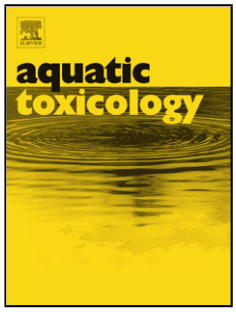

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Histological changes, apoptosis and metallothionein levels in *Triturus carnifex* (Amphibia, Urodela) exposed to environmental cadmium concentrations

Running Title: Cadmium-induced modifications in *Triturus carnifex*

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Highlights

- Specimens of the newt *Triturus carnifex* were exposed to environmental Cd doses.
- Newts exposed to Cd during 9 months accumulated Cd in their tissues.
- Cd induced histological alterations in the skin, liver and kidneys.
- Cd induced apoptosis only in the kidneys.
- Cd did not increase metallothionein levels in the skin and the liver, nor MTs mRNA.

Abstract

The aim of this study was to verify if the freshwater safety values established from the European Community (Council Directive 1998) and the Italian Ministry of Health (2001) for cadmium (44.5 nM/L in drinking water and 178 nM/L in sewage waters) were safe for amphibians, since at these same concentrations cadmium induced endocrine disruption in the newt *Triturus carnifex*. Adult male specimens of *T. carnifex* were exposed daily to cadmium (44.5 nM/L and 178 nM/L as $CdCl_2$, nominal concentrations), respectively, during 3- and 9-months; at the same time, control newts were exposed to tap water only. The accumulation of cadmium in the skin, liver and kidney, the levels of metallothioneins in the skin and the liver, the expression of metallothionein mRNA in the liver, as well as the presence of histological alterations and of apoptosis in the target organs were evaluated. The 9-months exposure induced cadmium accumulation in all the tissues examined; moreover, histological changes were observed in all the tissues examined, irrespective of the dose or the time of exposure. Apoptosis was only detected in the kidney, whereas metallothioneins and metallothionein mRNA did not increase. This study demonstrates that the existing chronic water quality criterion established for cadmium induces in the newt *T. carnifex* cadmium accumulation and histological alterations in the target organs examined. Together with our previous results, showing that, at these same concentrations, cadmium induced endocrine disruption, the present results suggest that the existing chronic water quality criterion for cadmium appears to be not protective of amphibians.

Keywords: amphibians; apoptosis; cadmium; environment; metallothionein; *Triturus carnifex*

1. Introduction

Cadmium (Cd) is a relatively rare metal in the Earth's crust (Hubner et al., 2010); it is not generally believed to have a biological function, except for marine diatoms (Lane and Morel, 2000). It enters the aquatic systems from natural and anthropogenic sources, as smelting, battery manufacture, paints, fertilizers, and plastic stabilizers. Cd is considered one of the most toxic environmental and industrial pollutants, since it is a teratogen, carcinogen, and a possible mutagen (Burger, 2008). It is not biodegradable and accumulates in vertebrates, particularly in freshwater organisms, damaging the physiological processes or tissues even at concentrations far below the lethal level (Vogiatzis and Loumbordis, 1997; He et al., 2005; Sura et al., 2006). When Cd is present in living tissues, it is mostly bound to metallothioneins (MTs), low molecular weight cysteine-rich proteins, involved in metal detoxication (Klaassen et al., 2009). Since Cd possesses chemical properties close to those of zinc and copper, enzymatic cofactors essential for metabolism, its cellular toxicity is thought to be based, at least partially, on its ability to displace these essential metals (Mounaji et al., 2003). Indeed, many adverse effects have been reported on ecosystems and human and animal health (Nawrot et al., 2010; Prozialeck and Edwards, 2010): embryotoxicity in mammals (Fein et al., 1997) as well as in amphibians (Flament et al., 2003; James and Little, 2003; Fridman et al., 2004; James et al., 2005) and reptiles (Simoniello et al., 2011), impairment of growth, reproduction, respiratory functions, and osmoregulation in fish (Hontela, 1997), genotoxicity in amphibians (Mouchet et al., 2006), endocrine disruption in many species (Garcia-Morales et al., 1994; Le Guével et al., 2000; Gay et al., 2013; Simoniello et al., 2013).

In freshwater, Cd concentration ranges from 17.8 nM/L to 267 nM/L (Audry et al., 2004). The European Community (Council Directive 1998) and the Italian Ministry of Health (2001) have established a freshwater safety value for Cd of 44.5 nM/L in drinking water and 178 nM/L in sewage waters. However, also at these low concentrations, Cd behaved like an endocrine disruptor, affecting the pituitary- adrenal activity of the newt *Triturus carnifex* (Gay et al., 2013), an urodele amphibian spending a great part of its life in the aquatic environment, and considered an ideal bioindicator, due to its high sensitivity to waterborne chemicals (Capaldo et al., 2006; 2012). These results let foresee further consequences on the newt physiology; therefore, in the present study, we evaluated some biological markers widely used to assess the kind and degree of metal contamination: the accumulation of Cd; the occurrence of histological

alterations in the skin, the liver and the kidney, well-known targets of Cd toxicity; the presence of apoptosis and the concentration of MTs, involved in metal detoxication (Vogiatzis and Loumbordis, 1998; Loumbordis and Vogiatzis, 2002; Tzirogiannis et al., 2003; Loumbordis, 2005; Mouchet et al., 2006; Giari et al., 2007; van Dick et al., 2007; Prozialeck and Edwards, 2010; Annabi et al., 2011).

2. Materials and methods

2.1. Reagents

Cd was applied as $CdCl_2$ (CAS no. 10108-64-2, ≥ 99.0%, SIGMA- Aldrich, St. Louis, MO 63103 USA). The stock solution of Cd was prepared by dissolving 0.4 g $CdCl_2$ in 1000 ml distilled water.

2.2. Animals

Adult male specimens of *T. carnifex* (mean mass 8.0 g.), captured in the field around Naples, were kept in 50 L glass tanks (10 newts per tank, with a loading rate of 1.6 g newt/L water), under a natural photoperiod, in dechlorinated, well-aerated tap water, at seasonal temperature, with the following physicochemical conditions: pH 7.4 ± 0.1, ammonia < 0.01 mg/L, dissolved oxygen 8.1 ± 0.2 mg/l, 16.5 ± 1°C mean temperature during February-May period; 20 ± 1°C mean temperature during February-November period. The animals were fed minced cow liver and used after an acclimation period of 2 weeks. The experiments were performed in accordance to EU Directive 2010/63/EU for animal experiments and authorized by the National Committee of the Italian Ministry of Health on in vivo experimentation (Department for Veterinary Public Health, Nutrition and Food Safety). Efforts were made to avoid animal suffering and minimize the number of specimens used. The animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals.

