of fish (Dang *et al.*, 2001; Alvarado *et al.*, 2006). Such an osmoregulatory disturbance or respiratory stress caused by exposure to Cu has been reported in various teleost species (Grosell *et al.*, 2007). Excess metals also would cause oxidative stresses in a variety of fish tissues especially including liver and kidney (Cho *et al.*, 2006). Unlike Cu and Zn, Cd is a non-essential heavy metal, which does not contribute to metabolic processes in living organisms. This metal also exhibited high binding affinity for certain cell types in fish gills: waterborne Cd has been known to compete with Ca^{2+} for Ca^{2+} uptake sites on the gills, which may disrupt Ca^{2+} homeostasis (Matsuo *et al.*, 2005). Many previous studies have reported toxic effects of this non-essential heavy metal on impaired physiology and/or performance at both sublethal and lethal levels in a number of fish species. It includes induced apoptosis in rainbow trout, *Oncorhynchus mykiss* (Risso-de Faverney *et al.*, 2001), adverse effects on the embryogenesis in zebrafish, *Danio rerio* (Meinelt *et al.*, 2001), impaired cortisol regulation in rainbow trout and yellow perch, *Perca flavescens* (Alexandra and Hontela, 2004) and lower body weight growth with the impairment of lipid storage in eel, *Anguilla anguilla* (Pierron *et al.*, 2007). In addition, a recent study illustrated the importance of cross-acclimation of different metals on acute Cd toxicity in rainbow trout (McGeer *et al.*, 2007).

2. Response of MT expression to metal exposures

From the first experimental exposure using three different metals, MT expression was significantly altered in response to all of three heavy metals when determined by RT-PCR analysis (Fig. 2). Among three metals, Cd was most potent in the sense of the highest induction of MT transcripts (up to more than five-fold as relative to non-exposed control), while Zn showed the least effect on the transcriptional stimulation of MT. Even at the lowest concentration of cadmium (0.2 ppm), MT expression in fry reached the maximal levels as evidenced by no further increase of MT transcripts with increase of Cd concentration up to 1 ppm ($p > 0.05$). Meanwhile, MT expressions in groups exposed to relatively higher concentration of Cu (0.5 and 1 ppm) were more pronounced than that observed in group treated with the lowest Cu dose (0.2 ppm) ($p < 0.05$). Zinc stimulated the MT expression moderately only, however the increase of MT transcripts was clearly dose-dependent ($p < 0.05$). From the second experiment (each 0.5 ppm for 48 hours), the potency of each heavy metal was clearly confirmed: MT

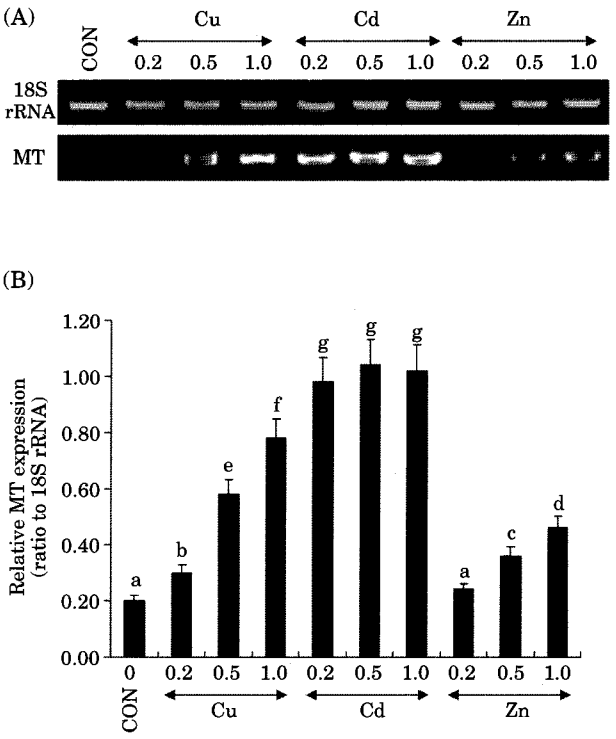

Fig. 2. Modulation of MT expression at mRNA level during exposures to three heavy metals at varying doses. (A) Representative semi-quantitative RT-PCR gels showing the expression of MT mRNA along with 18S rRNA internal control. (B) Densitometric analysis of RT-PCR bands using Quantity-One software (Bio-Rad, USA) based on triplicate assays. Mean±SDs (histograms with T bars) with same letters were not significantly different ($p > 0.05$).

expression in *H. mylodon* fry was the most responsive to Cd (up to 5-fold as relative to non-exposed control), followed by exposure to Cu (2.8-fold) and Zn (1.7-fold) (histograms not shown).

