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Immunolocalization of metallothioneins in different tissues of turbot (*Scophthalmus maximus*) exposed to Cd

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Summary. Metallothioneins (MT) were localized by immunochemistry in different organs and cell compartments of turbot exposed to sublethal concentrations (100 ppb) of Cd for 7 days. The polyclonal rabbit anti-cod MT antibody (NIVA, Norway) applied herein exhibited positive cross-reactivity with turbot MTs. Immunoreactive MTs were localized in the branchial epithelium, in the liver and in the kidney of turbot. In Cd exposed fishes MTs were demonstrated mainly in branchial chloride cells (CC) and to a lesser extend in the area where progenitor cells are located and in the cells of the respiratory epithelium (secondary lamellae). A higher staining intensity for MTs was observed in CC of the interlamelar space of the main branchial epithelium in comparison with control CC. MT-staining was also observed in the chondroblasts of the cartilage and in the erythrocytes within blood vessels both in control and Cd-exposed specimens. MT immunoreaction was high in the liver hepatocytes and weak in the epithelium of the proximal portion of the kidney in exposed turbot. The tegument, spleen and muscle were devoid of any immunolabelling in both treatments. Ultrastructural studies at the transmission electron microscope revealed that Cd-induced MTs were mainly located in the cytoplasm of gill CC, the lysosomes and the cytoplasm of hepatocytes and in the basal labyrinth of kidney proximal nephrocytes. The differential localization/induction of MTs in different cell types described hereby suggests that the quantification of the specific expression of MT may be used in biomonitoring programs as a biomarker of Cd exposure in aquatic environments.

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Introduction

The study of metallothionein (MTs) has greatly improved the understanding of metal body burdens, metal storage and detoxification processes in aquatic organisms subjected to contamination by toxic metals mainly belonging to groups IB and IIB (Kägi and Schäffer, 1988; Pavicic et al., 1991; Mackay et al., 1993; Roesijadi, 1994, 2000; Hylland et al., 1995; Olsson et al., 1998; Isani et al., 2000). They are also crucial in cell protection against oxidative stress caused by free radicals (Roesijadi, 1994; Klaasen et al., 1999; Viarengo et al. 2000). It is generally recognised that due to their biochemical and functional characteristics, MTs are able to protect cell structures from non-specific interactions with toxic metal cations (i.e. Cd^{2+}) and to detoxify intracellularly accumulated metal excess (Hamer, 1986; Viarengo and Canesi, 1991; Roesijadi, 1992; Viarengo and Nott, 1993; Nordberg, 1998). All these metal cations accumulated within the cells stimulate protein biosynthesis by enhancing MT gene transcription (Olsson et al., 1998; Viarengo et al., 1999, 2000). Due to their inducibility MTs are considered important specific biomarkers that help in the detection of organism exposure to metals such as Cd, Hg, Cu, Zn, etc., present in the aquatic environment (Addison and Clarke, 1990; Hylland et al., 1992; 1996; Geret et al., 1998; Dang et al., 1999; 2001; Amaral et al., 2002). Metallothionein levels have been shown to vary depending on body weight, gender, reproductive stage, size, season and water temperature (Hylland et al., 1992; Roesijadi, 1994; Serra et al., 1999; Serafim et al, 2002). Consequently, these natural fluctuations have strong implications for the use of MTs as biomarker for the determination of metal pollution (Olsson et al., 1998).

This induction of fish MTs has been quantified, applying differential pulse polarography (DPP) or enzyme-linked immunosorbent assays (ELISA) (Palmiter, 1987; Duquesne et al., 1995) among other methods. As an alternative, the use of immunohisto(cyto)chemical techniques recently has been proposed to specifically detect MT levels in cell compartments of target tissues. These immunochemical techniques are very sensitive and allow the detection of very low amounts of protein due to the use of specific antibodies. Immunochemical analyses might be of great importance allowing the detection of the specific cell-types expressing MTs in organs of great complexity. The quantification of MT levels in these selected cell-types could be a more accurate strategy than pure biochemical analyses that do not discriminate among different cellular compartments in certain experimental situations. Thus, previous studies reporting no or low induction in different tissues or organs after metal exposure may be misleading as whole-tissue homogenates were analysed (Burkhardt-Holm et al., 1999). However, the use of MT immunohistochemistry has been restricted to detect and quantify MT levels in several tissues of mammals (Mullins and Fuentealba, 1998; Tuccari et al., 2000), whilst data concerning aquatic organisms are scarce. Recent reports suggested the differential expression of MTs in selected cell-types of the gills of tilapia (*Oreochromis mossambicus*) after exposure to copper (Dang et al., 1999), in gills and kidney of salmon (*Salmo salar*) exposed to Cd (Berntssen et al., 2001; Dang et al., 2001), in liver and gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu and Zn (Amaral et al., 2002; Alvarado et al., 2005, 2006) and in the gills of brown and rainbow trouts (*Oncorynchus mykiss*) environmentally exposed to sewage treatment plant effluents (Burkhardt-Holm et al., 1999).