2.3. Experimental design

At the beginning of February, 60 adult male newts were divided into 6 groups, each containing 10 specimens. Two groups were exposed daily to a nominal concentration of 44.5 nM/L of Cd; the exposure lasted 3 months, for the first group, and ended at the beginning of May, and 9 months, for the second group, and ended at the beginning of November. The third and the fourth group were exposed daily to a nominal concentration of 178 nM/L of Cd; also in this case, the exposure lasted 3 months, for the third group,

and ended at the beginning of May, and 9 months, for the fourth group, and ended at the beginning of November. Two control groups, one for each of the 2 different exposure periods, were exposed to tap water only. Mortality and behaviour were observed daily in each exposure. The water was changed every day in all the tanks. All the experiments were carried out in triplicate. At the beginning of May (3-months exposure) and November (9-months exposure) the newts were anaesthetized by hypothermia, chilling them in chipped ice, within 5 min after removal from tank. The animals were killed by decapitation.

2.4. Cd analysis

Samples of liver, kidney and dorsal skin from each animal of each group were collected separately and analysed for Cd content. The samples were weighed, thoroughly washed in PBS, pH 7.4, and digested with concentrated nitric acid (65%, Ultrapure, Fluka), using 1 mL of acid every 50 mg of wet tissue. The mixture was heated for 30 min at 70 °C, cooled and centrifuged for 10 min at 12000*g*. Cd content in the supernatant was determined by the graphite furnace atomic absorption spectrophotometry, using a Varian AA220 equipped with Zeeman graphite furnace. Ultrapure water and stock standard solution of the metal (1 mg/mL) were from commercial source (Fluka). Working standards in 0.2% v/v HNO_3 were prepared daily by diluting known aliquots of the stock solution to the appropriate volume. Metal recovery, calculated as a percentage of the known aliquot of metal added, ranged from 90% to 95%. The detection limit in different samples was determined from the standard additions curve for Cd. It was based on the usual definition of the concentration of the analyte yielding a signal equivalent to three times the standard deviation of the reagent blank signal (n=5). The detection limit estimated was in the range 5-15 ng/g.

2.5. Histology

From each animal, samples of liver, kidney and sections of dorsal skin of nearly 2 cm^2, were fixed in Bouin's solution, dehydrated in graded alcohols, cleared in Histolemon and embedded in Paraplast. Serial 6 µm sections were processed for routine histological analysis and stained with Mallory trichromic stain for general histology. Moreover, the thickness of the epidermis was evaluated, utilising 10 longitudinal sections from each specimen of each experimental group, and therefore 100 longitudinal sections for each experimental group. Observations were performed using a Zeiss Axioskop microscope;

images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system (Zeiss, Oberkochen, Germany) and Adobe Photoshop.

2.6. Detection of apoptosis

A commercial terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay kit (Fragment End Labeling (FragEL™) DNA Fragmentation Detection Kits Calbiochem) was used, according to the manufacturer-provided protocol, to detect cell apoptosis in Paraplast-embedded sections of liver, kidney and dorsal skin, from each animal of each group. In this assay terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells. Observations were performed as previously described.

2.7. MTs quantification

The levels of total MT proteins were determined in the skin and the liver from each single animal of each group by a spectrophotometric method, using the Micro MT Spec kit (Ikzus Environment). This method is based on the chemical determination of cysteine residues by the Ellman reaction (1958) and represents an improvement of the method reported by Viarengo and coworkers (1997). Briefly, tissue samples were homogenised in dounce in three volumes of homogenising buffer with 0.1% β-mercaptoethanol and centrifuged at 10000g for 30 min and at 100000g for 60 min. The final supernatants were subjected to an ethanol/chloroform extraction followed by an overnight ethanol precipitation at -20°C. The MTs-enriched ethanol precipitates were collected by centrifugation at 20000g for 20 min, dried at room temperature and dissolved in 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) reaction buffer. The resulting samples were incubated with DTNB according to the kit protocol; finally, samples absorbance was read at 412 nm. The interpolation of the obtained value over the glutathione standard curve retrieves the nanomoles of cysteine residues, directly

referred to the MTs present in the sample. The concentration of MTs in nanomoles per gram of wet tissue was calculated considering that the number of cysteine residues present in newt MT is 20 (Trinchella et al., 2012). The conversion of MTs molar levels to mass partition (μg/g wet tissue) was calculated on the basis of the estimated molecular mass for the same MT, i.e. 6190 Da.

2.8. RNA extraction

Small sections of livers from control specimens and specimens exposed to 178 nM/L during 3 and 9 months were quickly removed and frozen at -80°C in RNAlater (Ambion). Total RNA was prepared using a pool of livers from five specimens. Total RNA was isolated using the TRI-REAGENT (SIGMA-Aldrich), according to the manufacturer's specifications and then subjected to a deoxyribonuclease treatment using the TURBO-DNA-*free* kit (Ambion). After DNase treatment, the concentration and purity of RNA samples were analysed spectrophotometrically and by ethidium bromide agarose gel electrophoresis.

2.9. Dot blot hybridization

Dot-blot analysis was used to determine MTs mRNA levels in the control and Cd-exposed livers. Five μg samples of total RNA were denatured in 500 μL solution of 10 mM NaOH/10 mM EDTA and then blotted onto a positively-charged Nylon membrane (Immobilion-Ny^{+}, Millipore) using a vacuum Bio-Dot apparatus (Bio-Rad Lab). The membrane was then UV-crosslinked and rinsed in 6x SSC before being used for hybridization. A cDNA probe labelled with [α-^{32}P]-dCTP, corresponding to the coding region of the *T. carnifex* MT (Trinchella et al., 2012, accession number HE681911), binding specifically only the MT transcripts, was prepared by PCR. The cDNA probe sequence was:
atggaccctaaagactgcggctgtgcctccggtggctcttgttcatgtgctgggtcgtgcaagtgcgagaactgcaaatgtacc
tcctgcaaaaaaagctgctgttcctgttgccctgccggatgcgataaatgtggccagggttgtgtgtgcaaaggagggtcgact
gagaaatgcagctgttgcacc.

