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Autometallography and metallothionein immunohistochemistry in hepatocytes of turbot (*Scophthalmus maximus* L.) after exposure to cadmium and depuration treatment

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In this study, autometallography and immunohistochemistry were used to localize and quantify cadmium and metallothionein (MT) levels, respectively, in cellular compartments of turbot liver on exposure to cadmium for 7 days and further depuration treatment for 14 days. Metals weakly bound to proteins (i.e. MTs) in hepatocyte lysosomes were visualized as black silver deposits (BSDs) using a light microscope. With the aid of a newly developed immunohistochemical procedure, MTs were localized and semi-quantified in both the cytosolic and the lysosomal compartments of hepatocytes. The BSD extent in the lysosomes of hepatocytes increased significantly as a result of cadmium exposure. This response was evidenced after 1 h. Further, a progressive increase in the volume density of BSDs occurred up to the seventh day. Total MT immunohistochemical levels increased at a lower rate, starting after 1 day of cadmium exposure. BSD extent values recovered after depuration, whilst MT levels remain unchanged. It is possible that the detoxification rate of metals via lysosomes was diminished, whilst MT levels remained unchanged, at least after 14 days of depuration. It can be concluded that autometallography and MT immunohistochemistry are good tools for clarifying metal and metal–MT trafficking routes in hepatocytes, and also that BSD extent and MT immunohistochemical levels in the lysosomes and cytosol of fish hepatocytes can be considered to be useful biomarkers of metal exposure.

Keywords: biomarkers, metal pollution, fish, liver, hepatocytes, lysosomes, metallothioneins, autometallography, immunohistochemistry

Introduction

Management and conservation of aquatic resources require the means to establish and predict the impact of single contaminants and contaminant mixtures on individual organisms, populations and communities (Hylland *et al.* 1992). At present, methods tend to involve the analysis of cellular biomarkers that reflect an early stage in a potentially harmful process, thus are measuring a

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short-term response to a specific pollutant or group of pollutants (Cajaraville *et al.* 2000).

Autometallography (AMG) allows the cell localization of metal ions, which appear as black silver deposits (BSDs) in different cell compartments of molluscan and fish tissues (Soto *et al.* 1996, Dang *et al.* 1999). This technique is very sensitive; only very few atoms of the metal are needed to catalyse the deposition of metallic silver around them (Danscher, 1981). Although AMG is not fully specific for particular metals, reports based on electron microprobe analysis have confirmed that certain metals, such as cadmium, are accumulated within the lysosomal compartment of different cell types when marine animals are exposed to the metal (Marigómez *et al.*, 2002). The combination of AMG and image analysis has allowed the quantification of metal levels in the cells and tissues of marine invertebrates, and is considered to be a useful tool for assessing the bioavailable fraction of metals (Soto and Marigómez 1997, Marigómez *et al.* 2002).

Environmental exposure of fish to metals has been shown to result in hepatic accumulation of the metals associated with metallothioneins (MTs) (Olsson *et al.* 1998). Metals are soft electron donors and will therefore readily bind to soft acceptors, such as sulphydryl groups. MTs are low molecular weight (6–7 kDa), cysteine-rich proteins found in all vertebrates and in the majority of eukaryotic organisms, which bind group 1B and 2B heavy metals (Olsson *et al.* 1998). This protein family is involved in the homeostasis and storage of copper and zinc, and, since its synthesis is induced by group 1B and 2B metals, it plays a protective role against the toxic effects of metals (Kägi and Kojima 1987, Kägi and Schäffer 1988, Roesijadi 1994, Nordberg 1998, Binz and Kägi 1999). In addition, MTs display *in vitro* oxyradical scavenging capacity, suggesting a crucial role in neutralizing hydroxyl radicals (Viarengo *et al.* 2000). However, free radicals are not as strong inducers of MTs as some metals are (Olsson *et al.* 1998). Therefore, the induction of fish hepatic MTs has been employed as a biomarker of heavy metal pollution in both freshwater and marine environments (Hylland *et al.* 1992), being quantified, usually, using differential pulse polarography (DPP) or enzyme-linked immunosorbent assays (ELISAs) (Duquesne *et al.* 1995). The use of MT immunohistochemistry has been hitherto restricted to the detection and quantification of MT levels in several tissues of mammals (Mullins and Fuentealba 1998, Tuccari *et al.* 2000); data concerning aquatic organisms are scarce (Dang *et al.* 1999, Soto *et al.* 2000). Additionally, it seems that AMG is not able to detect extralysosomal cadmium (present in cadmium–MT complexes), since cadmium tightly bound within the protein core is not accessible to autometallographic silver. Only when these stable cadmium–MT complexes are semi-digested in the digestive cell lysosomes, exposing the bound cadmium ions, can BSDs be formed to reveal the toxic metal (Soto *et al.* unpublished data).

