

Species-specific expression of metallothionein in the hepatopancreas of seawater fish from the Montenegrin Adriatic coastline

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ABSTRACT

In the present study we examined the protein expression of metallothioneins (MTs) in the hepatopancreas of several seawater fish species: Red mullet (Mullus barbatus), European hake (Merluccius merluccius), Tub gurnard (Trigla lucerna) and Thinlip mullet (Liza ramada), from the Montenegrin Adriatic coastline. Western blot analysis revealed the highest induction of MTs in the hepatopancreas of the examined fish species in the following order: M. merluccius, T. lucerna, M. barbatus and L. ramada. These results are in correlation with literature data showing that fish species differ in their detoxification capacities and amounts of accumulated metals. We conclude that the level of induction of MTs in the hepatopancreas of the examined fish species correlates with the level of their reliability as bioindicator species in heavy metal biomonitoring.

Keywords: fish, metallothionein, Montenegrin Adriatic coastline

INTRODUCTION

The use of molecular biomarkers in aquatic organisms is very important in order to address the broad spectrum of industrial, agricultural, commercial and domestic chemicals entering the aquatic environment and being taken up into the tissues of aquatic organisms. Among the various types of biomarkers the following have received special attention in ecotoxicological studies: cytochrome P450 (indicator of exposure to organic contaminants PAHs, PCBs, etc), DNA-damage (strand breaks) and bulky DNA-adducts due to exposure to mutagenic inorganic and organic xenobiotics, inhibition of acetyl cholinesterase (AChE) activity (organophosphorous, carbamates, Cd, Pb, Cu, etc), metallothionein synthesis in hepatic and other tissues (exposure to metals Zn, Cu, Cd, Hg, Fe, etc), antioxidant enzymes (superoxide dismutase, catalase, glutathione transferase) (exposure to ROS, free radicals,

pollutants causing oxidative stress, lipid peroxidation (oxidants, metals, etc), and vitellogenin induction (oestrogenic substances) (Stegeman & Hahn 1994, Van der Oost, 2003).

To prevent the toxic effect of some metals, in fish as well as in most aquatic organisms, one very important mechanism has been developed. Metallothioneins (MTs) constitute a family of low-molecular weight, cysteine-rich and heat stable proteins involved in the binding and regulation of essential metals, such as copper and zinc, and in the detoxification of these and other non-essential metals, such as cadmium and mercury (Bervoets *et al.*, 2013).

The aim of the present study was to examine the MTs protein expression in the hepatopancreas of several seawater fish species: Red mullet (*Mullus barbatus*), European hake (*Merluccius merluccius*), Tub gurnard (*Trigla lucerna*) and Thinlip mullet (*Liza ramada*), from the Montenegrin Adriatic coastline.

MATERIAL AND METHODS

Animals

Specimens of *M. merluccius*, *M. barbatus*, *T. lucerna* and *L. ramada* were collected in winter by trawling in the Adriatic Sea in Montenegro as shown in Figure 1. The fish were killed immediately by spinosectomy according to standard animal care regulations. The hepatopancreas was quickly removed, washed in ice-cold 0.15 M NaCl and frozen in liquid nitrogen. Individuals of the same size were selected to ensure uniformity of samples.

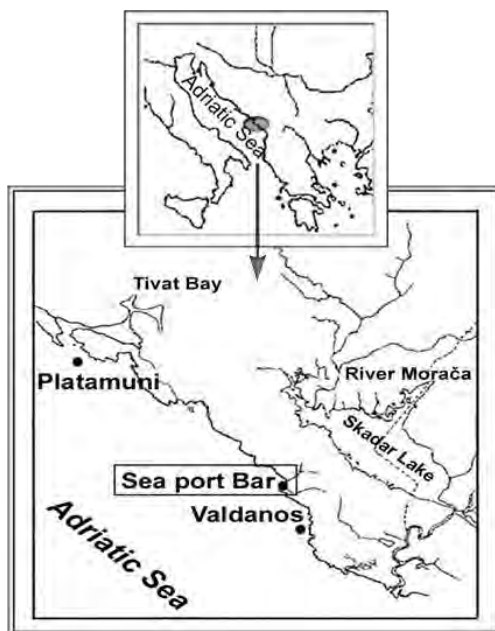


Figure 1. A map of the Adriatic coast of Montenegro, the locations where the fish caught are indicated (Platamuni, Valdanos and Bar).

