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Original Article

Cholinesterase activity and histopatological changes in the Mediterranean crab, *Carcinus maenas*, exposed to environmental contaminants

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Abstract: Marine environments are continuously being threatened by a large number of pollutants including heavy metals and organophosphorous pesticides from anthropogenic sources. These compounds can cause a serious environmental problem. The present study aimed: (1) to measure sensitivity of acetylcholinesterase (AChE) activity to *in vivo* exposure to the organophosphorous chlorpyriphos-ethyl (CPF) and to the heavy metals cadmium (Cd) and copper (Cu) and (2) to use the histopathological lesions as tissue biomarkers for biomonitoring of different contaminations. The results clearly showed that the AChE activity in different tissues (digestive gland, muscle and eyes) of *Carcinus maenas* was relatively sensitive to the concentrations of CPF and tended to have different patterns in response to Cd, Cu and Cu+Cd mixture exposure. The transfer of treated crabs to the clean sea water allowed to recover totally or partially the lost activity depending on selected tissues and contaminant exposure (metals or organophosphorous compounds). Histopathological biomarkers in *C. maenas* exposed to different contaminants showed the presence of different lesions which altered the digestive gland after 7 days of contamination.

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Introduction

The marine ecosystem is threatened by increasing levels of contaminants originating from anthropogenic activities (Ali and Sreekrishnan, 2001; Chouksey et al., 2004). This situation endangers the health of organisms and human. Among anthropogenic contaminants, pesticides are widely detected in freshwater and marine ecosystems. The organophosphates (OP) and carbamates (Cs) are modern synthetic insecticides and potent neurotoxic molecules (Ghedira et al., 2009). These compounds reach the sea through rivers and lead to the contamination of different marine ecosystems (water and sediments) (Mora et al., 1999). They can produce adverse effects on non-target aquatic organisms. The most widely used pesticide in agriculture to control insects and fungi is the OP insecticide chlorpyrifos (CPF). Because of its broad spectrum, CPF represents a threat to non-target species including aquatic organisms (Botté et al., 2012). This toxicity has been demonstrated by numerous field and laboratory studies on temperate species (Fulton and Key, 2001). The mode of CPF action is cholinesterase (ChE) inhibition. Chlorpyrifos induces irreversible ChE inhibition (Fulton and Key, 2001), triggering constant stimulation of the muscles which leads to paralysis and death (Botté et al., 2012) and this at high doses of OP exposure.

An increasing number of studies provide evidence that ChE activities may be affected by a wide range of contaminants other than OP and Cs, including heavy metals (Jebali et al., 2006; Vioque-Ferňandez et al., 2007; Elumalai et al., 2007; Bonacci et al., 2008). Heavy metals such as cadmium and copper have the capability to induce harmful effects on living organisms at ecological relevant concentrations and have been considered as important environmental contaminants (Cunha et al., 2007). Copper and cadmium are of particular concern in marine ecosystem because several species are able to bioaccumulate and/or bioconcentrate them in the body tissues where they may reach toxic concentrations that

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cause deleterious effects (Pereira et al., 2009).

Histopathological lesions are another environmental biomarker. However, whilst studies on finfish have shown that histopathology is a sensitive indicator of individual and population health status, and results from numerous controlled laboratory exposures of shellfish (crustaceans and molluscs) to toxicants have shown that histopathological changes also occur in the organ and tissue systems of these animals, relatively few field studies have included shellfish histopathology in the suite of employed monitoring tools (Stentiford et al., 2005).

In this study, we (1) measured the sensitivity of ChE versus AChE activity to *in vivo* exposure to the OPs (Chlorpyrifos-ethyl) and heavy metals (cadmium and copper) and (2) to show the histopathological alterations after exposure to different contaminants.

Materials and Methods

Chemicals: The following reagents were obtained from Sigma-Aldrich (Villefranche, France): acetylthiocholine (ASCh) and 5,5'-dithio-2-nitrobenzoate (DTNB).

Crabs collection: Crabs (*Carcinus maenas*) were collected with hand at the Kuriat Island, which is an uncontaminated area (Jebali et al., 2011) in September 2012. Specimens (male) were immediately transported to the laboratory in aerated buckets filled with seawater. Upon arrival at the laboratory, crabs were divided into 31 groups of 6 crabs and placed in plastic tanks with 10 L of natural sea water (36.89‰ salinity) and kept at 17.2°C. Crabs were fed regularly with fish every three days during the acclimation and the exposure period. After 7 days of acclimation, crabs were used to study the kinetic effect of Cd, Cu and chlorpyrifos-ethyl pesticide (CPF) on crab AChE activity and the histopathological biomarkers.

