

Acute Effects of Cadmium and Copper on Cytochemical Responses in the Polychaete *Hediste diversicolor*

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ABSTRACT: This study investigated the cytochemical responses in *Hediste diversicolor* exposed to the environmental concentrations of cadmium (1 μM Cd), copper (1 μM Cu) and to their equimolar mixture for 48h. Lysosomal membrane stability (LMS), neutral lipids (NL) level, lipofuscin (LF) content and Ca²⁺-ATPase activity were determined as early subcellular biomarkers of toxicity. The biomarker battery pointed out a high cytotoxicity on the polychaete treated with metals. Excessive induction of neutral lipids and lipofuscin accumulations were observed in polychaete treated with copper than cadmium. In addition, the highest level of neutral lipids and lipofuscin was noted in mixture treated group [Cu+Cd]. The increase of neutral lipids and lipofuscin accumulations were negatively correlated with the lysosomal membrane stability and Ca²⁺-ATPase activity alterations. Our study also demonstrated that the cytochemical biomarkers responses were very important to identify the cellular toxic effects of metals and promising biological tools in the assessment of biological effects of pollutants in natural marine environment.

Key word: Cadmium, Copper, Cytotoxicity, Cytochemical biomarkers, *Hediste diversicolor*

INTRODUCTION

Marine ecosystem is the final receptacle of various pollutants originating from human activities, urban, agricultural and industrial discharges. A considerable number of chemicals including metals have already been released into the environment and persist in sediment, water and biota. This situation endangers the health of organisms inhabiting this ecosystem (Levin *et al.*, 2001; Wiegner *et al.*, 2003). Metals have been of great concern in marine and coastal ecosystems, since they cause several biological alterations from molecular to tissue level depending on their concentrations and time exposure (Banni *et al.*, 2007; Ben Khedher *et al.*, 2014). Therefore, they may be also, accumulated in various tissues of living organisms and lead to several orders of magnitude higher than those of the surrounding water (Ghedira *et al.*, 2011; Jebali *et al.*, 2014).

Copper (Cu) is one of the most prevalent metals in coastal sediment (Salomons and Förstner, 1984) and at

high concentration, is highly toxic to marine life. Copper pollution in the aquatic environment results from natural and anthropogenic sources such as mine washing or agricultural leaching. Although copper is a trace element essential to life it is also one of the most toxic heavy metals (Tóth *et al.*, 1996). Its action alters intracellular protein machinery either directly via denaturing enzymes or indirectly via generation of reactive oxygen species (Pourahmad and O'Brien, 2002; Droge, 2002) and has also recently been shown to cause DNA damage in sperm (Caldwell *et al.*, 2011). Cadmium (Cd) is a heavy metal widely distributed in the environment as a result of natural and anthropogenic activities. Cadmium is commonly used in environmental studies because it is highly toxic (Lane and Morel, 2000) and can adversely affect the organisms at relatively low exposure concentrations (Waisberg *et al.*, 2003). Cd is a toxic metal because of its interference with essential metals such as Zn and Fe, thereby interfering with many biochemical

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processes that are regulated by metallic enzymes (Wright and Welbourn, 1994). In addition, Cd is also known to exert mutagenic (Mandel and Ryser, 1984) and carcinogenic (Degraeve, 1981) effects, and induce lipid peroxidation and DNA breakage (Ochi and Ohsawa, 1983). Cd toxicity is also manifest as perturbations in metabolism such as enhanced lipolysis (Pierron *et al.*, 2007), and lowered glycogen content (Teh *et al.*, 1999; Bernsten and Lundebye, 2001). By contrast, very few data are available on other sublethal effects including lysosomal membrane stability, cytotoxicity, oxidative stress that could result from the biochemical activity of cadmium and copper in the polychaete *H. diversicolor*.