In the present work, a commercial fish species (turbot, *Scophthalmus maximus*) was exposed to a sublethal concentration of Cd for 7 days. The main purpose of this study was to determine the cell-types that exhibited metal-induced MT expression in liver, gills, spleen, kidney and muscle, using MT-immunohistochemistry. The specific detection of MT expression may allow the accurate identification of metal responsive cell-type(s) that might usefully serve as subjects for biomarker assessments of water quality.

Materials and methods

Experimental design

Twenty juvenile turbots, *Scophthalmus maximus*, all within the weight range 250 to 350 g (approximate age: 6 months) were acquired at a fish farm (Culmanor, S.A. – Donostia). Turbots were maintained in a temperature controlled (17-19°C) semi-static flow system, with active charcoal- and glass-filtered seawater. Tanks were continuously aerated, water was changed every day and food was supplied ad libitum during the experiment. After acclimatisation to laboratory conditions for 5 days, two groups of 5 fish were exposed to 100 ppb Cd (as $CdCl_2$) for 7 d. Remaining 10 fish were used as controls (in two replicate series) and were not exposed to Cd. Five specimens per experimental group were removed from aquaria and anesthetized in ice after 7 days exposure.

Chemicals

All reagents employed were purchased from Sigma unless specified otherwise.

Polarographical detection of metallothioneins

MTs were analyzed by differential pulse polarography assay (DPP) based on –SH group quantification according to Bridcka reaction modified by Olafson and Olsson (1991). Gills, liver, kidney, spleen and muscle of 5 animals were pooled in 3 cryovials and frozen immediately in liquid nitrogen. Tissues were homogenized in four volumes of 20 mM Tris-HCl buffer pH 8.6 in an ice bath. The homogenate was centrifuged at 30000g for 2 hours at 4°C. The pellet was discarded and an aliquot of the supernatant (cytosolic fraction) was used for the determination of protein concentration using the DC protein assay (BioRad, Richmond, CA-USA) based in the method of Lowry et al. (1951) with gamma-globulin as standard. Another aliquot was diluted 10 times with saline solution (0.9% NaCl). The diluted sample was heated at 80°C for 10 min to precipitate high molecular weight proteins, immediately cooled on ice and centrifuged at 30000g for 1 hour at 4°C. Aliquots (50-250 µl) of the heat-treated cytosolic fraction were taken for MT quantification by DPP. Polarographic measurements were made with a 745 VA Computrace (Methrom, Switzerland) analyzer and a static mercury drop electrode (SMDE). Rabbit liver metallothioneins (MT-I and MT-II) were used as reference for the calibration curve according to the method of standard additions. Metallothionein concentration was expressed as µg MT/mg cytosolic protein.

Metallothionein immunochemistry

Western Blots

Excised organs (liver, gills, kidney, spleen and muscle) were individually homogenised in buffer (10 mM Tris-HCl, pH 8.1). Cell debris were discarded with a 2 h centrifugation step at 50000g. Supernatants were considered whole homogenates and were frozen at -80°C until they were required for western blot analysis. Protein concentration of the whole homogenates was determined according to Lowry as specified above. Polypeptides were separated by SDS-PAGE (16.5%) and electrotransferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK) by means of a moist blotting chamber (Bio-Rad, Hercules, CA, USA) for 60

min at a constant current of 190 mA. 12.18 µg protein were loaded per lane. Together with turbot samples, pure cod MT and liver cod samples were loaded in adjacent lanes. Membranes were blocked at room temperature for 1 h with 1% BSA in phosphate-buffered saline (PBS) with 0.05% Tween 20 followed by incubation with the polyclonal rabbit anti-cod MT antibody (MT 630, NIVA, Norway) (1:3000 in PBS Tween) overnight at 4°C. After washing the membranes in PBS-Tween they were incubated at room temperature for 1h with goat anti-rabbit (Sigma) secondary antibody (1:12000 in PBS-Tween). Immunoreactive bands were visualised by means of chemiluminescence (ECL, Amersham). The height of the bands (molecular weight) and their intensity (optical density) was calculated scanning the blots and applying the software 1D-Manager (v. 2.0).