The present study aimed to achieve two objectives: (1) to develop a immunohistochemical semi-quantitative procedure to determine MT levels in fish liver hepatocytes to be applied in metal pollution monitoring programmes based on biomarkers; and (2) to assess the degree of exposure to cadmium in *Scophthalmus maximus* through two complementary cytochemical approaches (MT immunohistochemistry and AMG) that reveal two different fractions of metals within cells – that bound to MTs (in cytosol and lysosomes), and that unbound or weakly bound to MTs (in lysosomes) (Soto *et al.* unpublished data).

Materials and methods

Experimental design

Sixty turbots, *Scophthalmus maximus*, all within the weight range 250–350 g, were acquired from a fish farm (Culmanor SA, Donostia) and taken to the laboratory. They were maintained in a temperature-controlled (17–19°C) semi-static flow system, with active charcoal- and glass-filtered seawater. Tanks were continuously aerated, the water was changed every second day, and food was supplied *ad libitum* during the experiment. After acclimatization to laboratory conditions for 5 days, two groups of 20 fishes each were exposed to two sublethal doses of cadmium – 10 µg $Cd\,l^{-1}$ and 100 µg $Cd\,l^{-1}$ (as $CdCl_2$ dissolved in water) – for 7 days. After 7 days of cadmium exposure, turbots were subjected to a 14-day depuration period. Twenty fishes were used as controls and were not exposed to cadmium. Liver samples from five different turbots per treatment (cadmium exposures and control) were excised after the first hour, the first day, the seventh day and after the 14-day depuration period. Individuals were anaesthetized with ice. Liver samples were fixed in Bouin's solution (Martoja and Martoja-Pierson 1970) for 24 h at 4°C, and processed normally before paraffin embedding. Sections (7 µm thick) were cut using a Leitz 1512 microtome (Leica). For metal and MT quantification purposes, sections were mounted with Mayer's albumin (Martoja and Martoja-Pierson 1970) and on silane-coated slides, respectively.

Metal quantification

The procedure used to demonstrate metals in the tissue sections was AMG, which is a histochemical technique based on principles of photography (Soto *et al.* 1998a). Paraffin sections were dewaxed in xylene, hydrated in ethanol–water mixtures, and left in an oven at 37°C until completely dried. Tissue sections were covered with a photographic emulsion (Ilford Nuclear Emulsion L4) under safety light conditions. After drying for 30 min in total darkness, sections were rinsed in a developer bath (1:5, b/w Ultrathin Tetenal) for 15 min, rinsed in a stop bath (1% acetic acid) for 1 min, and finally rinsed in a fixative bath (1:10, b/w Agefix Agfa) for 10 min (Soto *et al.* 1998a). Sections were mounted in Kaiser's glycerol gelatine (Merck). Metals were demonstrated as BSDs and were quantified by means of an image analysis system. The volume density of BSD (VD_{BSD}) was calculated as:

$$VD_{BSD} = V_{BSD}/V_{Ti}$$

where V_{BSD} is the volume of BSDs and V_{Ti} is the volume of tissue.