The polychaete *Hediste diversicolor*, is a marine annelid which lives in estuary sediments rich in microorganisms and toxic agents resulting from pollution. It has been the subject of numerous studies, focusing on different aspects of its biology and ecology, including a range of pollution related subjects. This polychaete is characterized by a high physiological tolerance to extreme variation of many environmental parameters such as temperature and salinity (Bartels-Hardege & Zeeck, 1990; Ait Alla *et al.*, 2006). Also it's regarded as good bioindicator of metal and organic contamination in estuaries due to their highly tolerance of metal pollutants (Scaps, 2002; King *et al.*, 2004; Bouraoui *et al.*, 2010). A previous study found that the LC50 of Cd in *H. diversicolor* is approximately 100 mg/L after 192 h of exposure (Bryan, 1976), which is much higher than that in most marine invertebrates (0.1-10 mg/L) (Cossa and Lassus, 1989). For copper, Yang *et al.* (2012) reported that the LC50 of Cu is approximately 475 µg/L after 96 h of exposure. So it inferred that polychaetes have complex and effective detoxification mechanisms to protect them from metal toxicity. Therefore, the use of polychaete worms as bioindicators for marine ecosystems has proved efficiency to be a useful tool in the assessment of environmental quality (Dean, 2008; Bouraoui *et al.*, 2010).

Aquatic organisms are invariably exposed to pollutants mixtures. Studies examining bioaccumulation and subsequent toxicity of contaminants have often focused on single pollutants (Selck *et al.*, 1999; Pechenik *et al.*, 2001; Sandrini *et al.*, 2008). However, the interactions between these pollutants are poorly known and need to be studied. Considering the facts cited above, this work aimed to investigate the acute subcellular effects of copper and cadmium on the polychaete *H. diversicolor*. A set of cellular stress markers was used including lysosomal membrane stability (LMS), lysosomal accumulation of lipofuscin (LF) and neutral lipids (NL); and plasma membrane Ca²⁺ ATPase activity.

MATERIALS & METHODS

Specimens of *H. diversicolor*, weighing between 0.4 and 0.6 g were collected in the intertidal zone at low tide from Teboulba (Tunisia) that was previously reported to be an unpolluted site (Banni *et al.*, 2005, 2007; Bouraoui *et al.*, 2009, 2014). The polychaetes and the surrounding sediments were placed in open polyethylene bottles and maintained at 14 °C to 16 °C. Once in the laboratory the polychaetes were separated from sediment, cleaned from debris then placed in glass petri dishes at 14 °C with aerated clean seawater to ambient photoperiod regimes for 3 days. This acclimatation period was used for excluding specimens with exoskeleton or skin infections.

After this period, polychaetes were exposed for 48 h to the environmental concentration of 1 µM of Cd (CdCl₂) or 1 µM of Cu (CuSO₄) and to their equimolar mixture. A control group was run in parallel, employing only saline water (10‰) with the same characteristics cited above. After 48 h of exposure, worms washed briefly in ice-cold buffer, flash-frozen in N-hexane, chilled in liquid nitrogen and then stored at -80 °C.

For Lysosomal membrane stability (LMS) assay, Pieces of excised worms were placed on two different aluminum cryostat chucks for a total of six individuals, and sections (10µM) were cut with a cryostat (Leica CM3050) and flash-dried by transferring them to room temperature. The cryostat sections were incubated at 37 °C in 50 mL of 0.1 M citrate buffer with 2.5% NaCl containing 20 mg naphthol AS-BI-N-acetyl- -glucosaminide previously dissolved in 2.5 mL 2-methoxyethanol and 3.5g of polypeptide, rinsed at 37 °C in 3% NaCl, treated at room temperature with 1 mg/mL Fast Violet B in 0.1 M phosphate buffer and fixed for 15 min in Baker's fixative at 4 °C (Moore, 1976). Staining intensity of lysosomes was determined by studying the slide at 400× magnification with an inverted Axiovert microscope (Zeiss), connected to an AxioCam digital camera (Zeiss). Digital image analysis was carried out using the Scion Image software package (Scion Corp).