Unincorporated nucleotides were separated from the labelled cDNA by MicroBioSpin P-30 column. The membrane was prehybridized for 30 min at 42 °C in the Ultrahyb buffer (Ambion) and hybridized with the probe in the same buffer for 16 h at 42 °C. The membrane was then washed under high stringency conditions (two final washes of 0.1X SSC, 0.1% SDS at 42 °C for 15 min) and exposed to X-ray film. The amount of probe

bound to each RNA was quantified by ImageQuant software (GE Healthcare). Normalization for unequal loading of total RNA was assessed by staining the membrane with a methylene blue solution as described by Sambrook and Russell (2001). Three distinct membranes were prepared and blotted with the pooled RNAs.

2.10. Statistical analysis

The values were expressed as means ± standard error of mean (SEM). All the data were first tested for normality and homogeneity of variance to meet statistical demands; the homogeneity of variance was assessed by the Bartlett test. The data were compared by both one- and two-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test and the Fisher's LSD post hoc comparison tests. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Visual observations

Death did not occur in either control or exposed newts; both groups showed a good health condition. The Cd-exposed newts, compared to controls, showed only an increase in the appetite and a frequent moulting.

3.2. Cadmium accumulation in newt tissues

The average Cd accumulation in tissues are reported in Table 1. After the 3-months exposure, for both doses of Cd (44.5 nM/L and 178 nM/L), one-way ANOVA analyses indicated that in all the tissues examined the Cd levels were not significantly different from controls (skin: DF2, F=0.57, P=0.58; liver: F=1.89, P=0.21; kidney: F=2.08, P=0.18). On the contrary, after the 9-months exposure, for both doses of Cd (44.5 nM/L and 178 nM/L) one-way ANOVA analyses indicated that in all the tissues examined the Cd levels were significantly higher than controls (skin: F=4.67, P=0.045; liver: F=10.2, P=0.006; kidney: F=4.43, P=0.05), with the highest concentrations being observed in the liver and the kidney. Two-way ANOVA analyses demonstrated that the principal source of variation in Cd accumulation was the time of exposure; a significant interaction between time and Cd concentration in the water was present only in liver (Table 2). Fisher's LSD post hoc comparison tests demonstrated a significant accumulation of Cd over the time for all the tissues examined (liver and kidney, P<0.0001; skin, P=0.0003); when comparing the three treatment groups (control, 44.5 nM/L Cd and 178 nM/L Cd), significant differences in Cd content were found only in

liver, between control and 44.5 nM/L Cd (P=0.004) and control and 178 nM/L Cd (P=0.0002). In all the other comparisons, tests retrieved P values > 0.05. Interestingly, in the tissues of control animals, housed in tap water, Cd ions were always present and their concentrations increased over the time (Table 1).

3.3. Histology of newt tissues

3.3.1. Skin

The morphology of the skin in control specimens (Figs. 1A, B) showed the features typical of this species (Duellman and Trueb, 1986). The skin of *T. carnifex* consisted of the outer epidermis and the underlying dermis, separated by a basement membrane of collagenous fibers. The epidermis was basophilic and consisted of a basal layer, with columnar cells; an intermediate layer, with progressively shorter cells, and an outer thin keratinized stratum corneum, eosinophilic, consisting of a single layer of flattened cells. In the dermis, mucous, granular, and mucous-granular glands, were present. After the 3-months exposure, both doses of Cd (44.5 nM/L: Figs. 1C, D and 178 nM/L: Figs 1E, F), decreased the thickness of the epidermis, that appeared eosinophilic. It consisted of few rows of cells, roundish or flattened, mostly arranged in the inner layers of the epidermis, whereas the superficial layers appeared almost devoid of cells. The same changes were observed after the 9-months exposure, for both doses (44.5 nM/L: Figs. 1G, H and 178 nM/L: Figs 1I, L). The evaluation of the thickness of the epidermis confirmed the morphological observations. After the 3-months exposure, the average thickness of the epidermis in the specimens exposed to 44.5 nM/L Cd (20.16 ± 0.3, 10 specimens) and to 178 nM/L Cd (21.50 ± 0.4, 10 specimens) was significantly ($p < 0.05$) lower than control specimens (41.13 ± 0.8, 10 specimens). After the 9-months exposure, the average thickness of the epidermis in the specimens exposed to 44.5 nM/L Cd (18.61 ± 0.3, 10 specimens) and to 178 nM/L Cd (18.61 ± 0.4, 10 specimens) was significantly ($p < 0.05$) lower than control specimens (41.16 ± 0.7, 10 specimens). No differences were found between the two doses of Cd (44.5 nM/L Cd and 178 nM/L), and the two different exposures (3-months and 9-months).

3.3.2. Liver

The morphology of the liver in control specimens (Figs. 2A, B, C) showed the features typical of this species (Duellman and Trueb, 1986; Frangioni et al., 2000). The hepatic parenchyma was formed by rounded hepatocytes arranged in cords-like structures

separated each other from blood sinusoids; moreover, many small melanomacrophages, the so-called Kuppfer cells, were present (Fig. 2A,C). The hepatocytes had large rounded nuclei with evident nucleoli, and well-stained cytoplasms (Fig. 2B). In the specimens exposed to 44.5 nM/L of Cd during 3 months (Figs. 2D, E, F) the sinusoids appeared dilated; the number and the size of Kuppfer cells increased; the cytoplasm of the hepatocytes was cloudy and granular, signs of hydropic degeneration; karyolysis and karyorrhexis were observed. In the specimens exposed to 178 nM/L of Cd during 3 months (Figs. 2G, H, I), the same changes, together with blood congestion, were observed. In the specimens exposed to 44.5 nM/L (Figs. 2L, M, N) and 178 nM/L (Figs. 2O, P, Q) of Cd during 9 months, the hepatocytes showed the same changes observed after the 3-months exposure to 44.5 nM/L of Cd, and hepatic necrosis as well.