MT quantification

Sections were dewaxed in xylene, hydrated in acetone, rinsed in distilled water and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by shortly incubating the sections in 3% hydrogen peroxide. Sections were then washed in PBS and incubated at room temperature, inside a moist chamber, for 30 min with a blocking solution consisting of 5% normal goat serum and 1% bovine serum albumin (BSA) diluted in PBS. After a brief rinse in PBS, sections were incubated overnight, inside a moist chamber, at 4°C with rabbit anti-cod-MT antibody (630 NIVA) diluted (1:500) in 1% BSA-PBS. Previous immunoblots showed that turbot liver MT exhibited positive cross-reactivity with the anti-cod MT polyclonal antiserum (Hylland *et al.* 1995, Soto *et al.* 2000). After several baths in PBS, sections were incubated for 1 h at 4°C, inside a moist chamber, with biotinylated goat anti-rabbit IgG antibody (A-6154 Sigma) diluted (1:20) in 1% BSA-PBS. They were then rinsed in PBS and incubated with ExtrAvidin-Peroxidase (Extra 3 Sigma) (1:20) in PBS for 30 min. Following several rinses in PBS, the visualization of peroxidase activity was achieved with 3-amino-9-ethylcarbazole (AEC) (A-6926 Sigma), using 200 µl AEC (8 mg ml^{-1} in *N*,*N*-dimethylformamide) in 4 ml of 0.05 M sodium acetate (pH 5.2) plus 2 µl of 30% hydrogen peroxide. Finally, after a brief rinse in PBS, sections were counterstained with haematoxylin (5–10 s), washed in running tap water, and mounted in Kaiser's glycerol gelatine (Merck). PBS was used instead of the primary antibody solution for the control sections.

The semi-quantitative assessment of the MT immunohistochemical levels found in the liver sections was performed on a consensus basis by two observers (A.A. and N.A.) using a Laborlux S (Leitz) light microscope. After establishing the criteria on which to base a consensus, a trial was done performed which showed no significant differences between the results obtained by the two observers using one-way analysis of variance (ANOVA) ($p < 0.001$). According to the method described by Tuccari *et al.* (2000), the percentage of stained hepatocytes (staining score) was graded as follows: 0, no staining; 1, >0 to 5%; 2, >5 to 50%; 3, >50%. Additionally, an intensity distribution index (IDI) was calculated by multiplying, for each case, the staining score by the staining intensity (1, weak; 2, moderate; 3, strong).

Statistical methods

Data from microscopic observations of MT immunoreaction were normalized using the arcsine transformation (Sokal and Rohlf 1995) before proceeding with the statistical analysis. A two-way ANOVA was performed (Sokal and Rohlf 1995) in order to determine the effect of concentration and time of exposure on the BSD extent and MT immunohistochemical levels in hepatocytes. This was complemented by the Duncan test for multiple comparisons between pairs of means ($p \leq 0.05$) using SPSS/PC+ (SPSS Inc., Microsoft) software.

Results

Autometallography revealed the presence of BSDs in hepatocyte lysosomes of both control and cadmium-exposed turbots (figure 1A–B). There were no differences in the distribution of hepatocytes with BSDs in the different liver zones. The BSD extent and BSD size were higher in cadmium-exposed turbots than in controls (figure 1A–B).

According to the quantitative analyses, both exposure time (T) and concentration (C) significantly affected the VD_{BSD} (table 1). VD_{BSD} was significantly raised after 1 h of exposure to both cadmium concentrations used (Duncan's test, $p \leq 0.05$). Further increasing trends at longer exposure times are not statistically significant, although the highest VD_{BSD} values were recorded in the 100 µg Cd l^{-1}