Neutral lipids content was made from sections prepared the same as for the lysosomal membrane stability assay by fixing the sections in calcium-formaldehyde (2% Ca-acetate (w/v), 10% formaldehyde (v/v)) for 15 min at 4°C, followed by a rinsing step with de-ionised water, and incubation with 60% triethylphosphate (TEP) for 3 min. The sections were then stained with Oil Red-O (1% in 60% TEP) for 30 s, rinsed with de-ionised water, and mounted in 20% (v/v) glycerol (Moore, 1988). Neutral lipid content was quantified by digital image analysis, as described for the LMS assay.

For Lipofuscin content, Cryostat sections were fixed in calcium-formaldehyde and rinsed with de-ionised water, as described for the neutral lipid assay, followed by a 5 min incubation step with 1% Fe_2Cl_3 , 1% potassium ferrocyanide in a 3:1 ratio (Moore, 1988). The sections were rinsed with 1% acetic acid and mounted in 20% (v/v) glycerol. Lipofuscin content was quantified by digital image analysis of stained sections, as described for the LMS assay.

The Ca^{2+} -ATPase activity enzyme was quantified from cryostat sections obtained as for the LMS assay, using the histochemical method described by Pons *et al.*, (2002). Cryostat sections were washed in 0.05M cacodylate buffer (pH 7.4), fixed in 1% paraformaldehyde (pFA) in 0.05 M cacodylate buffer (pH 7.4) for 30 min at 4 °C and washed again in 0.05M cacodylate buffer. Samples were then dehydrated in increasing acetone concentrations at 4 °C and embedded in Technovit 7100 resin. Serial cross sections (2 μm) were cut using a microtome, transferred onto glass slides and incubated for 6 h at room temperature in a medium containing 2.4 mM ATP, 18 mM CaCl_2 , 8 mM levamisole, 0.2 mM ouabain, 1 mM $\text{Pb}(\text{NO}_3)_2$, and 20 mM sodium barbiturate. After incubation, the medium was removed and slides washed in water and rinsed in an ammonium sulfide-saturated water solution (3 min) to reveal the brown lead sulfide. $\text{Pb}_3(\text{PO}_4)_2$ precipitates stained with ammonium sulfide was quantified on sections by digital imaging as described above.

The results were expressed as means \pm SD. SPSS software (version 20.0) was used for statistical analysis. The data were first tested for normality and homogeneity of variance to meet statistical demands. Data from different groups were compared by a one-way analysis of variance (ANOVA). Gabriel's test was used to analyze raw biomarker data for comparison of responses between treatments. All differences were considered significant at $p < 0.05$. Different letters a, b, c and d indicated significant differences between groups.

RESULTS & DISCUSSION

No significant worm mortality (<10%) was observed during the exposure period at different treatments with 1 μM Cd, 1 μM Cu or with their equimolar mixture of Cd and Cu. The effect of the tested single metal of Cu or Cd and their mixture on lysosomal membrane stability (LMS) is reported in Fig.1. A significant membrane destabilization with respect to untreated group was observed after exposure to each treatment with a percentage inhibition rising to 55% in copper group, 56% in cadmium group and 65% in mixture group. On the other hand, no significant

differences ($p < 0.05$) in terms of LMS was observed between treated groups. Representative images of intestinal cells staining of control and exposed animals are reported in Fig. 1.

The neutral lipid (NL) and lipofuscin (LF) contents in the treated *H. diversicolor* are reported in Fig 2 and 3. Lysosomal contents of NL and LF showed similar trend. In comparison to non exposed polychaetes, contents of NL significantly increased in Cu-, Cd- and mixture- treated group.