3.3.3. Kidney

The morphology of the kidney in control specimens (Figs. 3A, B, C) showed the features typical of this species (Bellocci et al., 1971). The kidney had well organized renal corpuscles, with the glomeruli, consisting in tufts of capillaries, Bowman's capsules and well defined Bowman's spaces. The proximal tubules had columnar epithelial cells with a basally displaced nucleus and an apical brush border; the distal tubules had cuboidal cells, with a relatively clear cytoplasm and without brush border. In the specimens exposed to 44.5 nM/L (Figs. 3D, E, F) and 178 nM/L (Figs. 3G, H, I) of Cd during 3 months, the kidney showed glomerular expansion, resulting in reduction of Bowman's space. The renal tubules, mainly the proximal ones, appeared dilated, showing degeneration in the epithelial cells, with karyolysis and karyorrhexis. In the specimens exposed to 44.5 nM/L (Figs. 3L, M, N) and 178 nM/L (Figs. 3O, P, Q) of Cd during 9 months, the kidney showed the same changes observed after the 3-months exposure; moreover, at the highest dose (178 nM/L of Cd), also tubular necrosis was observed.

3.4. Apoptosis

Nick end-labeling for detection of apoptotic cells revealed a high percentage of TUNEL-positive cells only in the kidney (Fig. 4), whereas the skin and the liver failed to exhibit significant levels of apoptosis (data not shown). Instead, all the specimens of kidney exposed to Cd (44.5 nM/L during 3 months: Fig. 4A; 178 nM/L during 3 months: Fig. 4B; 44.5 nM /L during 9 months: Fig. 4C; 178 nM/L during 9 months: Fig.

4D) showed TUNEL-positive cells, whereas no evidence of apoptosis was observed in control specimens (Fig. 4E).

3.5. MTs accumulation in newt tissues

After the 3-months and 9-months exposure, one-way ANOVA analysis indicated, for both doses of Cd (44.5 nM/L and 178 nM/L), no significant differences in MTs levels between control and Cd-exposed specimens, in both liver and skin (Table 3). Unfortunately, due to the smallness of the organ, it has not been possible to perform this kind of analysis on the kidney. Two-way ANOVA analysis (Table 4) followed by Fisher's LSD test retrieved statistically significant differences in the MTs content between the two different exposures (3 months *vs* 9 months, $P<0.0001$) in both liver and skin; the time of exposure was the only factor affecting MTs concentration (Table 4). Fisher's LSD test retrieved P values >0.05, for all the considered pairwise comparisons. The analysis of the level of MTs mRNA gave similar results (Fig. 5).

4. Discussion

The results of the present study show that the newt *T. carnifex*, chronically exposed to Cd concentrations corresponding to the existing chronic water quality criterion (EU Council Directive, 1998; Italian Ministry of Health, 2001), accumulates Cd in its tissues and shows histological alterations and evidence of apoptosis. These results agree with the endocrine disruption, evidenced in this species after 3- and 9-months exposure to the same Cd concentrations (Gay et al., 2013).

As regards Cd accumulation, the results showed that the 9-months exposure induced all the tissues examined to accumulate Cd, with the highest concentrations being observed in the liver and the kidney, whereas the 3-months exposure was ineffective. Our results showed also that the Cd accumulation was more influenced from the time of exposure than the dose administered. Indeed the two Cd concentrations, 44.5 nM/L and 178 nM/L, had the same effect on Cd accumulation in all the tissues except the liver, where a significant interaction between time and Cd concentration in the water was present. This time-dependent pattern of Cd accumulation has been already demonstrated in amphibians (Vogiatzis and Loumbordis, 1998).

The Cd accumulation observed in the newt tissues after a chronic Cd exposure, is in agreement with the Cd accumulation observed in fish, amphibians and reptiles when they are chronically exposed to this metal (Vogiatzis and Loumbordis, 1997; 1998;

Dobrovolijc, 2003; Sura et al., 2006; Trinchella et al., 2006; Annabi et al., 2011; Ezemonye and Enuneku, 2012). In these studies, the specimens, exposed to Cd concentrations in the order of µM/L, accumulated Cd in their tissues after exposures of several hours or days. The long time spent from the newts to accumulate Cd is probably related to the low, environmental concentrations (in the order of nM/L) used in our study. Also the highest Cd levels found in the liver and kidney of *T. carnifex* agree with the role of these organs as main body stores of Cd (Vogiatzis and Loumbordis, 1997; 1998; Dobrovolijc et al., 2003; Godt et al., 2006; Burger, 2008; Prozialeck and Edwards, 2010).

A noteworthy finding of our study was the accumulation of Cd also in control specimens, not exposed to the metal. To explain this result, we measured the Cd content in the tap water, used for the experiment, and in the cow liver, used to feed the animals. Whereas in the tap water Cd was below detection (<0.03 µg/L), the cow liver had a small quantity of Cd (0.039 μg/g ± 0.002), likely sufficient to induce a Cd accumulation also in the tissues of the control newts. This result showed therefore that the Cd-exposed newts took up Cd also via oral route, as well as through the skin and the gills, the main ways of entrance. However, the low Cd concentrations detected in control animals, compared to the Cd-exposed specimens, suggest that the oral accumulation represented only a small part of total accumulation.

As regards the histological lesions, they were observed in all the tissues examined, whereas evidence of apoptosis was observed only in the kidney.

In the skin, Cd exposure deeply influenced the epidermis, that appeared decreased in thickness, eosinophilic, and showed few rows of roundish or flattened cells, mostly arranged in the inner layers of the epidermis, whereas the superficial layers appeared almost devoid of cells. These changes occurred irrespective of the dose or the time of exposure. The features of the epidermis suggest a process of keratinisation, Cd-induced, probably mediated through aldosterone, whose levels increased following Cd exposure (Gay et al., 2013). In amphibians, aldosterone induces conversion of the stratum granulosum into a cornified cell layer and transformation of the cell layer below into a new stratum granulosum (Smith 1975), and promotes moulting, the process whereby the outer parts of the skin is cast off and replaced, as a result of the activity of the underlying cells (Hanke 1978). Therefore, aldosterone could be involved in the frequent moulting and the resulting decreased thickness of the epidermis, observed in Cd-exposed specimens. However, it cannot be excluded also a direct Cd action on the newt

epidermis, since many studies showed alterations in the structure and physiology of the skin of Cd-exposed amphibians (Selvi et al., 2003; Simoncelli et al., 2015). These studies, using Cd concentrations in the order of μM/L, showed also an increase in mucus secretion following Cd exposure, suggesting mucus involvement in protective mechanisms (Part and Lock, 1983). The lack of increase in mucus secretion, observed in our study, was probably due to the lower Cd concentrations used, in the order of nM/L.