Immunohistochemistry (Light Microscopy)

Samples from liver, gills, kidney, spleen and muscle were fixed in Carnoy's fluid for 1h, dehydrated in alcohol and embedded in paraffin for immuno-histochemical studies. Sections (4 µm thick) were cut using a Leitz 1512 microtome (Leica-Austria), placed on silane-coated slides, dewaxed in xylene, hydrated in acetone, brought to distilled water and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by briefly incubating the sections in 3% hydrogen peroxide. Sections were then washed in PBS and incubated at room temperature inside a moist chamber for 1 h, with a blocking solution that was made of 5% normal goat serum and 1% bovine serum albumin (BSA) diluted in PBS. After a brief rinse in PBS, sections were incubated, inside a moist chamber at 4°C overnight, with the rabbit anti-cod MT antibody (MT 630, NIVA, Norway) (1:500 in PBS containing 1% BSA). After several washes in PBS, sections were incubated, inside a moist chamber at 4°C for 1 h, with a biotinylated goat anti-rabbit antibody (Sigma) (1:20 in PBS containing 1% BSA). Then sections were 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 using a chromogen solution containing 3-amino-9-ethylcarbazole (AEC, 0.5 mg/ mL), (200 µl de 8 mgAEC/ml in N,N-dimethylformamide in 4 ml of 0.05 M sodium acetate (pH 5.2) plus 2 ml of 30% hydrogen peroxide). Finally, after a brief rinse in PBS, sections were counterstained with haematoxylin (5-10 sec), washed in running tap water and mounted in Kaiser's glycerol gelatine (Merck). In control sections PBS was used instead of the primary antibody solution. Micrographs were obtained using an Olympus PM-20 camera attached to an Olympus BX50 light microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Immunocytochemistry (Electron microscopy)

Portions of the same organs were fixed for 2 h by immersion in 4% formaldehyde and 0.25% glutaraldehyde in 0.1 sodium cacodylate buffer (pH 7.2, 4°C), washed in the same buffer, dehydrated in alcohols and embedded in LR-white resin. Sections were cut in a Reichert-Jung ultramicrotome and collected on Formvar coated Ni or Cu grids (200 mesh). Ultrathin sections were blocked with TBS containing BSA (20 mM Tris, 0.1% BSA in physiological saline, pH 7.2) and incubated overnight on a drop of rabbit anti-cod MT antibody containing solution. After several washes on drops of TBS, sections were incubated for 60 min with a colloidal gold (particle size, 10 nm) labelled protein A (1:10 in TBS). Sections were than washed in distilled water, air-dried and counterstained with uranyl acetate. Electron micrographs were obtained with a JEOL JEM-100SX electron microscope (Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 60 keV.

Statistical analysis

Statistical analyses were carried out with the aid of the statistical package SPSS® 10.0 for Windows (SPSS Inc., Chicago, Illinois). Results were reported as mean values ± standard deviations (SD). Data were analyzed by the t test at a significance level of $p<0.05$. Data were analyzed for homogenity of variances (Levene's test) and normality (Kolmogorov-Smirnov test). Data were logarithmically transformed when required in order to obtain a normal distribution of the data and one-way analysis of variance (ANOVA) was performed. Significant differences between pairs of means were established at $p<0.05$ level using Duncan's test.

Results

Polarographic quantification of metallothioneins

The sublethal waterborne Cd-concentration used significantly induced MT synthesis in liver, gills and kidney ($p<0.05$). In liver and gills the Induction Factor in comparison with the same tissues of control individuals was higher than 2 while in the kidney it was 1.2 (Table 1). MT levels obtained for muscle and spleen were below or very close to the detection limit of the polarographer after 7 days of Cd-exposure (Table 1).

Western blot analysis of metallothioneins

Western blot analysis (Fig. 1) revealed that the selected anti-cod MT antibody cross-reacted specifically with turbot MT. Pure MT from cod liver and cod liver samples (used as positive controls) exhibited two bands at 6.12 kDa and 12.14 kDa while only the second band appeared in turbot liver, gills and kidney. The intensity of the bands (optical density) was higher in Cd-exposed turbots than in controls for liver and gills. In the case of kidney Cd-exposed specimens and control ones exhibited similar values, while no reaction was observed in samples from muscle and spleen.

Immunolocalization of immunoreactive MT

The cross-reactivity found with the anti-cod MT antibody used allowed the immunolocalization of MTs in tissue sections of organs from control and Cd-exposed turbot. At the light-microscope the presence of immunoreactive MTs (*ir*MT) was visualized as an easily distinguished brownish precipitate (Fig. 2) and at the transmission electron microscope as electrondense gold particles of 10 nm (Fig. 3). The specificity of the immunostaining was confirmed in control sections that were incubated without specific antibody being consistently negative. *ir*MT were easily observed in liver and gills, and up to a lesser extent in the kidney, of either control and Cd-exposed turbot. The cross reactivity exhibited by spleen and muscle sections was very weak or null both in controls and in exposed fishes.