The highest neutral lipid (NL) and lipofuscin (LF) accumulations levels were observed in (Cd+Cu) group (5.53-times higher than the control, $P < 0.05$). NL levels were 4.04-fold and 2.83-fold higher than the control group after single exposure respectively to Cu and Cd (Fig.2). When compared to control values, LF contents tended to increase in *H. diversicolor* exposed to contaminants (Fig. 3), but only the Cu and mixture exposure caused a significant increase in this parameter with more than respectively 435% and 1937%.

The Ca^{2+} ATPase activity, showed small changes between treatments tested with Cd and Cu (Fig. 4). However, the highest decrease in the activity was noted in worms exposed to the mixture with respect to the control reaching a percentage-value of 59%.

It has been accepted that aquatic ecosystems often contain mixtures of toxicants, including organic and inorganic compounds. It is important then to investigate the joint effects of more than one chemical pollutant on living organisms. In fact, individual components are likely to produce different responses within the same organisms and/or interact with other chemical pollutant producing additive, synergistic or antagonistic toxic effects (Barata *et al.*, 2006). The influences of toxicant mixtures on natural communities are poorly understood and little is known about their interaction. However, the vast majority of available toxicity data, deals with the effects of single pure chemicals (Zhou, 1995). In the other hand, the joint effect of marine toxicants on the cytochemical markers in invertebrates was rarely investigated except for a few species (Koehler, 1992; Moore *et al.*, 2004; Dondero *et al.* 2006 a,b; Gastaldi *et al.*, 2007). In this work, we aimed to investigate the acute subcellular effects of copper and cadmium on the polychaeta *H. diversicolor*. A set of cellular stress markers were used including lysosomal membrane stability (LMS), lysosomal accumulation of lipofuscin (LF) and neutral lipids (NL); and plasma membrane Ca^{2+} ATPase activity.

The lysosomal system of many marine organisms including molluscs, annelids, crustaceans and fish, is known to be particularly sensitive to environmental perturbations and is generally thought to provide early

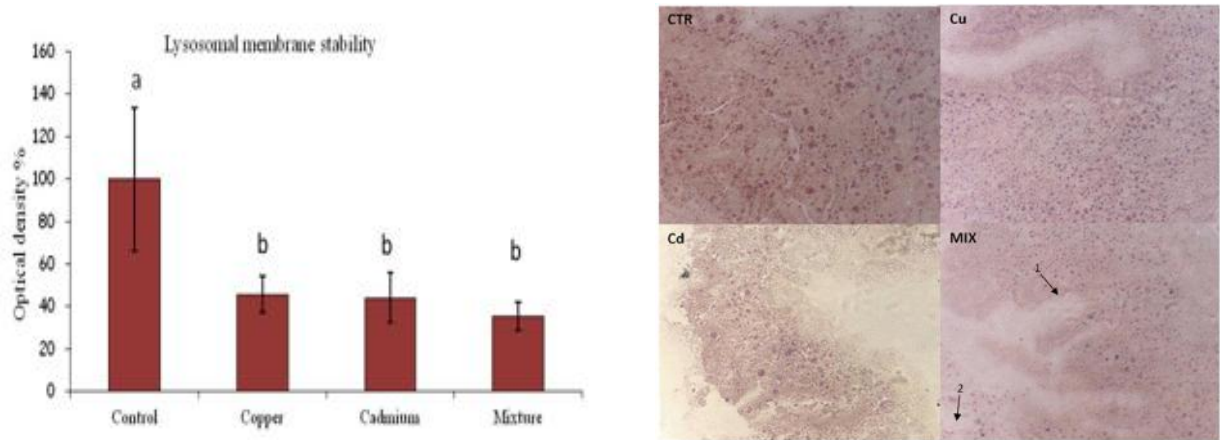


Fig 1. Acute effect of Cu, Cd and their mixture on LMS in *H. diversicolor* after 48 h exposure. Equal letters indicate absence of significant difference between means of different treatments. Representative images of cryostat tissue sections from control and exposed worms are reported. 1: epithelium, 2: coelome