The skin of amphibians provides the first biological barrier against chemical and physical pollutants. It is involved in the physiological exchange of respiratory gasses, ions and water, and is therefore one of the major routes of exposure to environmental contaminants. Moreover, it is a direct route of Cd uptake (Vogiatzis and Loumbordis, 1997). The keratinisation, observed in the epidermis of Cd-exposed newts, could be a protective mechanism, induced by the presence of the metal, and limiting Cd-uptake in the organism.

In the liver, Cd influenced both the hepatic parenchyma and the melanomacrophages, the so-called Kuppfer cells, irrespective of the dose or the time of exposure. The liver showed signs of toxicity: dilated sinusoids, blood congestion and necrotic areas. Cloudy cytoplasm, karyolysis and karyorrhexis were observed in the hepatocytes. The liver is well known as the primary organ for Cd accumulation; indeed, after absorption and distribution in the bloodstream, Cd is transported to the liver, taken up by hepatocytes, where induces the synthesis of MTs, which bind Cd and buffer its toxic effects in the cell. When the hepatocytes die off, either through normal turnover or as a result of Cd injury, the Cd-MTs complex can be released into the bloodstream . Even though this complex is nontoxic to most organs, it can be filtered at the glomerulus and taken up by the epithelial cells of the proximal tubule, mediating some of the toxic effects of Cd in the proximal tubule (Prozialeck and Edwards, 2012). Moreover, the liver is also the primary site for detoxification mechanisms (Thopon et al., 2003). The liver toxicity, induced by Cd in *T. carnifex*, agrees with the vascular and cellular alterations observed in the liver of Cd-exposed fish (Thopon et al., 2003; Giari et al., 2007; van Dyk et al., 2007; Annabi et al., 2011), amphibians (Loumbordis, 2005; Ezemonye and Enuneku, 2012), reptiles (Simoniello et al., 2010) and mammals (Tzirogiannis et al., 2003). Moreover, in the liver of Cd-exposed newts, the Kuppfer cells increased in number and size, as previously shown in the liver of rat (Meiss et al., 1982) and the frog *Rana ridibunda* (Loumbordis and Vogiatzis, 2002; Loumbordis, 2005) after Cd exposure. The Kuppfer cells are involved in scavenging and phagocytosing foreign

substances and cell debris (Loumbordis and Vogiatzis, 2002) and in survival in hypoxia, when the newt's oxygen supply is inadequate to meet its metabolic needs (Frangioni et al., 2000). Moreover, they have been shown to play a negative role in the liver damage, since they participate, together with other inflammatory cells, in the pathogenesis of tissue injury by releasing cytotoxic mediators such as cytokines, superoxide anion, nitric oxide and proteases. $CdCl_2$ was found to significantly enhance the activity of Kuppfer cells (Sauer et al., 1997), potentiating therefore the hepatotoxicity of Cd. However, because all these mediators are non-specific, their release in excess quantities or in an uncontrolled manner, can also destroy normal tissues (Loumbordis, 2005). It is possible that also in the newt *T. carnifex*, the Kuppfer cells could be involved in liver damage.

In the kidney, Cd induced pathological changes as glomerular expansion and reduction of Bowman's space, changes in the renal tubules, mainly the proximal ones, as tubular necrosis, degeneration in the epithelial cells of the renal tubule, karyolysis and karyorrhexis. These results agree with the adverse effects observed in kidney of Cd-exposed fish (Thopon et al., 2003; Giari et al., 2007; Annabi et al., 2011), amphibians (Loumbordis, 2005; Ezemonye and Enuneku, 2012) and humans (Johri et al., 2010; Prozialeck and Edwards, 2010). Kidneys are one of the main targets of Cd accumulation and the major organs from which Cd is excreted from an organism; they are also the first organs to show toxic effects (Vogiatzis and Loumbordis, 1998; Burger, 2008). The portion of kidney mainly affected by Cd is the proximal tubule; the current hypotheses ascribe the pathogenesis of nephrotoxicity to the Cd originating from MTs-Cd complex, released from the liver into the plasma and taken up by the proximal tubules of the kidneys. However, a great deal of evidence indicates that ionic cadmium ($Cd2^{+}$), and not CD-MTs complex, injures proximal tubule epithelial cells. According to this second hypothesis, Cd not bound to MTs, released into the bloodstream, reaches the proximal tubular lumen and splits by an unknown mechanism into Cd ions and holo-thionein on the brush border membrane of the proximal tubular cells. Free radicals, which are initiated by Cd ion, may injure the brush border membranes (Nomiyama and Nomiyama,1998). Also the sensitivity of MTs-null animals to cadmium-induced proximal tubule injury provides evidence that Cd-MTs complex does not play a critical role in directly mediating Cd nephrotoxic effects (Prozialeck and Edwards, 2012). The exact mechanism of nephrotoxicity in *T. carnifex* needs further investigation; however the nephrotoxicity, Cd-induced, occurred in absence of an increase in MTs or MTs

mRNA, evidenced after Cd exposure and discussed below. This observation could therefore confirm this second hypothesis.

In the kidneys of Cd-exposed newts, apoptosis was observed, irrespective of the dose or the time of exposure. Many studies showed Cd-induced apoptosis in both liver and kidney of amphibians (Loumbordis, 2005), mammals (Tzirogiannis et al., 2003) and fish (Gao et al., 2013). Apoptosis is a physiological, critical process occurring during amphibian development and metamorphosis (Ishizuya-oka et al., 2010), but has also been suggested as a sensitive and early indicator of chronic chemical stress, loss of cellular function and structure, and organism death; under toxic exposure, apoptosis is suggested to remove critically damaged cells followed by compensatory cell regeneration to maintain tissue structure and function (Loumbordis, 2005). Moreover, apoptosis is also considered a well-known cellular biomarker of Cd exposure in aquatic organisms. The presence of apoptosis indicates that the kidneys were the most damaged organs in Cd-exposed newts, consistently with their role as critical organs in Cd toxicity (Burger, 2008; Johri et al., 2010).

Cd has been shown to induce apoptosis also in the liver (Tzirogiannis et al., 2003; Loumbordis, 2005; Gao et al., 2013). The absence of apoptosis in the liver of Cd-exposed newts could be related to the low Cd concentrations used in our study and/or the lack in MTs and MTs mRNAincrease, discussed below. Recent data, indeed, demonstrated, in human hepatocellular metastatic carcinoma, a relationship between absence of MTs and appearance of apoptotic cells (Deng et al., 1998; Cui et al., 2003).