Gills

The gills of control animals exhibited very few immunoreactive cells that corresponded to chloride cells (CC) located only in the central layer of the filament epithelium (Fig. 2a). As a result of Cd-exposure the number of chloride cells expressing *ir*MT in the filament epithelium was much more numerous than in control animals (Fig. 2b). This increase of CC in the gill epithelium enhances migration of CC up to the edge of the secondary lamellae and provokes the thickening of the epithelium and the fusion of secondary lamellae (Fig. 2b). The immunolabelling observed in the other cell-types (pavement cells, mucocytes, respiratory cells and undifferentiated cells) of the gill epithelium was very weak and did not change as a result of metal exposure (Fig. 2a,b). Some chondroblasts in the cartilage (Fig. 2a) as well as some erythrocytes in the blood vessels, interspersed within lamellae (inset of Fig. 2b), also exhibited irMT in both control and treated specimens.

Liver

Immunoreactive MTs were mainly localized in hepatocytes and, to a lesser extent in erythrocytes of control and Cd exposed turbots (Fig. 2c,d). As observed under the transmission electron microscope (Fig. 3) *ir*MTs were specifically localised in the lysosomes (Fig. 3a) and in the cytoplasm of hepatocytes (Fig. 3b). The immunolabelling produced in hepatocytes after Cd-exposure was stronger than in controls. *ir*MTs have been also localised in macrophages and blood cells present in the liver sinusoids (results not shown).

Kidney

Low levels of *ir*MT were localised in the kidney of both control and Cd- exposed turbots. The immunolabelling was mainly detected in the nephrocytes placed in the proximal tubules of the nephron (Fig. 2e,f) and more precisely in their basal labyrinth (Fig. 3c).

Spleen and muscle

MT immunoreactivity was very weak in spleen and muscle of control and Cd-exposed turbots.

Discussion

MT-induction was shown under present exposure conditions through the use of differential pulse polarography. It has been observed that MT levels increased more than two folds in liver (2.4) and gills

Table 1. MT concentrations (µg MT/mg total cytosolic protein) measured by difference pulse polarography (DPP) in liver and gill samples of control and Cd-exposed turbot (7d). Mean ± Standard deviation, n = 3. IF, induction factor.

ORGAN	MT CONCENTRATION		IF
	Control	Cd-exposed (7 d)	
Liver	0.58±0.24	1.42±0.45	2.44
Gills	0.42±0.12	0.92±0.23	2.19
Kidney	0.37±0.17	0.45±0.21	1.21
Muscle	0.21±0.12	0.15±0.39	0.71
Spleen	b.d.l.	b.d.l.	-

b.d.l., below detectable limit.

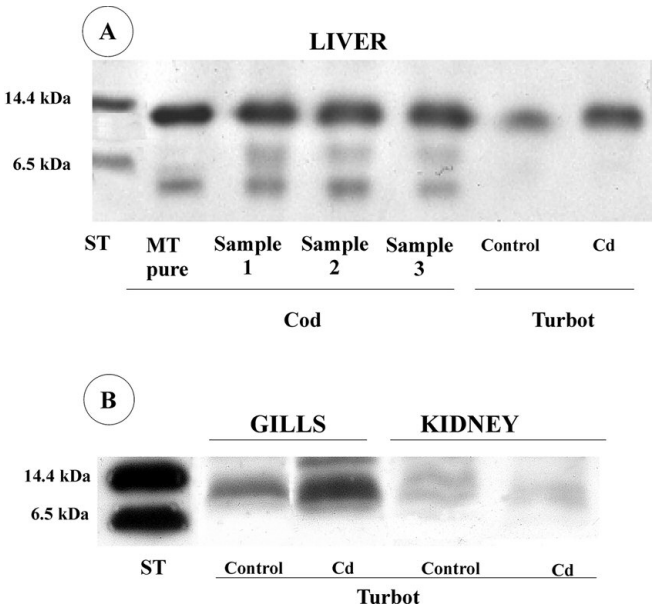

Fig. 1. Results of the immunoblot analysis of SDS-polyacrilamide gels from (**A**) liver, (**B**) gills and kidney homogenates showing cross-reactivity of the cod-anti MT antibody against turbot MT. ST, standards; MT pure, cod pure metallothionein; Cod 1, 2 and 3, cod samples with known exposure to Cd concentrations (inner controls) from a previous intercallibration exercise; C, control turbot; Cd, Cd-exposed (100 ppb) turbot after 7d.