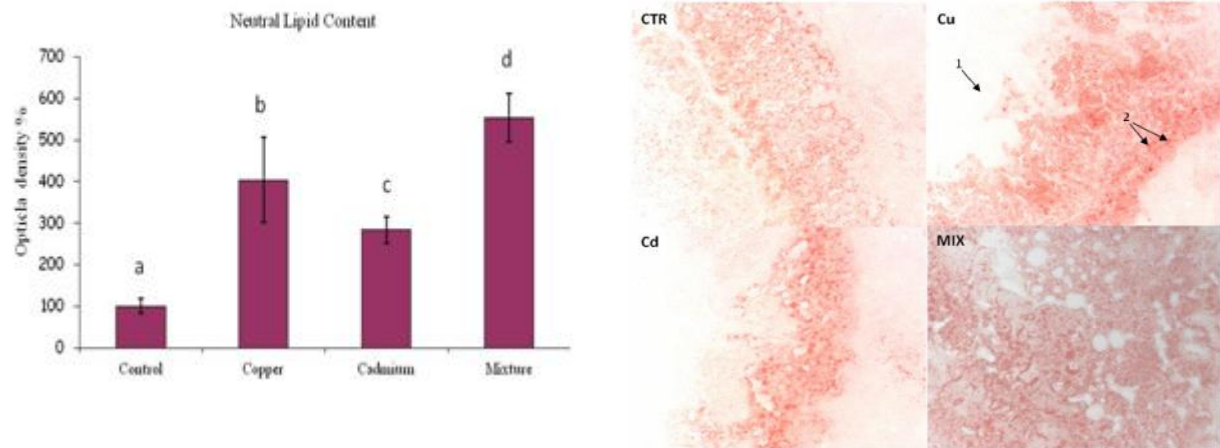


Fig 2. Acute effect of Cu, Cd and their mixture on NL accumulation in *H. diversicolor* after 48 h exposure. Equal letters indicate absence of significant difference between means of different treatments. Representative images of cryostat tissue sections from control and exposed worms are reported. 1: coelome, 2: Neutral Lipid

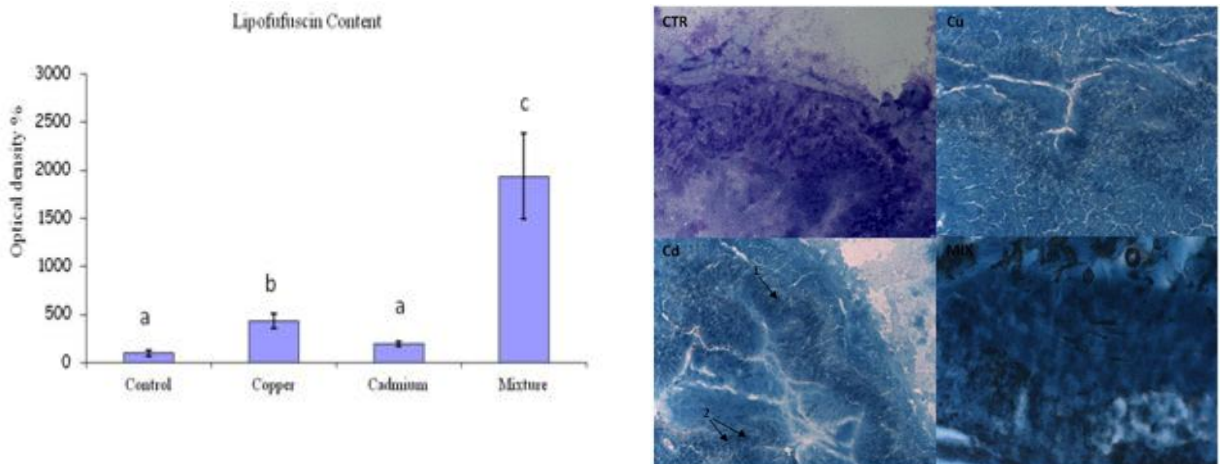


Fig. 3. Acute effect of Cu, Cd and their mixture on lipofuscin (LF) content in *H. diversicolor* after 48 h exposure. Equal letters indicate absence of significant difference between means of different treatments. Representative images of cryostat tissue sections from control and exposed worms are reported. 1: epithelium, 2: lipofuscin