***In vivo* effects of environmental contaminants:** After 7 days of acclimation, groups of crabs were exposed to chlorpyrifos-ethyl (CPF) (4.8 µg/L) (Gagnaire et al., 2008), Cd (200 µg/L) (Blasco et al., 1999), Cu (200 µg/L) (Roméo et al., 2006; Vieira et al., 2009) and to their mixture Cu+Cd (200 µg/L Cd+200 µg/L Cu) for 0.5, 1, 2, 4 and 7 days (d); after each time, 6 crabs of treated and relative control groups were sacrificed and the main organs i.e. digestive gland, muscle and eyes, were carefully removed and frozen at -80°C until analysis. Exposure to CPF, Cd, Cu and (Cd+Cu) was renewed every 3 days. Chlorpyrifos-etyl is the active ingredient of pesticide Dursban®, it was prepared by adding an organic solvent i.e. acetone. All treatments and controls received the same acetone concentration (0.01%) (Tu et al., 2009). This acetone concentration is below the no-observed-effect concentration (NOEC) of 0.1% reported by Mayer (1987). Cadmium and copper were prepared by adding distilled water. In order to study the capacity of crabs to recover the AChE activity; at the end of contamination period (7 day), six treated crabs of CPF, Cu, Cd and their mixture (Cd+Cu) groups were transferred into the clean water. After 7 days, crabs were sacrificed and the same organs were carefully removed and frozen at -80°C until analysis.

Biochemical determinations

Cell-free extract preparations: All steps for cell-free extract preparation were carried out at 4°C. Organs (digestive gland, muscle and eyes) were homogenized in ice-cold phosphate buffer (100 mmol/L; pH 7.5; 1 mmol/L EDTA; 1 mmol/L reduced glutathione; GSH) at a rate of 3 mL/g (buffer volume/tissue weight). Homogenates were then centrifuged at 9,000×g for 15 min. The supernatant of each sample was stored at –20°C, for no longer than a week, until enzyme activity determination. Total protein content in the supernatant (S9) was measured following the Bradford method (Bradford, 1976), at 595 nm, using bovine serum albumin as standard protein.

AChE activity to *in vivo* exposure to environmental contaminants: ChE activities were estimated by the method of Ellman et al. (1961). Acetylthiocholine (ASCh) was used as substrate for preliminary screening of ChE types in tissues. Basal conditions in the reaction mixture (final volume 1150 µl) of the ChE assay were as follows: 100 mM phosphate buffer, pH 7.5; 50 µl of 8 mM dithiobisnitrobenzoate (DTNB) and 40 µl supernatant (enzyme solution). The reaction was started by adding 51.28 µl of 45 mM substrate (2 mM final concentration).