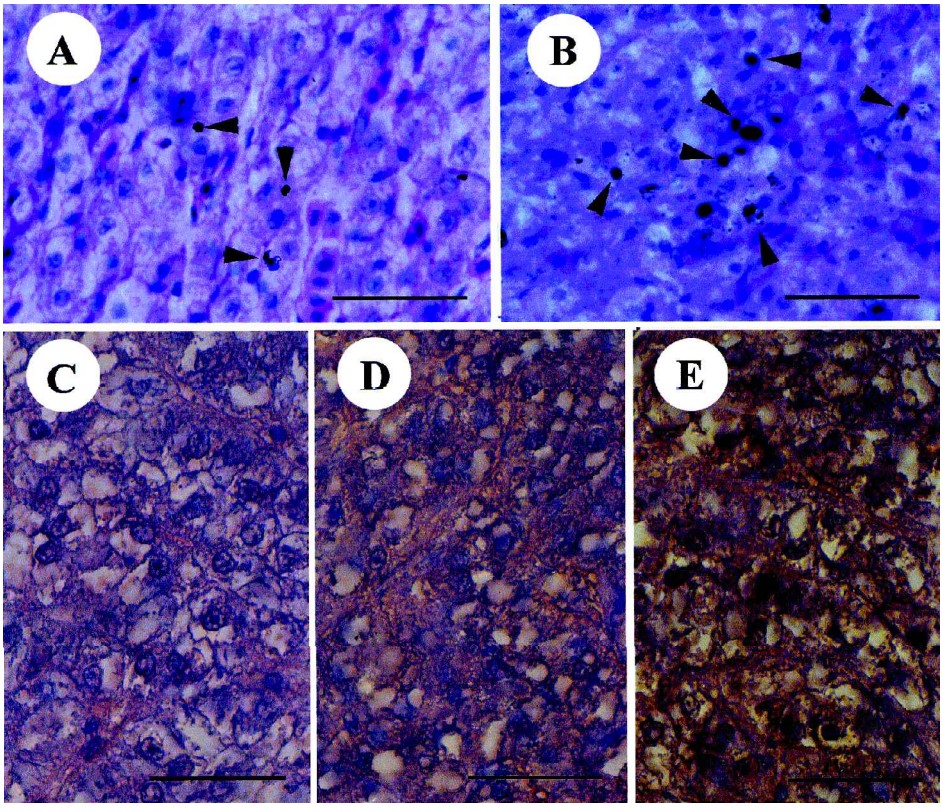

Figure 1. A–B. Autometallographical localization of metals after 1 h in hepatocytes of controls (A) and cadmium-exposed turbot (100 µg Cd l^{-1}) (B). C–E. Immunolocalization of MTs in liver sections of controls after 1 h (C) and cadmium-exposed turbot (D, 10 µg Cd l^{-1} for 1 h; E, 100 µg Cd l^{-1} for 7 days). Scale bars: 30 µm.

Table 1. Summaries of the F ratios from two-way ANOVAs carried out to assess the effect of exposure time (T) (d.f. = 2), concentration (C) (d.f. = 2) and their interaction ($C \times T$) (d.f. = 4) on VD_{BSD} and IDI_{MT} in *S. maximus* exposed to $CdCl_2$ (residual d.f. = 36).

	F ratio		
Biomarker	T	C	$C \times T$
VD_{BSD}	4.879*	54.906**	0.569
IDI_{MT}	3.518*	18.210**	3.840*

d.f., degrees of freedom; *, $p \leq 0.05$; **, $p \leq 0.001$.

group. Accordingly, ANOVA revealed that both C and T but not $C \times T$ affected the BSD extent in hepatocyte lysosomes. It is therefore shown that a threshold in BSD extent is reached very promptly after cadmium exposure (figure 2). It also appears that the BSD extent was quite rapidly reduced after cadmium exposure ceased, as the VD_{BSD} values were similar between the control and cadmium-exposed groups after 14 days of depuration (figure 2; Duncan's test, $p \leq 0.05$). Moreover, these VD_{BSD} values were not statistically different from those of the controls at the beginning of the experiment (figure 2).

