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Age- and tissue-dependent metallothionein and cytosolic metal distribution in a native Mediterranean fish, *Mullus barbatus*, from the Eastern Adriatic Sea

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Abstract

The levels of metallothionein (MT), a biomarker of metal exposure, and of cytosolic metals (Zn, Cu, Cd), known as MT inducers, were investigated as variables of age (1 to 8 years) and tissue mass (liver, kidney, brain) of red mullet (*Mullus barbatus*). Within the age from 1 to 8 years the most significant increase is evident for cytosolic Cd in liver (43-fold) and in kidney (5-fold). MT and essential metals are constant with age or slightly increased. Over the growth period, statistically significant MT and metal increase is evident only between 1 and 6–8 years old specimens, while for Cd in liver and kidney cytosol significant increase already exists at 4 years old specimens. Metal distribution in all tissues follows the order: Zn>Cu>Cd, with even 500–800 times lower Cd levels than essential metal levels. Consequently, MTs follow the levels of essential metals, Zn and Cu, indicating MT involvement in homeostasis of essential metals. In contrast to kidney and brain, hepatic MT levels are not age-dependent. Inclusion of hepatic MT measurements and the associated cytosolic metals will be useful in the assessment of long-term metal effects in demersal fish *M. barbatus*.

Keywords: Metallothionein; Zinc; Copper; Cadmium; Teleost; *Mullus barbatus*; Tissue distribution; Natural variation; Age; Growth

1. Introduction

Applicability of metallothionein (MT) as a biomarker of biological effects of metals in the field study firstly requires suitable selection of indicator organs and species. Secondly, many abiotic and biotic factors, like water pH, temperature, salinity, fish age, size, sex, season, feeding habits, reproductive cycle, influence metal uptake and accumulation in different tissues and consequently, MT levels (Al-Yousuf et al., 2000; Langston et al., 2002; Canli and Atli, 2003). Thus, before applying MT in a biomonitoring program, more information is needed on endo- and exogenous factors influencing cellular MT and metal content (Hamza-Chaffai et al., 1995; Handy et al., 2003).

Fish represent species at the top of the aquatic food chain, which as vertebrates respond strongly to stress conditions (Weber et al., 1992). Therefore, they are often used as indicator species of pollutant exposure in the aquatic environment. Metal absorption in fish is carried out along gill surface and gut tract, followed by blood transport to other organs (Chevreuil et al., 1995). Higher levels of heavy metals are accumulated particularly in target organs of fish species (Bustamante et al., 2004). Cadmium predominantly accumulates in liver and kidney (Dallinger et al., 1997; Olsvik et al., 2001; Van Campenhout et al., 2004), Zn in muscle, skin and bone, while Cu in liver (Olsson et al., 1998). Metal accumulation depends on tissue metabolism and differences in their chemical environment, including presence of various ligands, each with different metal-binding characteristics (Kito et al., 1986). In fish, trace metal detoxification processes depend mainly on metal binding to MTs. As a consequence, any relationship between MT and metal exposure is easier to demonstrate in fish (Mason and Jenkins, 1995), but the double role of MTs in homeostasis of essential metals (Zn, Cu) and detoxification of nonessential metals (Cd, Hg, Ag) requires cautious interpretation of field data.

Our previous paper (Filipović and Raspor, 2003) referred to the preliminary interpretation of data on *Mullus surmuletus* specimens of 1 to 2 years old, only. MT and cytosolic Zn

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levels were significantly and highly correlated to fish length and body mass, but the age classes were too limited for statistical comparison. Significant progress achieved in this paper refers to an extensive database of overall 74 specimens of *Mullus barbatus*, which enables reliable interpretation of MT and cytosolic metal fluctuations over the age range from 1 to 8 years. Age-related levels of total cytosolic proteins, specific cytosolic proteins like MTs and MTs inducers (Zn, Cu and Cd) in liver, kidney and brain of *M. barbatus*, a representative benthic, Mediterranean fish recommended by UNEP (1999), are selected for the assessment of chronic metal pollution. Liver and kidney are chosen as usual indicator organs, responsible for metal detoxification and excretion, unlike brain which has different metabolic characteristics without significant role in metal detoxification, and therefore, in our study serves as a "reference" tissue.