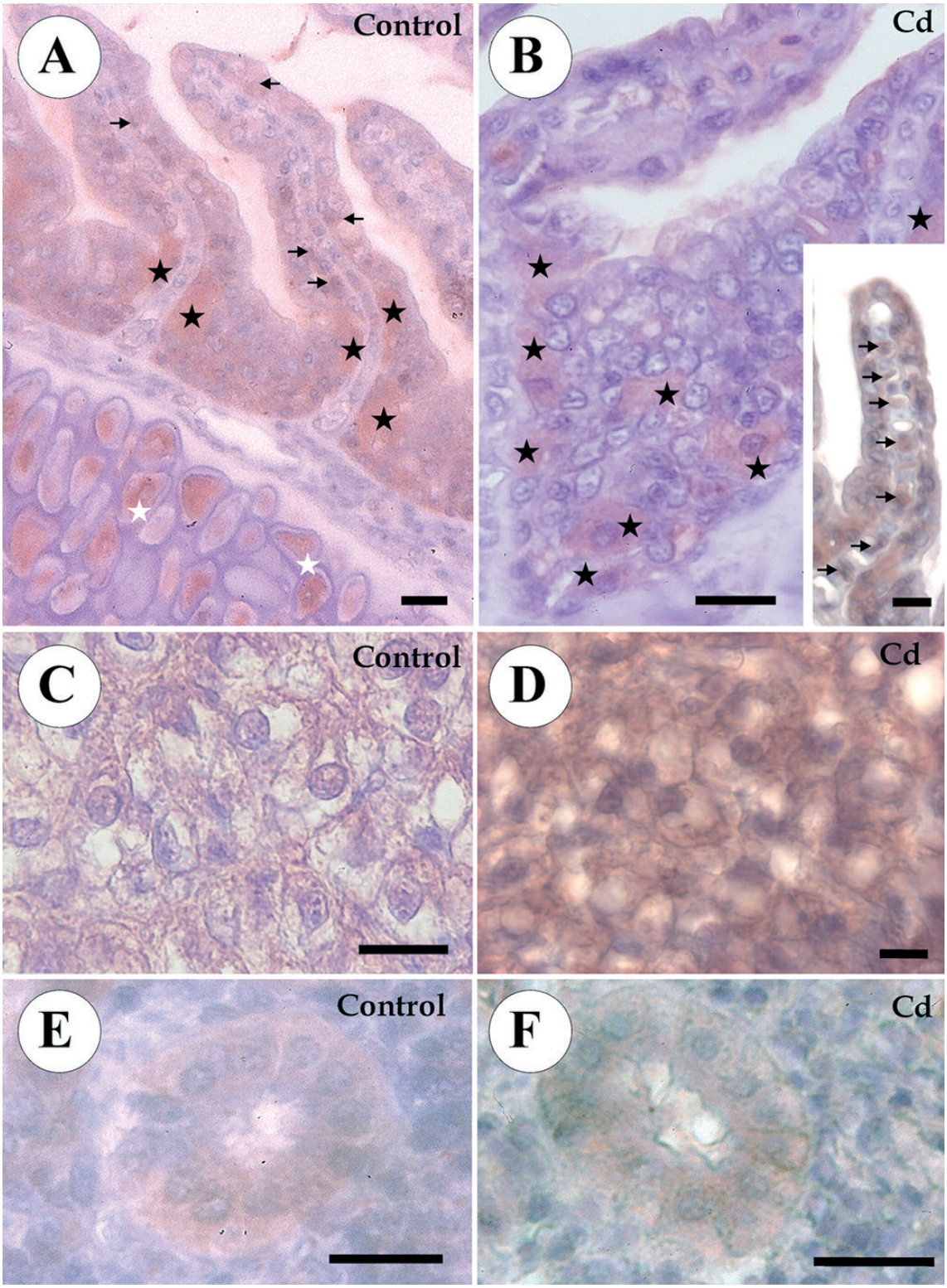

Fig. 2. Light micrographs showing immunolocalisation of metallothioneins in gills **(A-B)**, liver **(C-D)** and kidney **(E-F)** of control and Cd-exposed turbots (100 ppb) after 7 d of treatment. **(A)** Gills of control turbot (7 d). Positive labelling is located in the cytoplasm of CC (asterisks), in the chondrocytes of the cartilage (white asterisks) and in erythrocytes (arrows). **(B)** Intense immunolabelling in the cytoplasm of CC (asterisks) after Cd-exposure. Note the increased number of CC, the thickening of filament epithelium, and immunolabelling in erythrocytes (arrows). **(C)** Hepatocytes of control turbot. **(D)** Intense immunolabelling in the cytoplasm of hepatocytes after Cd-exposure. **(E)** Nephrocytes of control turbot showing weak immunolabelling in the distal tubules, as well as in **(F)** Cd-exposed turbot. Scale bars: A, B, D, inset: 10 µm; C, E, F: 20 µm