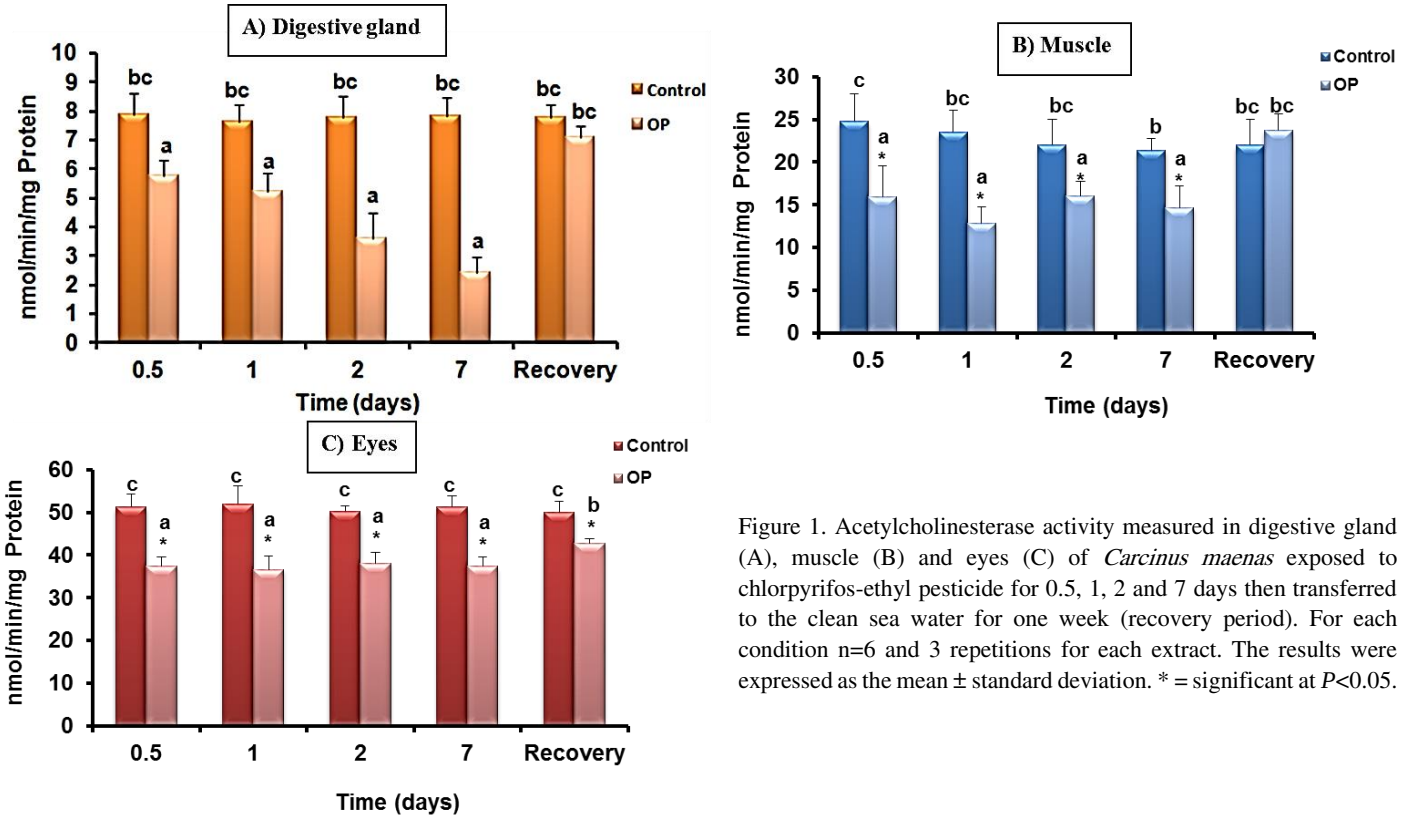

Figure 1. Acetylcholinesterase activity measured in digestive gland (A), muscle (B) and eyes (C) of *Carcinus maenas* exposed to chlorpyrifos-ethyl pesticide for 0.5, 1, 2 and 7 days then transferred to the clean sea water for one week (recovery period). For each condition n=6 and 3 repetitions for each extract. The results were expressed as the mean ± standard deviation. * = significant at $P<0.05$.

The enzymatic reaction rate was quantified spectrophotometrically at 412 nm against a blank without substrate for each activity measurement. In order to subtract the spontaneous hydrolysis of substrate, a second blank was performed without sample. Enzyme activity was recorded over 10 min after adding substrate. The AChE activity was expressed as specific activity (nmol substrate hydrolysed/min/mg protein).

Histology: The digestive gland of the 6 crabs of treated and control groups sacrificed after 7 days of exposure to metals and CPF and after 7 days of recovery was removed and fixed in Bouin's fixative for 48 hrs. The preserved tissue was processed by a routine histological method; dehydrated in graded ethanol solution and embedded in paraffin. Embedded tissues were cut into sections of 5 µm thickness by a rotary microtome (Leitz WETZLAR 1512). The thin sections of the digestive gland tissue were stained by Trichrome Masson and Hematoxylin-Eosin for observation by the light microscope. Sections were photographed with a microscope (LEICA 132 DM 750) provided with a numerical camera (Leica ICC50 HD) and examined for lesions.

Statistical analysis: Statistical analyses were performed using SPSS software. Significant differences between means were determined using one-way ANOVA followed by the Duncan's test. In order to determine kinetic parameters such as the apparent Michaelis–Menten constant (Km) and the maximum substrate hydrolysis velocity (Vmax), we used GraphPad Prism version 4 for Windows (GraphPad Software).