Immunohistochemistry using rabbit anti cod-MT revealed the presence of MTs in the hepatocytes of control and cadmium-exposed turbots as a brownish precipitate (figure 1C–E), which was evenly distributed in the liver tissue sections. MTs have been also localized in macrophages and blood cells present in the liver sinusoids. In hepatocytes, MTs appear to occur in both the cytosolic and the lysosomal compartments. The extent and intensity of the immunohistochemical staining was higher in cadmium-exposed turbots than in controls (figures 1C–E). The semi-quantitative assessment was restricted to determining the MT levels in

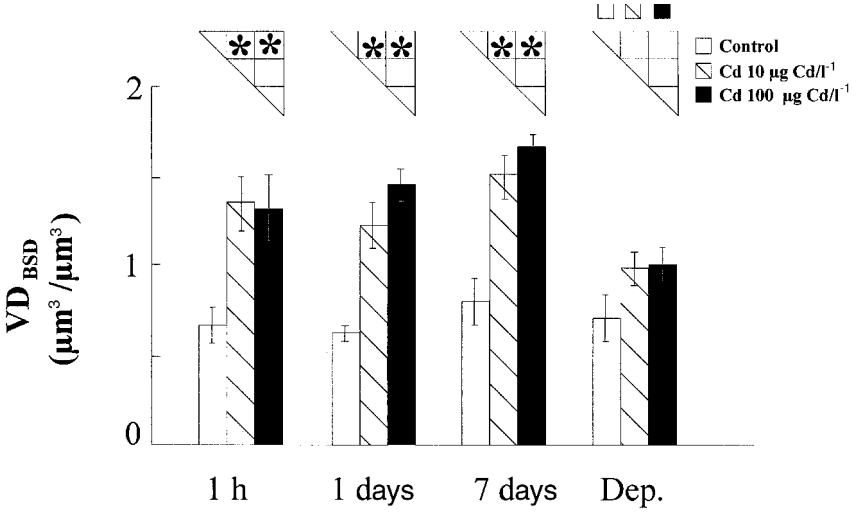

Figure 2. Quantification of metal ions by means of the VD_{BSD} (mean ± SD) in hepatocytes of *S. maximus* after 1 h, 1 day and 7 days of cadmium exposure and then subjected to depuration (Dep.) (14 days). The asterisks in the upper matrix indicate significant differences between pairs of means based on Duncan's tests ($p \leq 0.05$).

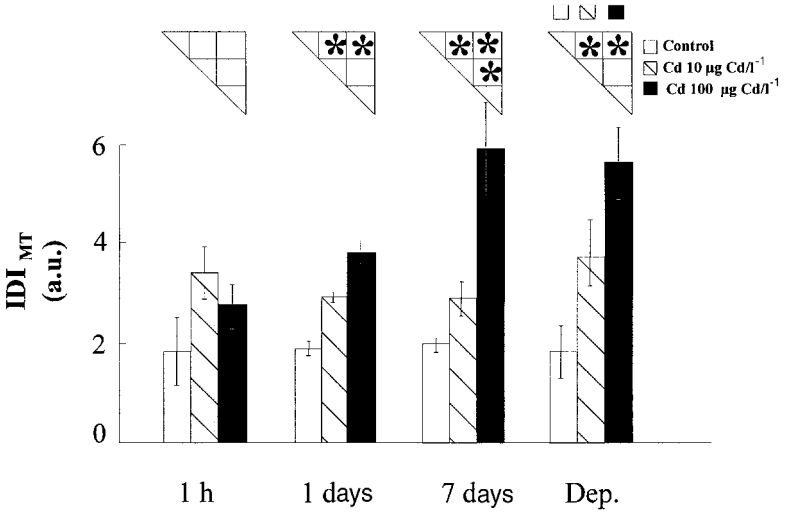

Figure 3. Semi-quantification of MT levels as the IDI_{MT} (mean ± SD) in hepatocytes of *S. maximus*. The asterisks in the upper matrix indicate significant differences between pairs of means based on Duncan's tests. a.u., arbitrary units.