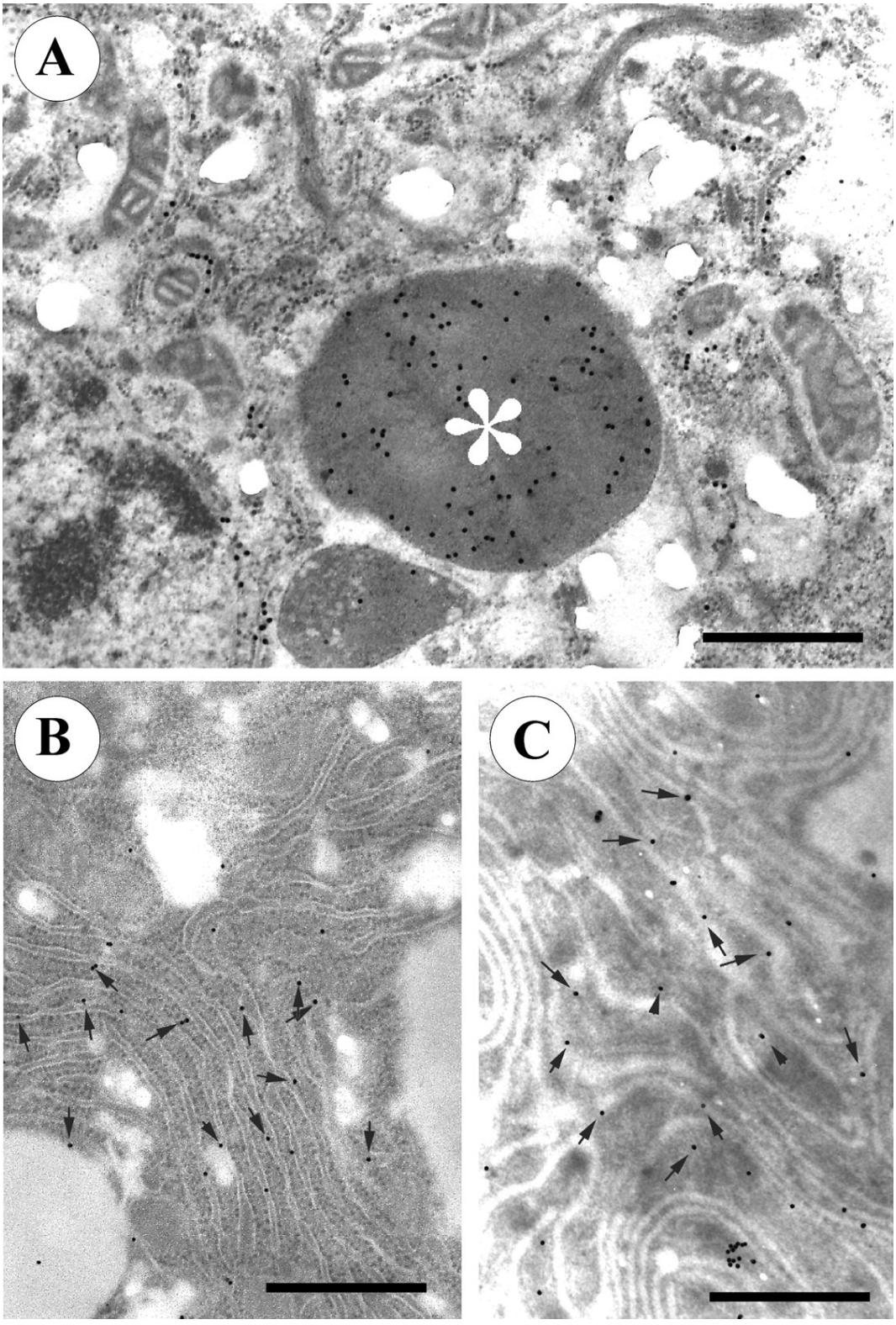

Fig. 3. Immunogold staining showing MT for localization in liver **(A-B)** and kidney **(C)** of Cd-exposed turbots **(A)** Gold particles within a hepatic lysosome (white asterisk). Note the presence of gold particles in the cytosol and **(B)** associated to the endoplasmic reticulum. **C.** Gold particles (arrows) in the basal labyrinth of nephrocytes in the distal tubules of the kidney. Scale bars: 20 µm.