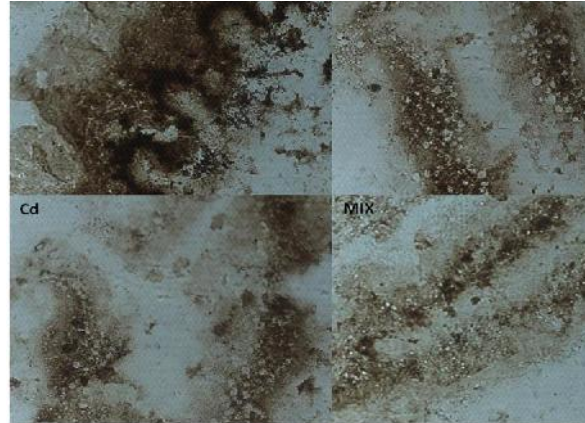
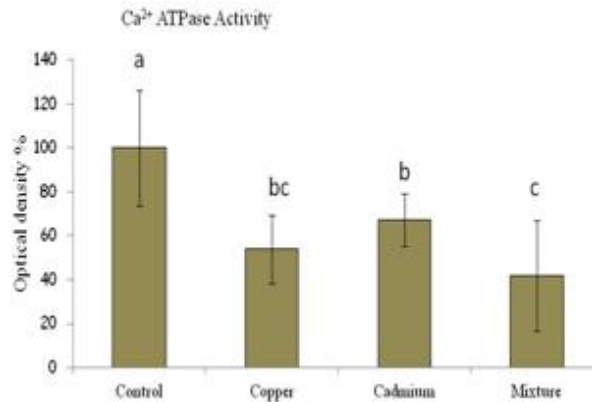


Fig. 4. Acute effect of Cu, Cd and their mixture on Ca²⁺ATPase activity in *H. diversicolor* after 48 h exposure. Equal letters indicate absence of significant difference between means of different treatments. Representative images of cryostat tissue sections from control and exposed worms are reported

response to pollutant exposure (Moore *et al.*, 2006; Gastaldi *et al.*, 2007; Bouraoui *et al.*, 2014; Moschino *et al.*, 2014). Lysosomal membrane stability (LMS) is a routinely used biomarker acting as an early indicator of the adverse effects of environmental contaminants both in invertebrates and vertebrates (Cajaraville *et al.*, 2000; Galloway *et al.*, 2004; Hankard *et al.*, 2004; Gastaldi *et al.*, 2006; Moore *et al.*, 2006; Catalano *et al.*, 2012). The effects on lysosomal membrane stability in *H. diversicolor* were evaluated in digestive cells following the reaction for N-acetyl- α -hexosaminidase using histochemical procedures applied on frozen tissue sections. The obtained results demonstrated that both compounds (Cu 1 μ M; Cd 1 μ M and [Cd+Cu] 1 μ M) induced a significant decrease in LMS after 48h of exposure underlining the high toxicity of these metals and confirming that lysosomes are a subcellular target for the action of both metal treatment. Similarly, previous studies in the polychaetes *H. diversicolor* including both laboratory exposure experiments and field surveys reported a destabilization of lysosomal membrane under Cd, Cu and polycyclic aromatic hydrocarbons (PAHs) gradient (Catalano *et al.*, 2012; Bouraoui *et al.*, 2014). Interestingly, lysosomal membrane destabilisation was associated with a decrease of the Ca²⁺ATPase activity in *H. diversicolor* exposed to Cd or/and Cu. Ca²⁺ATPases are membrane SH-containing proteins and fundamental in the maintenance of cytosolic calcium homeostasis (Carafoli, 1994; Berridge *et al.*, 1998). Marchi *et al.* (2004), have demonstrated that destabilisation of lysosomal membranes is linked to an increase in [Ca²⁺] and an activation of calcium-dependent phospholipase A2 (cPLA2). Also cPLA2 enzyme activity was the main factor for destabilisation of lysosomal membranes (Dondero *et al.*, 2006). In other hand, several authors have demonstrated an inhibition of Ca²⁺ATPase under