hepatocytes. According to the two-way ANOVA (table 1), T, C and the $C \times T$ interaction influenced significantly the IDI of MT (IDI_{MT}) in hepatocytes (table 1). IDI_{MT} values were always low and constant in the control groups throughout the experiment, and increased with increasing exposure time in the 100 µg $Cd\,l^{-1}$ group; values for the 10 µg $Cd\,l^{-1}$ group were in between (figure 3). It was observed that the increase in IDI_{MT} values was not as prompt as the increase in BSD extent. Moreover, no threshold appeared to occur for IDI_{MT} values (figure 3), in contrast to VD_{BSD} values. Interestingly, IDI_{MT} values after 14 days of depuration are similar to those recorded after 7 days of cadmium exposure (figure 3).

Discussion

AMG has been previously reported to be a useful technique for the localization and quantification of metal deposits in cellular compartments of marine animals (Hemelraad and Herwig 1988, Holwerda 1991, Marigómez *et al.* 1996, Soto *et al.* 1996, 1998b, 2000, Alvarado *et al.* 2000). In the present study, metals weakly bound to proteins were readily visualized using a light microscope as BSDs in the lysosomes of turbot hepatocytes. In contrast, the cytosol was devoid of BSDs. These observations are in agreement with previous ones reporting BSDs restricted to the endo-lysosomal compartment in the digestive cells of molluscs (Cajaraville *et al.* 1995, Marigómez *et al.* 1996, 2002, Soto *et al.* 1996, 1999).

The BSD extent in the lysosomal compartment has been proposed as a metal exposure biomarker that reflects the levels of bioavailable metals in the environment (Soto and Marigómez 1997, Soto *et al.* 1996, 1998b, 1999, 2000, Da Ros *et al.* 2000, Porte *et al.* 2001). In the present study the BSD extent (VD_{BSD}) in the lysosomes of hepatocytes increased significantly as a result of cadmium exposure. This response is extremely prompt and was already evidenced after

1 h. Furthermore, a progressive increase in VD_{BSD} values occurred up to the seventh day in both the 10 μg $Cd\,l^{-1}$ and the 100 μg $Cd\,l^{-1}$ treatment groups. Moreover, lysosomal BSD extent is seemingly dependent on both metal concentration and exposure time, as was previously reported for another piscine species (Cinier *et al.* 1999, Tayal *et al.* 2000, Smet *et al.* 2001). The increase in lysosomal BSD extent appears to be a reversible process: 14 days after the cadmium exposure ceased, the VD_{BSD} values are not significantly dissimilar between controls and the 10 μg $Cd\,l^{-1}$ or the 100 μg $Cd\,l^{-1}$ treatment groups. Therefore, it can be concluded that the BSD extent in the lysosomes of turbot hepatocytes increases in one hour in the presence of cadmium and decreases after the end of the exposure period. Thus, as previously proposed for digestive cell lysosomes in molluscs (Soto and Marigómez 1997, Da Ros *et al.* 2000, Marigómez *et al.* 2002), the lysosomal BSD extent in turbot hepatocytes might be considered as a responsive and reliable biomarker of metal exposure.

Induction of MT synthesis has been reported to be a specific and highly sensitive response to heavy metal pollution, as evidenced by different techniques (Sindermann 1995, Viarengo *et al.* 1999, 2000, Cosson 2000). However, in order to minimize sample collection and processing, most biomarkers are determined by applying the same technology. Our research group's aim was to study different biomarkers in tissue sections of the same sample by means of cytochemistry (Cajaraville *et al.* 2000). For this purpose, an important achievement of the present investigation was to localize MTs in turbot liver sections using a rabbit anti-cod-MT antibody that cross-reacted with turbot MTs (Duquesne *et al.* 1995, Hylland *et al.* 1995). MTs occur in both the cytosol and the lysosomes of hepatocytes after cadmium exposure. Thus, whereas MTs and BSD co-localize within lysosomes, it seems that cytosolic cadmium bound to MTs is not revealed by AMG. Accordingly, our group (Soto *et al.* unpublished data) has suggested that, in mussel digestive cells, AMG does not show the fraction of metals tightly bound to MTs in the cytosol but reveals MT–metal complexes in lysosomes when strong linkages are semi-digested by the action of acid hydrolases.