(2.1), and by nearly 20% (approx.) in the kidney of Cd-exposed turbot in comparison with controls after 7d treatment. In contrast, MT induction was not detected in the muscle and in the spleen. However, this method does not allow the determination which specific cell-type(s) express significant induction of MT in the selected organs as a result of metal exposure. As a complement, the use of antibodies against piscine MT may be a good approach to allow the in situ localization of metallothioneins in selected cell-types (Duquesne et al., 1995; Burhardt-Holm et al., 1999). However, antibodies against piscine MTs are scarce and include catfish (*Heteropneustes fossilis*), cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*), dab (*Limanda limanda*) and perch (*Perca fluviatilis*) (Duquesne et al., 1995; Hylland et al., 1995). In the present study and due to the lack of MT antibodies against turbot MT, we used the anti-cod MT antibody that Hylland et al. (1995) demonstrated to cross-react with turbot samples. Western blot analyses carried out herein confirmed those previous results. Alignment of cod MT amino acid sequence with the two complete MT sequences of the order Pleuronectiformes (*Pleuronectes platessa* and *Pseudopleuronectes americanus*) available in the GeneBank database revealed a significant homology (88%) among Pleuronectiformes and Gadiformes MT proteins. This suggests that the MT sequences of gadifomes (cod), and pleuronectiformes (turbot) are similar enough to allow the immunolocalization of turbot MTs employing the antibody raised against cod MT.

The gills are one of the main routes of entry for dissolved metals into the organism (Karlsson-Norrgren et al., 1985; Evans, 1987; Dang et al., 1999, 2001; Mazon et al., 2002) and form a very complex organ formed by different cell types that do not behave in the same way against metal insult. It has been previously reported that when dissolved metals (i.e. Cd, Cu or Zn) enter the gills, at sublethal levels, most of the metal binds to MTs (i.e. about half of the branchial cadmium) thus protecting the cells against metal ion toxicity (Olsson and Hogstrand, 1987; Hogstrand and Wood, 1988; Olsson et al., 1998 Burhardt-Holm et al., 1999; Dang et al., 1999, 2001; Mazon et al., 2002; Jezierska and Witeska, 2004; Alvarado et al., 2006). Interestingly, *ir*MT was mainly localized in the cytoplasm of chloride cells in control and in Cd-exposed turbot. The involvement of this cell type in active ion transport through the gill epithelium (Perry, 1997; Dang et al., 1999) might explain the presence of stable levels of *ir*MT in controls. The presence of chloride cells in control specimens was confined to the trailing edge of the filament epithelium and in the inter-lamellar regions. However, as a result of Cd-exposure, a series of related changes have been observed in the gill epithelium. First, 7 d of exposure to waterborne Cd induced increased immunolabelling (enhanced MT-synthesis) due to increased number of larger CC. Dang et al. (1999, 2001) also found that exposure to waterborne copper and dietary cadmium evoked both an upregulation of *ir*MT expression and increased numbers of CC in two teleost fish species. Second, we observed that the distribution pattern of CC was changed by cadmium exposure. CC from the filament epithelium migrated towards the apex of the epithelium of the secondary lamellae after exposure to Cd. Both proliferation and migration responses are not only produced by metals (Evans and More, 1988; Jezierska and Witeska, 2004; Alvarado et al., 2006), but other toxicants such as acid water and sewage treatment plant effluents also (Leino and McCormick, 1984; Pawert et al., 1998; Burkhardt-Holm et al., 1999).

The other cell types within the gill epithelium (pavement cells, chloride cells, mucocytes, respiratory cells and undifferentiated cells) either did not express *ir*MT or expressed the protein at very low rates even in the presence of Cd. It could be hypothesized that the Cd-concentrations used herein (100 ppb) and the exposure time (7 days) were not high and long enough to produce neosynthesis of MTs in these cell types. In any case, Dang et al. (1999, 2001) did not observe immunolabelled cell-types apart from chloride cells in the gills of tilapia exposed to 200 ppb waterborne Cu and in the same fish fed with Cd (from 0.2 to 125 mg/kg dry weight). Within the gills, but outside the gill epithelium, the chondroblasts of the supporting cartilage also exhibited immunoreactive MTs both in Cd exposed and control specimens suggesting that this expression was not related to Cd-exposure. There is evidence that Zn, incorporated in scales, originates from lysosomes in osteoblasts and chondroblasts (Saur and Watabe, 1989; Olsson et al., 1998). Zinc coexists in this cell compartment with sulphur-rich compounds (i.e. metallothioneins) (Olsson et al., 1998). It can be concluded that, under present exposure conditions, Cd-exposure induced the synthesis of *ir*MT in the gills. However, in some studies observed no increase in MT levels after metal exposure (Farag et al., 1995) whereas others reported that MT levels were significantly induced in the gills (George et al., 1996; see review by Olsson et al., 1998). This controversy may be related to the functional and physical heterogeneity (cell types and associated specific functions) of this organ that results in dissimilar cell MT expression levels (chloride cells vs. other cell-types). The studies reporting no MT induction in gills may be misleading since the analyses of whole gill homogenates may "dilute" MT levels by pooling together cell types expressing MTs with cell types that do not express the protein. It can be concluded that the assessment of total MT levels in the gill may be more reliable by measuring *ir*MT in the chloride cells and could be used as a useful biomarker of metal exposure.