Isolation of the microsomal fraction

The microsomal fraction of the hepatopancreas was prepared following the procedure of Krauss *et al.* (1983). The tissues were excised and homogenized (1g liver/1ml) in STM buffer: 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 1 mM PMSF) and pelleted at 10,000 x g, 4°C for 25 min. The obtained post mitochondrial supernatant was then centrifuged at 150,000 x g, 4°C for 60 min. The obtained supernatant was used for analysis of MT.

SDS-PAGE

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 20 µg of proteins were loaded onto 4% stacking/12% separating slab gels as described by Laemmli (1970). The gels were stained using Coomassie Brilliant Blue R-250. Protein concentrations were determined according to Lowry *et al.* (1951).

Immunoblot analysis

Twenty µg of the supernatant protein fractions were separated by SDS-PAGE and electroblotted onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). Immunoblot analysis was performed according to Towbin *et al.* (1979) using a polyclonal antibody to fish MT (Biosense laboratories, Norway). Immunoreactive bands were identified by an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. Antigen-antibody complexes were analysed with TotalLab (Phoretix) electrophoresis software and changes of the relative concentrations of MT in different samples were compared.

Statistics methods

The data were expressed as the mean ± S.E.M. (standard error of mean). Statistical differences between groups were analyzed using one-way Analysis of Variance (ANOVA), followed by Duncan's multiple range test. The difference was considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The presence of xenobiotic compounds in aquatic ecosystem does not always imply injurious effects. Connections between the levels of exposure, the degree of tissue contamination and the early adverse effects in living organisms need to be established. Fish play a major ecological role in the aquatic food-webs as carriers of energy through the trophic levels. The sudden death of large numbers of fish often indicates heavy pollution. The effects of exposure to sublethal levels of pollutants can be measured in terms of biochemical, physiological or histological responses of the fish organism (Van der Oost *et al.*, 2003).

To prevent the toxic effect of some metals, in fish as well as in most aquatic organisms, one very important mechanism has been developed. MTs have four main functions in aquatic vertebrate: bioaccumulation of toxic metals and detoxification, homeostatic regulation of metals, protection against oxidative stress and neuroprotective mechanism (Dziegiel, 2004). MTs have variety distributions in different tissues of aquatic vertebrate. Experimental animals treated with high doses of Cd led to morphological and

functional changes in hepatopancreas and high MTs amount in liver (Wang *et al.*, 2014). Heavy metal accumulation and MTs induction in fish gills have been revealed to be very species-specific. Kidney was a universal target to investigate metal toxicity and MTs expression. Even low-level exposure to Cd also results in kidney destroy (Wang *et al.*, 2014).

In the present study we examined protein expression of MTs in the hepatopancreas of several seawater fish species: Red mullet (*M. barbatus*), European hake (*M. merluccius*), Tub gurnard (*T. lucerna*) and Thinlip mullet (*L. ramada*), from the Montenegrin Adriatic coastline. The locations were selected in view of their different characteristics: Platamuni is an open sea locality, Valdanos, a locality of low anthropogenic and industrial influence and Bar is a locality of intense industrial and urban activity. As can be seen on Figure 2A, within the same species, as judged by Coomassie-staining, neither qualitative nor quantitative differences in the protein profiles were observed regardless of whether comparisons were made between samples that were obtained from different localities. However, interspecies differences in the protein profiles were evident.