As regards MTs, our results showed that Cd exposure did not increase MTs levels in the skin and the liver, nor MTs mRNA, irrespective of the dose or the time of exposure. On the contrary, significant differences were observed in MTs levels between the 3-months and the 9-months values. Such differences could be an effect of feeding the newts with cow liver containing small quantities of Cd, leading to a progressive increase in MTs levels with the extending of the exposure. MTs are a family of cysteine-rich, metal-binding proteins, identified in a wide range of phyla from invertebrates to humans, involved in zinc and copper homeostasis and Cd detoxification (Henry et al., 1994; Goyer et al., 1995). They have been shown to increase after Cd exposure in fish (Cope et al., 1994), amphibians (Vogiatzis and Loumbordis, 1997; 1998), reptiles (Trinchella et al., 2006; Simoniello et al., 2010; 2013; Scudiero et al., 2011) and mammals (Henry et al., 1994; Johri et al., 2010; Prozialeck et al., 2010). Our

results, the lack of MTs and mRNAs increase in Cd-exposed newts needs further studies to be explained.

5. Conclusion

In conclusion, our results show that Cd, at concentrations corresponding to the existing chronic water quality criterion, induced in the newt *T. carnifex* Cd accumulation and histological changes in the skin, the liver and the kidney; in the last tissue, also apoptosis was observed. Considering the physiological role of the tissues examined and the results of our previous studies, showing that the same concentrations and the same time of exposure to Cd induced endocrine disruption, these results suggest that the existing chronic water quality criterion for cadmium appears to be not protective of amphibians.

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Figure legends

Figure 1. Light micrographs of the skin of *Triturus carnifex*. Mallory staining. (A, B) Control specimens. The epidermis (E) appears basophilic, blue stained, and shows a basal layer (B), with columnar cells; an intermediate layer (I), with progressively shorter cells, and an outer thin keratinized stratum corneum (SC), consisting of a single layer of flattened cells. Mucous (M), granular (G), and mucous-granular (MG) glands are embedded in the dermis (D). (C, D) Specimens exposed to 44.5 nM/L of Cd during 3 months. (E, F) Specimens exposed to 178 nM/L of Cd during 3 months. The epidermis (E) appears eosinophilic, yellow stained, and decreased in thickness. Few rows of roundish or flattened cells are arranged in the inner layers of the epidermis, whereas the superficial layers appeared almost devoid of cells. (G, H) Specimens exposed to 44.5 nM/L of Cd during 9 months. (I, L) Specimens exposed to 178 nM/L of Cd during 9 months. The skin shows the same features observed in the specimens after the 3-months exposure to Cd. Scale bar. Figs 1A, C, E, G, I: 100 µm. Figs. 1B, D, F, H, L: 25 µm.

Figure 2. Light micrographs of the liver of *Triturus carnifex*. Mallory staining. (A,B,C): Control specimens. The hepatic parenchyma (HC) is formed by rounded hepatocytes separated by blood sinusoids (BS); many small Kuppfer (K) cells are present. The hepatocytes have large nuclei (N) and well-stained cytoplasms (C). (D, E, F) Specimens exposed to 44.5 nM/L of Cd during 3 months. The blood sinusoids (BS) appear dilated; the number and the size of Kuppfer cells (K) is increased. The cytoplasm (C) of the hepatocytes is cloudy and granular; karyolysis (arrow) and karyorrhexis (white arrow) are present. (G, H, I) Specimens exposed to 178 nM/L of Cd during 3 months. Blood congestion (BC), many small Kuppfer cells (K), karyolysis (arrow) and karyorrhexis (white arrow) are evident. (L, M, N) Specimens exposed to 44.5 nM/L of Cd during 9 months. (O, P, Q) Specimens exposed to 178 nM/L of Cd during 9 months.

The hepatocytes show the same changes observed in the specimens exposed to 44.5 nM/L of Cd during 3 months; moreover, hepatic necrosis (N) can be observed. Scale bar. Figs. 2A, D, G, L, O: 100 µm. Figs. 2B, E, H, M, P: 25 µm. Figs 2C, F, I, N, Q: 10 µm.

Figure 3. Light micrographs of the kidney of *Triturus carnifex*. Mallory staining. (A, B, C) Control specimens. The kidney shows well organized glomeruli (G), consisting in tufts of capillaries, Bowman's capsules and well defined Bowman's spaces (B). The proximal tubules (arrow) have columnar epithelial cells with a basally displaced nucleus and an apical brush border; the distal tubules (asterisk) haved cuboidal cells, with a relatively clear cytoplasm and without brush border. (D, E, F) Specimens exposed to 44.5 nM/L of Cd during 3 months. (G, H, I) Specimens exposed to 178 nM/L of Cd during 3 months. Glomerular (G) expansion, resulting in reduction of Bowman's space; dilated renal tubules (double asterisks); karyolysis (arrow) and karyorrhexis (white arrow) are evident. (L, M, N) Specimens exposed to 44.5 nM/L of Cd during 9 months. (O, P, Q) Specimens exposed to 178 nM/L of Cd during 9 months. The kidney showed the same changes observed in the specimens exposed to Cd during 3 months, and tubular necrosis (N) at the highest dose (178 nM/L of Cd). Scale bar. Figs. 2A, D, G, L, O: 100 µm. Figs. 2B, E, H, M, P: 25 µm. Figs 2C, F, I, N, Q: 10 µm.

Figure 4. Light micrographs of the kidney of *Triturus carnifex*. TUNEL reaction. (A) Specimens exposed to 44.5 nM/L of Cd during 3 months. (B) Specimens exposed to 178 nM/L of Cd during 3 months. (C) Specimens exposed to 44.5 nM/L of Cd during 9 months. (D) Specimens exposed to 178 nM/L of Cd during 9 months. Positive cells (asterisk) are evident in all the specimens exposed. (E) Control specimens showing no evidence of apoptosis. Scale bar: 100 µm.

Figure 5. Expression of the MT gene in *Triturus carnifex* liver exposed to 178 nM/L of Cd during 3 and 9 months. Dot blot of total RNA from livers of control and Cd exposed specimens were probed with a cDNA fragment corresponding to the *T. carnifex* MT coding sequence (upper panel). Visualization of loading consistency of RNAs (lower panel) was assessed by staining the membranes with a methylene blue solution. The histogram shows the amounts of MT mRNA estimated by the ImageJ Software. Each bar is the average of measurements carried out on three distinct blots.