2. Materials and methods

2.1. Fish sampling

Sampling was performed in 2002 and 2003 in the Croatian coastal area of the Eastern Adriatic Sea called Kaštela Bay and Split Channel (Fig. 1). This part of Eastern Adriatic is densely populated and urbanized. It receives urban, agricultural and industrial wastewaters. Assessment of the past and present contamination of the Kaštela Bay was the objective of many studies reporting the distribution of trace metals (Mn, Cr, Cd, Pb) in the sediments of different grain size fractions (Ujević et al., 2000). The decrease of Cd, Pb, Cu and Zn concentrations along the sediment depth-profile indicates their anthropogenic origin (Bogner et al., 1998). Native specimens of *M. barbatus* (overall $n=74$) were sampled in a period after spawning (October of two successive years), to minimize the influences of additional factors caused by enzymatic changes, especially in females (Hamza-Chaffai et al., 1995). Fish sampling was performed by a trawl. Captured fish was kept alive in aerated water tank until further processing in the laboratory. First, morphometric data were collected, including measurement of total length, body, liver, kidney and brain mass and isolation of scales and otoliths for age determination. Sex was not determined histologically, as would be appropriate. Visual observation was not reliable due to mostly empty gonads in the postspawning period. Tissue dissection included liver, kidney and brain for total cytosolic protein, MT and metal (Zn, Cu, Cd) analysis.

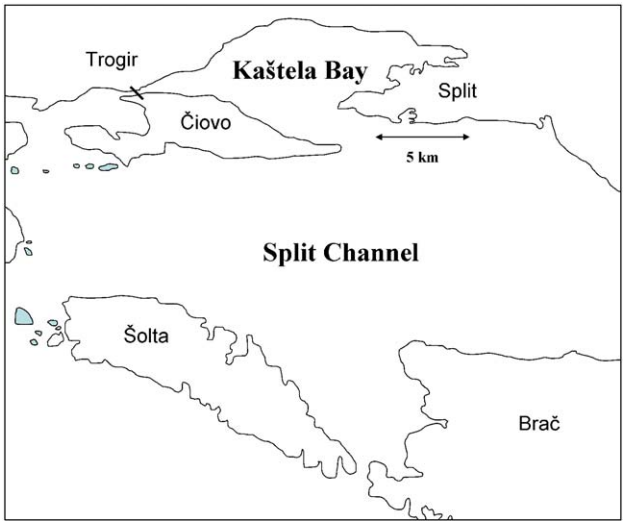

Fig. 1. Fish sampling locations in the Kaštela Bay and Split Channel (Eastern Adriatic Sea). *M. barbatus* was caught by the trawl in October 2002 and 2003.

2.2. Age determination

Age was determined by counting summer and winter growth zones which appear on calcified structures of fish body, scales and otoliths. Scales were taken dorsolaterally below the dorsal fin and otoliths from the inner ear. Determination was performed under stereo microscope (Carl Zeiss).

2.3. Tissue preparation

Tissue of liver, kidney and brain was homogenized by Potter–Elverhjem glass homogenizer with PTFE piston at 4 °C in 5 volumes of buffer, pH=7.5 (at 25 °C). Homogenizing buffer contained 100 mM Tris/Base (Merck), 1 mM DTT (Sigma) and 150 mM KCl (Kemika). In order to isolate cytosolic supernatant (S50), the homogenates were centrifuged in the Sorval RC28S centrifuge by Du Pont at 50,000×*g* for 2 h at 4 °C. Follows ten times dilution with 0.9% NaCl, Suprapur (Merck) and heat treatment of the supernatant (S50) in The Dri Block (Techne) at 85 °C for 10 min (Erk et al., 2002). Heat treated S50 were left on ice for 30 min at 4 °C, then centrifuged at 10,000×*g* for 15 min at 4 °C and stored at −80 °C until MT and metal analysis.

2.4. Total cytosolic protein determination

Total cytosolic proteins were measured according to Lowry et al. (1951). Reagent A (copper tartrate, Bio-Rad) and Reagent B (Folin reagent, Bio-Rad) were added in 10 (brain tissue) or 15 times (liver and kidney tissue) diluted cytosolic fractions (S50) and measured in a Varian DMS 80, Cary 4 spectrophotometer at 750 nm with 0.2−2 mg ml^{-1} of bovine serum albumin (Serva) as a standard.

2.5. MT determination

MTs were determined in heat treated S50 cytosolic fractions of indicator organs by differential pulse polarography on μAutolab (Eco Chemie, The Netherlands), according to the modified Brdička procedure (Raspor et al., 2001). Solution was thermostated at 7±0.1 °C and thoroughly deaerated with extra pure nitrogen. MTs were quantified from the calibration straight line using commercially available >95% pure native zinc-MT (I+II) from rabbit liver (Ikzus), in the concentration range from 0.02 to 0.15 μg mL^{-1}. Applying specific isolation procedure (Dabrio et al., 2002) rabbit liver MT (I+II) is operationally defined as an appropriate standard for intra- and inter-laboratory comparison.