Environmentally realistic doses of cadmium (0.01 to 0.05 ppm) were also proven to be able to stimulate the expression of MT in *H. mylodon* fry (Fig. 3). Levels of 18S rRNA were steady-state regardless samples without significant fluctuations in RT-PCR (gel not shown). However, MT transcripts in Cd-exposed groups were clearly modulated toward up-regulation with increasing doses of Cd. Relative MT transcript levels were sharply increased with increased concentrations of cadmium up to more than 3-fold at the highest dose (0.05 ppm). Other two lower doses (0.01 and 0.02 ppm) also represented 1.5- and 2-fold of MT mRNA levels, respectively when compared to that of non-exposed control (Fig. 3).

In the present study, Cd was the most potent stimulator for MT induction in *H. mylodon* fry, which is similar with previous observations made

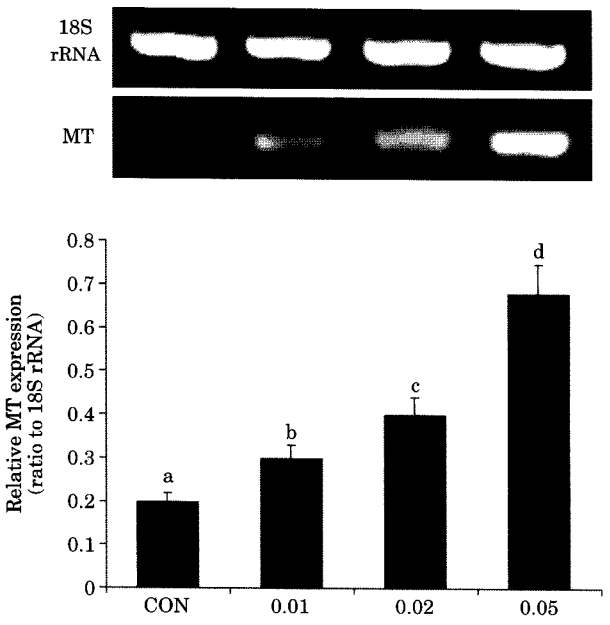

Fig. 3. Induction of MT mRNA in *H. mylodon* fry by exposure to low doses of cadmium. Mean±SDs were based on triplicate amplifications followed by densitometric analysis. Means with different letters were statistically different based on ANOVA ($p<0.05$). MT mRNA level in each group was normalized against 18S rRNA level in order to estimate the relative MT expression. Representative RT-PCR gels showing the amplification of MT mRNA and 18S rRNA are also shown at top.

on other fish species (Hermesz *et al.*, 2001). Differential regulation of MT gene by different metal ions is not surprising, which could generally been explained by the different availability of each metal ion in each organism. Actually, the induction of MTs by metals has been affected readily by the rate of accumulation and the binding affinity of metals to other biomolecules such as ligands (Kock *et al.*, 1995; Olsvik *et al.*, 2001). Because MT is known to play an important role in reserving excess metals for other metalloenzymes, expressed levels of pre-existing other metal-binding proteins may also influence the modulation of MT expression (Cho *et al.*, 2006). In addition, stimulation of MT transcripts could also be associated with the species-specific adaptive capacity (or degree of tolerance) to different metals (De Boeck *et al.*, 2003). From many previous literatures, the MT expressions have been provoked during metal exposures under sublethal conditions (Olsson *et al.*, 1998). However, in the present exposures using relatively high concentrations of metals, it was found that fish exposed to lethal doses also might represent considerable induction of MT mRNAs, although there was no direct relationship between MT mRNA levels and mortality of fish.

As a result of the third exposure experiment using low doses of Cd, the sharp induction of MT was possible as early as 24 hours after exposure even at the environmentally relevant doses. Such an expression pattern might be considered as an indicative for the rapid accumulation of metals in this species (Olsson *et al.*, 1998; Basha and Rani, 2003). Consequently our results suggest that biological stress associated with metal contamination could efficiently be detected even at very low concentration by means of examining the changes of MT expression. Further study to examine effects of many other biological factors (age, sex and tissues) or physicochemical parameters (temperature and dissolved oxygen) on the modulation of MT would be valuable to extend knowledge on the metal-caused stress physiology or adaptive capacity of this endangered fish species.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Environment of Korea as 'The Eco-technopia 21

project'.

LITERATURE CITED

Alexandra, L. and A. Hontela. 2004. A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch, *Perca flavescens*. *Aquat. Toxicol.* **67**: 13-21.

Alvarado, N.E., I. Quesada, K. Hylland, I. Marigómeza and M. Soto. 2006. Quantitative changes in metallothionein expression in target cell-types in the gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment. *Aquat. Toxicol.* **77**: 64-77.

Basha, P.S. and A.U. Rani. 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). *Ecotoxicol. Environ. Saf.* **56**: 218-221.