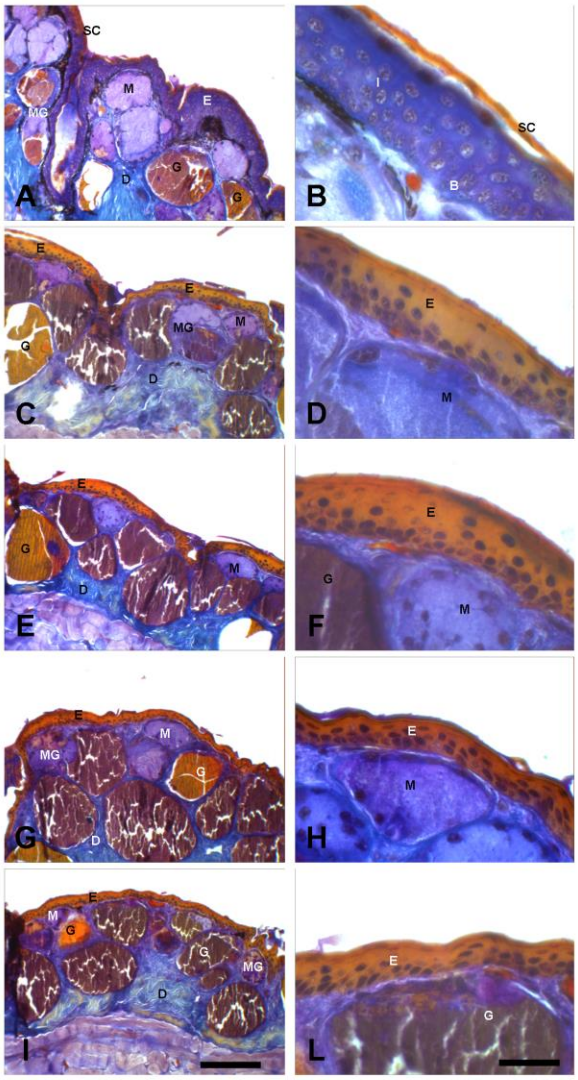

FIG.1

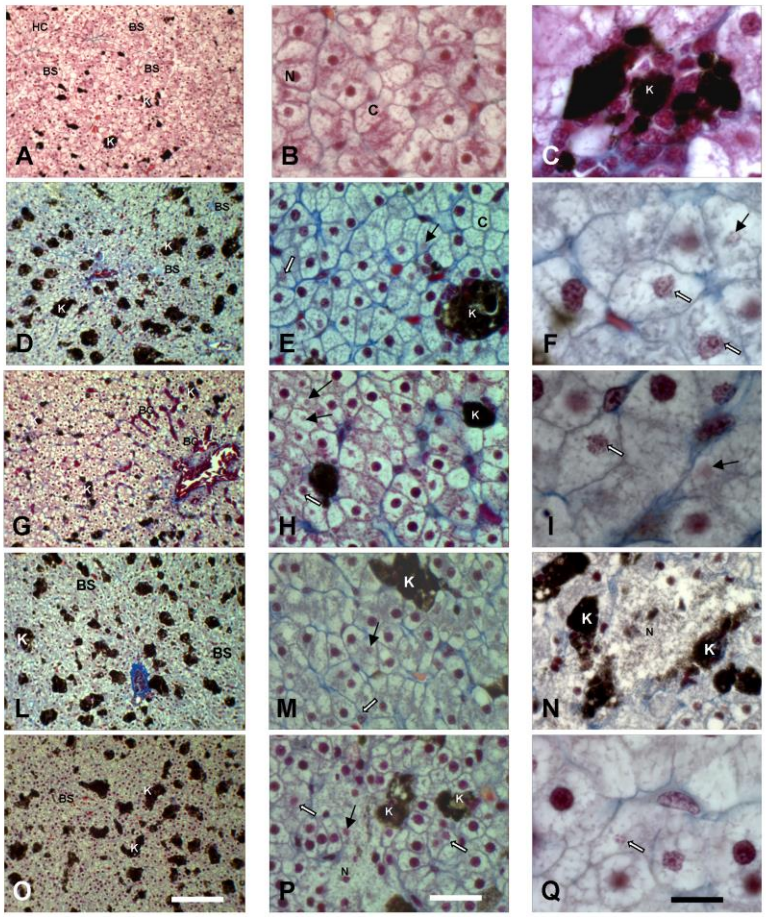

FIG.2

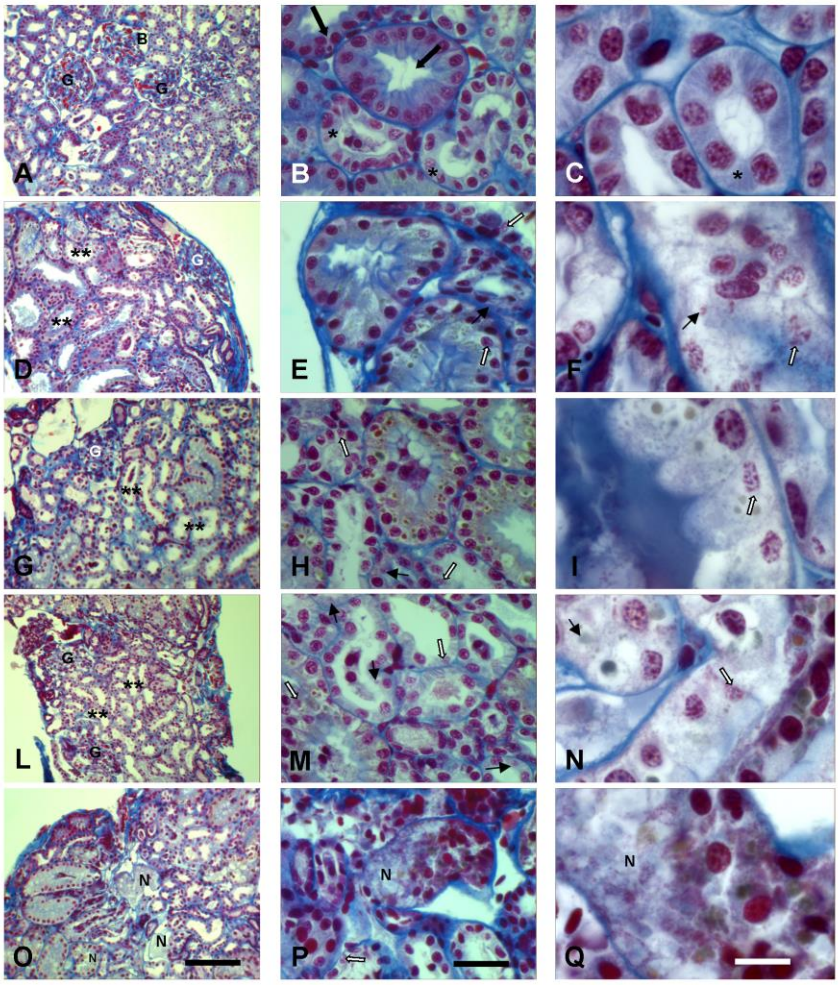

FIG.3

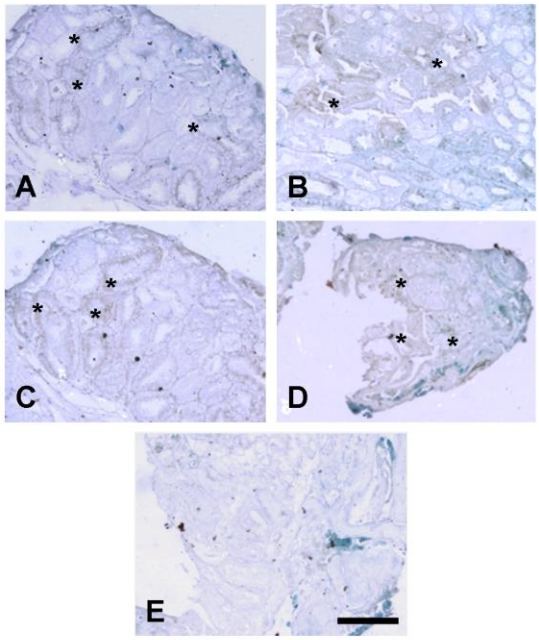

FIG.4

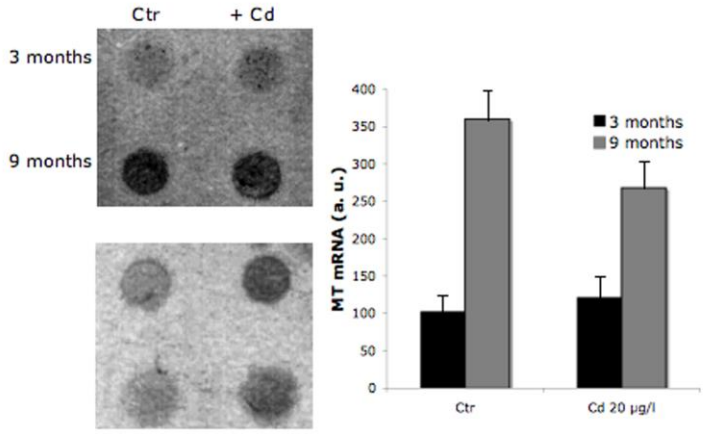

Table 1. Cadmium levels in the skin, liver and kidney of the newt *T. carnifex* exposed to 44.5 nM/L and 178 nM/L of Cd, respectively, during 3 and 9 months. Cd levels were expressed in nM/g wet tissue. Values are means ± SE of the mean. Differences were considered significant when $p < 0.05$

Tissues	Exposure					
	3 months			*9 months*		
	Control	**Cd 44.5 nM/L**	**Cd 178 nM/L**	**Control**	**Cd 44.5 nM/L**	**Cd 178 nM/L**
Skin	0.36 ± 0.27	0.45 ± 0.18	0.53 ± 0.23	1.33 ± 0.09 [a]	2.31 ± 0.82[a,b]	4.36 ± 0.89 [a,b]
Liver	2.49 ± 0.18	2.67 ± 0.89	4.36 ± 2.13	5.69 ± 1.78 [a]	19.7 ± 4.45[a,b]	23.2 ± 5.07 [a,b]
Kidney	4.09 ± 2.7	3.20 ± 1.78	3.38 ± 1.51	14.8 ± 2.67 [a]	23.3 ± 1.78[a,b]	20.6 ± 1.60 [a,b]

a= p <0.05, 3 months *vs* 9 months; b= p <0.05, Control *vs* Cd exposure. See text for details.

Table 2. Results from the two-way ANOVA analysis. Significant values are indicated by the asterisk.

Factors	**Tissues**								
	Skin			*Liver*			*Kidney*		
	DF	**F value**	**P value**	**DF**	**F value**	**P value**	**DF**	**F value**	**P value**