Results

***In vivo* effects of chlorpyrifos-ethyl on AChE activity:** During the seven days of experiment, no mortality was reported in controls. *In vivo* exposure to lower concentration of chlorpyrifos-ethyl (CPF) (4.8 µg/L) led to inhibition of AChE activity in all tissues (Fig. 1). A decrease of activity was not time dependent in any organ. In eyes, activity was decreased 29.46%. In muscle, the maximum decrease of activity was observed at 1 day (45.29%) and maintained lower than that of control. In digestive gland, the inhibition of AChE activity reached its maximum at 7d (68.71%).

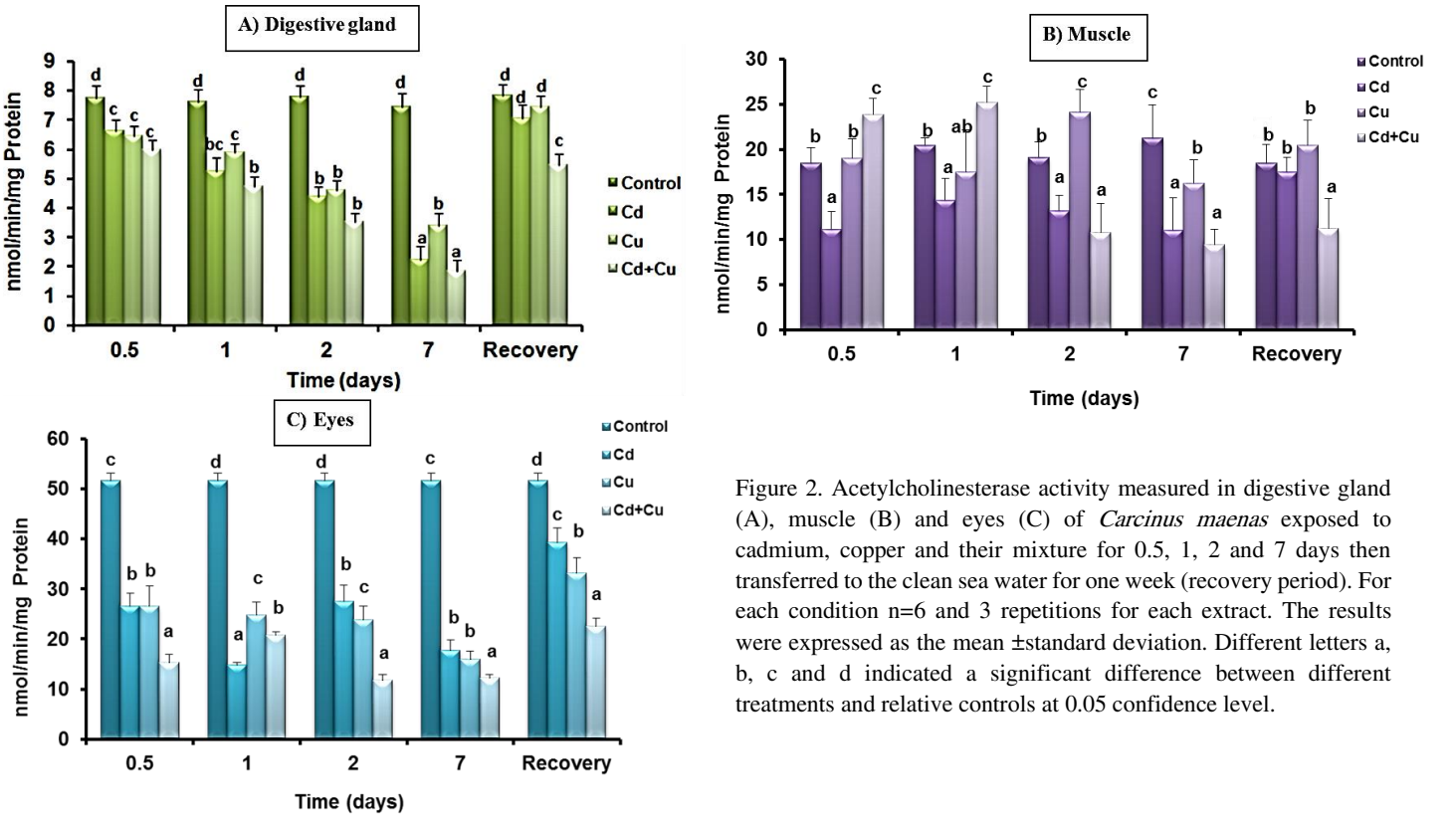

Figure 2. Acetylcholinesterase activity measured in digestive gland (A), muscle (B) and eyes (C) of *Carcinus maenas* exposed to cadmium, copper and their mixture for 0.5, 1, 2 and 7 days then transferred to the clean sea water for one week (recovery period). For each condition n=6 and 3 repetitions for each extract. The results were expressed as the mean ±standard deviation. Different letters a, b, c and d indicated a significant difference between different treatments and relative controls at 0.05 confidence level.

Table 1. A semi-quantitative evaluation of the histopathological lesions in digestive gland of control *Carcinus maenas* (C) and (C CPF) and crabs exposed to cadmium (Cd), copper (Cu), (Cd+Cu) mixture and chlorpyriphos-ethyl (CPF).

	C	Cd	Cu	Cd + Cu	C OP	OP
B cells	+	+[a]	+[b]	+	+	+
E cells	+	+	+	+	+	+
F cells	+	+	+	+	+	+
R cells	+	+	+	+	+	+
Abnormal lumen (ALU)	-	++	+++	+++	-	+++