Considering the overall MT immunohistochemical levels in both the cytosol and the lysosomes, a significant increase occurred that was dependent on the cadmium concentration and the exposure time, individually and interacting, which is consistent with MT synthesis induction reported by Roesijadi (1994) and Muto *et al.* (1999) in fish liver. A rapid raise in MT immunohistochemical levels (IDI_{MT}) took place after only 1 day of cadmium exposure, and the highest IDI_{MT} values were found under the highest exposure conditions presently studied (7 days, 100 μg $Cd\,l^{-1}$).

When AMG and MT immunocytochemical results are compared, it is readily obvious that both VD_{BSD} and IDI_{MT} values exhibit an increasing trend as a result of cadmium exposure. However, the IDI_{MT} presents an initially less pronounced slope, which becomes more marked as treatment continues. This attenuated initial response might be attributed to the existence of MT mRNA transcription controls causing a MT synthesis delay, which does not mean that induction is not a fast event (George *et al.* 1996). It seems that most of the inherent MT pool in the cytosol is not completely saturated prior to new MT synthesis (Cosson 2000, Isani *et al.* 2000), and when metal-binding sites on MT are fully occupied by incoming cadmium, uncomplexed metal stimulates the production of MT mRNA and further protein (shown by the increased levels of IDI_{MT} after 1 day of exposure).

However, the inherent non-saturated MT pool may form protein–metal complexes from the very beginning of metal exposure, which are degraded for partial excretion in lysosomes (shown by the increased levels of VD_{BSD} after 1 hour), stimulating further MT production (Roesijadi 1994, Langston *et al.* 1998, Cosson 2000). VD_{BSD} values reached a maximum after 1 day of cadmium exposure, suggesting a saturation threshold of metal sequestration by lysosomes, whilst MT synthesis increased in the cytosol.

Metal ions in excess might be removed by macrophages and blood cells that possess MTs, which would be consistent with the immunohistochemical results obtained. Such removal might contribute to a stabilization of the metal contents in the hepatocytes of exposed fishes beyond certain exposure conditions. Dang *et al.* (1999), also using AMG and MT immunohistochemistry data, concluded that macrophages and blood cells play a crucial role in metal detoxification in freshwater fishes (*Oreochromis mossambicus*) exposed to copper.

As a result of depuration, while the VD_{BSD} decreased to values similar to those of the control group, IDI_{MT} levels did not change. Correspondingly, George *et al.* (1996) treated turbots with a single intraperitoneal injection of cadmium (500 µg Cd kg^{-1}) and observed high MT levels after depuration (MT half-life >21 days). Indeed, Roesijadi and Fellingham (1987) and Olsson *et al.* (1998) demonstrated that pre-exposure of mussels and fishes to low levels of metals provokes an increase in the MT pool and renders the animal more resistant to further exposures to toxic levels of metals and protects against cell damage induced by free radicals (Viarengo *et al.* 2000).

In conclusion, the present results suggest that AMG and MT immunohistochemistry are good tools for clarifying metal and metal–MT trafficking routes in hepatocytes. In addition, the combination of VD_{BSD} and IDI_{MT} values reflect the level of exposure to cadmium in different cell compartments of fish hepatocytes, which renders them useful biomarkers of metal exposure. Their cost-effectiveness deserves comment, since both parameters (VD_{BSD} and IDI_{MT}) are determined on tissue sections (a minuscule sample) taken from the same tissue block, requiring only single processing of samples.

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