Cho, Y.S., B.N. Choi, E.M. Ha, K.H. Kim, S.K. Kim, D.S. Kim and Y.K. Nam. 2005. Shark (*Scyliorhinus torazame*) metallothionein: cDNA cloning, genomic sequence, and expression analysis. *Mar. Biotechnol.* (NY) **7**: 350-362.

Cho, Y.S., B.N. Choi, K.H. Kim, S.K. Kim, D.S. Kim, I.C. Bang and Y.K. Nam. 2006. Differential expression of Cu/Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (*Oplegnathus fasciatus*). *Aquaculture* **253**: 667-679.

Coyle, P., J.C. Philcox, L.C. Carey and A.M. Rofe. 2002. Metallothionein: the multipurpose protein. *Cell. Mol. Life Sci.* **59**: 627-647.

Dang, Z., M.H.G. Berntssen, A.K. Lundebye, G. Flik, S.E. Wendelaar Bonga and R.A.C. Lock. 2001. Metallothionein and cortisol receptors expression in gills of Atlantic salmon, *Salmo salar*, exposed to dietary cadmium. *Aquat. Toxicol.* **53**: 91-101.

De Boeck, G., T.T.H. Ngo, K.V. Campenhout and R. Blust. 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquat. Toxicol.* **65**: 413-424.

Grosell, M., J. Blanchard, K.V. Brix. and R. Gerdes. 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. *Aquat. Toxicol.* **84**: 162-172.

Haq, F., M. Mahoney and J. Koropatnick. 2003. Signaling events for metallothionein induction. *Mutation Res.* **533**: 211-266.

Hermesz, E., M. Abraham and J. Nemcsok. 2001. Tissue-specific expression of two metallothionein genes in common carp during cadmium exposure and temperature shock. *Comp. Biochem. Physiol.* **128C**: 457-465.

Jang, M.-H., M.C. Lucas and G.-J. Joo. 2003. The fish fauna of mountain streams in South Korean national parks and its significance to conservation of regional freshwater fish biodiversity. *Biol. Conservation* **114**: 115-126.

Kim, K.Y., S.Y. Lee, Y.S. Cho, I.C. Bang, D.S. Kim and Y.K. Nam. 2007. Characterization and phylogeny of two β-cytoskeletal actins from *Hemibarbus mylodon* (Cyprinidae, Cypriniformes), a threatened fish species in Korea. *DNA Seq.* (in press; DOI: 10.1080/10425170701445691)

Kock, G., R. Hoper and S. Wograth. 1995. Accumulation of trace metals Cd, Pb, Cu, Zn in arctic char *Salvelinus alpinus* from oligotrophic alpine lakes: relation to alkalinity. *Can. J. Fish. Aquat. Sci.* **52**: 2367-2376.

Matsuo, A.Y.O., C.M. Wood and A.L. Val. 2005. Effects of copper and cadmium on ion transport and gill metal binding in the Amazonian teleost tambaqui (*Colossoma macropomum*) in extremely soft water. *Aquat. Toxicol.* **74**: 351-364.

McGeer, J.C., S. Nadella, D.H. Alsop, L. Hollis, L.N. Taylor, D.G. McDonald and C.M. Wood. 2007. Influence of acclimation and cross-acclimation of metals on acute Cd toxicity and Cd uptake and distribution in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **84**: 190-197.

Meinelt, T. R.C. Playle, M. Pietrock, B.K. Burnison, A. Wienke and C.E.W. Steinberg. 2001. Interaction of cadmium toxicity in embryos and larvae of zebrafish (*Danio rerio*) with calcium and humic substances. *Aquat. Toxicol.* **54**: 205-215.

Olsson, P-E., P. Kling and C. Hogstrand. 1998. Mechanisms of heavy metal accumulation and toxicity in fish. In: Langston, W.J., M.J. Bebiano (Eds.), *Metal Metabolism in Aquatic Environments*. Chapman and Hall, London, p. 321-350.

Olsvik, P.A., P. Gundersen, R.A. Andersen and K.E. Zachariassen. 2001. Metal accumulation and metallothionein in brown trout, *Salmo trutta*, from two Norwegian rivers differently contaminated with Cd, Cu and Zn. *Comp. Biochem. Physiol.* **128C**: 189-201.

Pierron, F., M. Baudrimont, A. Bossy, J.P. Bourdineaud, D. Brethes, P. Elie and J.C. Massbuau. 2007. Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*). *Aquat. Toxicol.* **81**: 304-311.

Risso-de Faverney, C., A. Devaux, M. Lafaurie, J.P. Girad, B. Bailly and R. Rahmani. 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygen sepecies. *Aquat. Toxicol.* **53**: 65-76.

(Manuscript received 31 August 2007,
Revision accepted 7 December 2007)