Haemocytic infiltration in the interstitial sinus (HI)	-	+++	+++	+++	-	+++
Necrotic tubules of hepatopancreas (NT)	-	++	-	+++	-	++
Thickened basal laminae (TBL)	-	++	++	++	-	++
Coagulation in the thickened basal laminae (CO)	-	-	-	+	-	-
Walling off of the tubules by haemocytes around the thickened basal laminae	-	+	+	++	-	++
Reserve inclusion (RI)	++	++	++	++	++	++

The following symbols were adopted to describe the lesions severity and structural changes: (-): no alteration, (+): low, (++): moderate and (+ + +): high alteration.

[a] Decrease of the number of B cells

[b] Cells with large vacuoles

After short time (7 days) of depuration, the digestive gland and the muscle were able to recuperate AChE activity, while in the eyes, an increase of the AChE activity observed as 42.77±1.06 nmol/min/mg protein. ***In vivo* effects of Cd, Cu or their mixture on AChE activity:** AChE activity in tissues (digestive gland, muscle and eyes) after *in vivo* exposure to cadmium, copper and their mixture for seven days is summarized in Figure 2. ChE activity was affected by exposure to Cd, Cu and their mixture (Cu+Cd), but significant differences were observed compared to different metals. Exposure to Cd reduced AChE activity in all tissues and the maximum reductions were observed at 7d (respectively 69.79% in digestive gland, 48.12% in muscle and 71.44% in eyes). In contrast to higher inhibition of AChE activity in Cd-treated crab, Cu induced activity after 2 days (24.16±2.44 nmol/min/mg protein in muscle), then the activity