When Cd enters the blood after crossing the gills it is present in the plasma pool and it is gradually transferred to the erythrocytes until an equilibrium is reached between the Cd pools in erythrocytes and plasma (Watkins et al., 1977; Tanaka et al., 1985). Accordingly, immunolabelled erythrocytes were clearly observed

under present exposure conditions confirming the presence of MTs and their function as Cd-binding proteins in these cells (Tanaka et al., 1985). Cd is primarily distributed to the liver where, during chronic exposure to sublethal levels from 60 to 70% of the metal is sequestered by MTs, and to a lesser extent to the kidney (Olsson and Haux, 1986; Hogstrand et al., 1991). The induction of fish hepatic MTs has been employed as a biomarker of exposure to metals in both freshwater and marine environments (Hylland et al., 1992; Roesijadi, 1994; Muto et al., 1999). As a general rule, most of the metal stored in the liver is within the cytosol of hepatocytes (Wicklund Glynn et al., 1992) since the primary metal-binding protein, MT, is cytosolic (Olsson et al., 1998). Immunohistochemistry revealed an increase in MT protein production in Cd-exposed hepatocytes, in agreement with the MT protein levels measured by polarographic methods in the hepatic cytosolic fraction. In addition, together with this cytosolic MT localisation, the lysosomal population of hepatocytes also exhibited a strong MT labeling after Cd-exposure. The lysosomes constitute a major compartment for metal accumulation and sequestration (Fowler, 1987) allowing a reduction of the (toxico)-availability of Cd, at least transiently. Lysosomes can contain degradation products of MTs and serve as a final storage site of degraded MTs and possibly, of other metal-binding proteins (Klaassen and Lehman-McKeeman, 1989; Dallinger, 1995; Klaasen et al., 1999). Subsequently, lysosomes loaded with MT bound metals and extruded from the hepatocytes *via* exocytosis, or transported by blood cells or phagocytosed by specialized liver macrophages. Such removal might contribute to a stabilisation of the metal contents in piscine hepatocytes below certain exposure conditions (Amaral et al., 2002). However, under the present experimental conditions the level of immunolabelling observed in the macrophages of both control and Cd-exposed turbots was very weak and, thus, did not indicate a crucial role of this cell type in metal handling.

Metals can not be degraded metabolically so they have to be excreted via gills, skin, intestine liver or kidney (Harrison and Klaverkamp, 1989; Filipovic and Raspor, 2003). The main cell-type of the kidney is the nephrocyte which contains numerous, invaginations of the plasma membrane, often in the form of well-developed basal labyrinth. In this study, very low levels of MT were localised in the kidney after Cd-exposure. The presence of *ir*MT was mainly restricted to the basal part of the nephrocytes that form the proximal tubules. No differences in MT labelling among control and treated specimens were observed. The sparse MT immunolabelling of MT in kidney may be due to the fact that the main routes for Cd, Cu and Zn excretion are the gills and liver while very little is lost via kidney (Harrison and Klaverkamp, 1989; Olsson et al., 1998) because of the low filtration rate in this organ in marine species (Handy, 1996).

After exposure to waterborne Cd the presence of *ir*MT in the rest of the studied organs, spleen and muscle, was very low. This might suggest that MT induction did not occur in both organs of exposed turbot. Similarly, Long and Wang (2005) found very low concentration and induction rates of MT in the muscle of the marine black bream after Cd exposure. Sun and Jeng (1998) and De Boeck et al. (2003) reported very low levels of metals (and associated MT) in the muscle and spleen of a great variety of freshwater, brackish and seawater species exposed to Cu and Zn.

In conclusion, MT expression induced after Cd-exposure can be observed using MT-immunohistochemistry mainly in the branchial chloride cells and the hepatocytes of turbot. The specific expression of MTs in chloride cells may clarify the involvement of the gills in metal trafficking after metal exposure. The immunochemical approach in liver sections might give an accurate assessment of the total MT fraction since allows the determination of both the cytosolic and lysosomal pools, while the conventional chemical methods routinely employed only assess cytosolic MTs. Further investigations to compare MT induction quantified by immunohistochemistry and by differential pulse polarography are needed to study whether changes in the distribution pattern of given cell-types affect MT induction. These changes might be of great importance in complex organs (i.e. gills) where MT induction is restricted to certain cell-types (i.e. chloride cells). In the case of the liver, where abundant hepatocytes exhibited *ir*MT cell-type specific induction should not be the factor affecting possible discrepancies among methods. Therefore, the quantification of *ir*MT levels in both target cell-types could be used in biomonitoring programs as accurate measurements of MT induction as a biomarker of metal exposure.

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