effect of oxidative stress and heavy metals in both mammalian cells (Viarengo and Nicotera, 1991) and the cells of aquatic invertebrates such as mussels (Ermak and Davies, 2001; Burlando *et al.*, 2004), earthworms (Gastaldi *et al.*, 2007) and sea worms (Catalano *et al.*, 2012; Bouraoui *et al.*, 2014).

Toxic effects of metals are often depending on their capacity to increase the cellular levels of reactive oxygen species (ROS). When ROS levels production exceeds antioxidant defences, cells experience oxidative stress which causes, among others, membrane lipid peroxidation (Viarengo, 1989). Lipofuscins are end-products of lipid peroxidation which, due to their non-degradable nature, tend to accumulate in the lysosome vacuolar system. Our results showed that 1 μ M Cd, 1 μ M Cu and their equimolar mixture stimulate lipofuscinogenesis in *H. diversicolor*. It is well established that Cu is able to create an oxidative stress status by the Harber-Weiss and Fenton reaction of Cu cations (Suzuki *et al.*, 1996), resulting in cellular damages due to hydroxyl radicals (HO•). For cadmium, many authors have already shown that Cd induce lipid peroxidation and DNA breakage (Ochi and Ohsawa, 1983; Bouraoui *et al.*, 2009). Cd toxicity also manifest perturbations in metabolism such as enhanced lipolysis (Pierron *et al.*, 2007) and decrease of adenylate energy charge (Den Besten *et al.*, 1991). This holds even though it is well known that Cu is far more toxic than Cd in aquatic organisms such as fish, bivalves, and polychaete... (Cheng, 1990; Won *et al.*, 2008).

Accumulation of neutral lipids (NL) in lysosomes is often associated with the effects of pollutants as a measure of unbalanced fatty acid metabolism (Catalano *et al.*, 2012). As reported by Moore (Moore, 1988), a build-up of these substances in digestive cells may be

described as a form of lipidosis induced by toxic chemicals. In fact, our cytochemical data clearly indicate a stockpiling of neutral lipids in the cytoplasm of cells of organisms exposed to 1µM Cd, 1µM Cu and then peaked in those treated with equimolar mixture. Data reported in the present work show a strong and constant response of the cytochemical parameters in worms exposed either to only Cu/ Cd or to the mixture (Cu+ Cd). Our results have highlighted an additive effect of the binary mixtures (Cu+Cd) as already observed in previous studies at similar concentrations and time on enzymatic biomarkers and lipoperoxidation level in *H. diversicolor*. (Banni et al., 2009; Bouraoui et al., 2009).

CONCLUSIONS

This work demonstrates clearly, the subcellular negative effects of the environmental concentration of copper and cadmium in the polychaete in *H. diversicolor*. Remarkable alterations of the lysosomal membrane stability, lipid peroxidation and Ca²⁺-ATPase activity were observed. Excessive induction of neutral lipids and lipofuscin accumulations were observed in polychaete treated with copper than cadmium. The increase of neutral lipids and lipofuscin accumulations were negatively correlated with the lysosomal membrane stability and Ca²⁺-ATPase activity alterations. In addition, our results indicate that the polychaete *H. diversicolor* should be a valuable benthic organism for studying risks of marine pollutants using cytochemical tests.

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