Time (3 months vs 9 months)	1	12.54	0.002*	1	66.26	<0.0001*	1	229.67	<0.0001*
Treatment (44.5 nM/L vs 178 nM/L Cd)	2	2.515	0.110	2	11.05	0.001*	2	1.26	0.309
Time*Treatment	2	2.02	0.163	2	7.50	0.005*	2	5.68	0.137

Table 3. Metallothionein levels in the skin and liver of the newt *T. carnifex* exposed to 44.5 nM/L and 178 nM/L of Cd, respectively, during 3 and 9 months. Metallothionein levels were expressed in µg/g wet tissue. Values are means ± SE of the mean. Differences were considered significant when $P < 0.05$.

Tissues	**Exposure**					
	3 months			*9 months*		
	Control	**Cd 44.5 nM/L**	**Cd 178 nM/L**	**Control**	**Cd 44.5 nM/L**	**Cd 178 nM/L**
Skin	71±10	73.1 ± 9	75.2 ± 12	92.1 ± 11 [a]	99.8 ± 14 [a]	101±18 [a]
Liver	76.1± 9	62.3 ± 8	75.2 ±11	92.1 ± 12 [a]	100 ±15 [a]	101 ± 14 [a]

a = p <0.05, 3 months *vs* 9 months.

Table 4. Results from the two-way ANOVA analysis. Significant values are indicated by the asterisk.

Factors	**Tissues**					
	Skin			*Liver*		
	DF	**F value**	**P value**	**DF**	**F value**	**P value**
Time (3 months vs 9 months)	1	22.92	0.0002*	1	2296.7	<0.0001*
Treatment (44.5 nM/L vs 178 nM/L Cd)	2	1.11	0.354	2	2.09	0.244
Time*Treatment	2	1.96	0.174	2	5.32	0.069