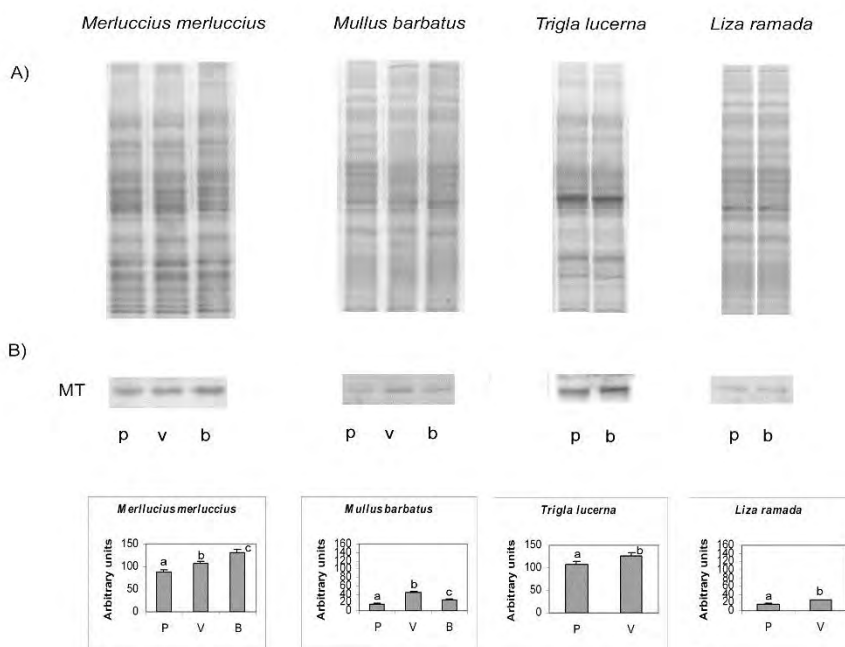


Figure 2. Electrophoretic profiles (A) and immunoblot analysis with anti-metallothionein antibody (B) of the supernatant protein fraction prepared from the hepatopancreas of *Merluccius merluccius*, *Mullus barbatus*, *Trigla lucerna* and *Liza ramada*; P – Platamuni, V – Valdanos, B – Bar. The values are presented as the mean \pm S.E.M.; values not sharing a common superscript letter differ significantly at $P < 0.05$.

According to the immunoblot analysis comparison between localities showed the highest MTs induction in the hepatopancreas in fish from Bar and the lowest in Platamuni (Fig. 2B). Previously we showed that the induction of MTs in *M. merluccius* and *M. barbatus* correlated with elevated concentrations of lead (Pb) (0.022 mg/L) and copper (Cu) (0.009 mg/L) determined by chemical analysis of the seawater from Valdanos and Bar (Mihailovic *et al.*, 2010). According to the Environmental Quality Standards for the Mediterranean Sea in Israel for the examined metals the recommended average concentration for both Pb and Cu is 0.005 mg/L. The minimal concentrations of Pb and Cu that have a negative impact on marine organisms are 0.005 and 0.003 mg/L, respectively. MTs concentrations increase in correlation with the extent of Cu accumulation in fish. The excess of Cu ions disrupt ion homeostasis, especially in gills of fish species, which might lead to a significant adverse effect on the viability of the fish (Dang *et al.*, 2001). Pb compounds could result in oxidative stress in various tissues along with the generation of reactive oxygen species, causing defects of cellular functions. Pb treatment has been known to associate with decreased sperm count, motility, and increased morphological abnormalities in animals and humans (Hsu *et al.*, 1997).

Western blot analysis also revealed the highest induction of MTs in the hepatopancreas of the examined fish species in the following order: *M. merluccius*, *T. lucerna*, *M. barbatus* and *L. ramada* (Fig. 2B). Considering that MTs play a role in the metabolism of essential metals, they are constitutively expressed. The presence of elevated concentrations of both essential and toxic metals provokes the induction of MTs (Muto *et al.*, 1999). These results are in correlation with literature data showing that fish species differ in their detoxification capacities and amounts of accumulated metals (Siscar *et al.*, 2014).

We conclude that the level of induction of MTs in the hepatopancreas of the examined fish species correlates with the level of their reliability as bioindicator species in heavy metal biomonitoring.

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