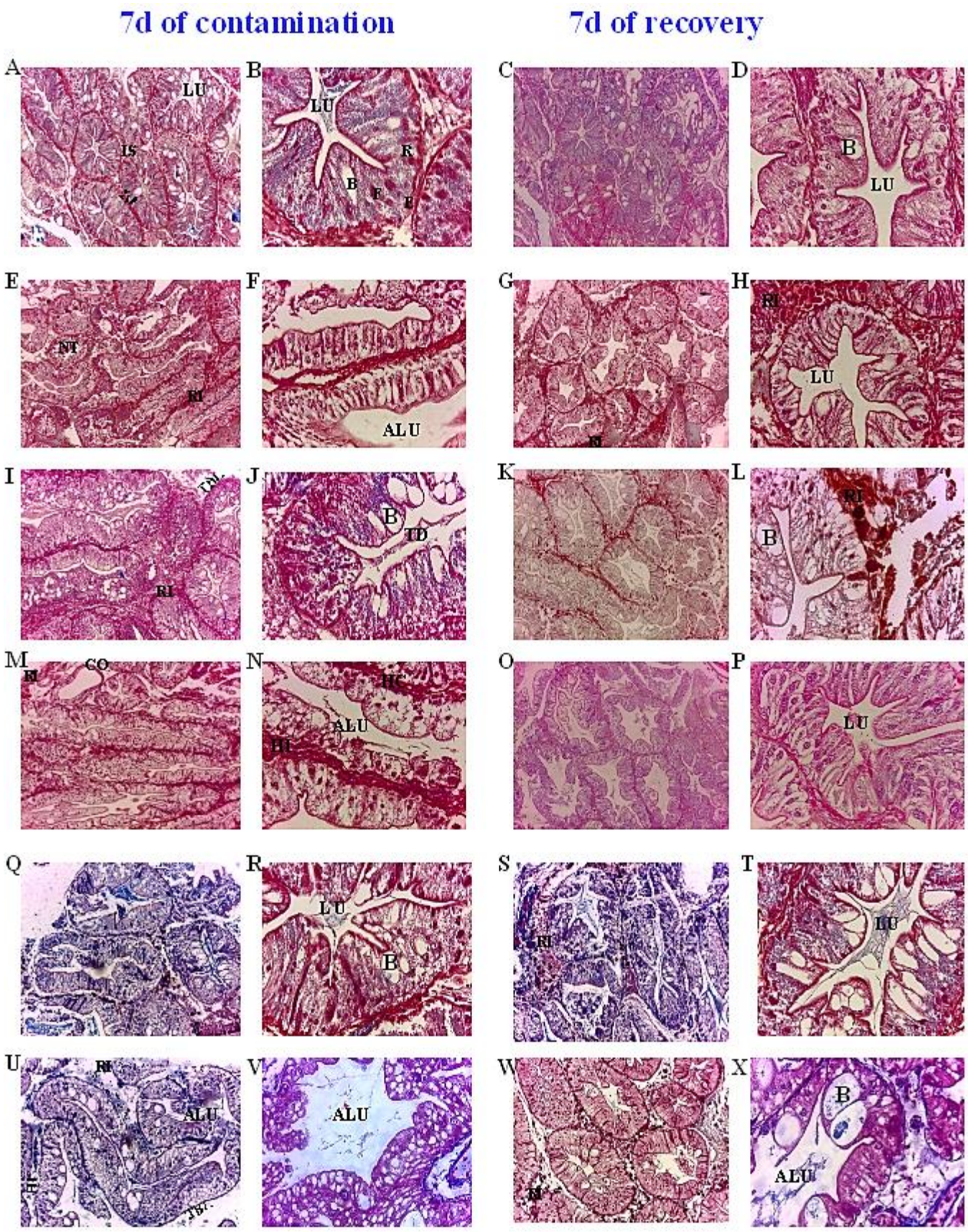
7d of contamination
7d of recovery
A
LU
IS
B
LU
B
C
D
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Figure 3. Digestive gland histopathology of *Carcinus maenas* after 7 days of contamination and 7 days of recovery. A-D (Control of metals) and Q-T (Control of CPF) Typical organization of the digestive gland of control *C. maenas*. Arrangement of digestive gland tubules and its cells. Distal tubule tips with E cells (embryonic cells), B cells (Blasenzellen cells), R cells (Restzellen cells) and F cells (Fibrillenzellen cells). The interstitial sinuses (IS) between tubules were normal (A, C bar=X10; B, D bar=X40). E-P and U-X Alterations in the histoarchitecture of the digestive gland of *C. maenas* exposed to cadmiuim (Cd) E-H, copper (Cu) I-L, Cd+Cu M-P and CPF (U-X). Formation of abnormal lumen (ALU) (F, N, V bar=X40) and haemocytic infiltration (HI) in the interstitial sinus (IS) (N, R, U bar=X40). Necrotic tubules (NT) (E bar=X10) of the digestive gland containing tissue debris (TD) in the lumen (J bar=X40). The thickened basal laminae (TBL) (I, U bar=X10) and the walling off of the tubules by haemocytes (HC) around the thickened basal laminae (N bar=X40).The coagulation (CO) in the thickened basal laminae (M bar=X10). The presence of reserve inclusion (RI) (A, E, I, M, U bar=X10) in control and exposed crabs.

decreased after 7days of exposure (16.2±2.62 nmol/min/mg protein). In digestive gland and eyes, different patterns in AChE response were observed compared to muscle. The inhibition of AChE activity was detected at 0.5d (16.77% and 48.53% in digestive gland and muscle, respectively), 1 day (22.7% and 51.89% in digestive gland and eyes respectively) and 2d (40.79% and 53.89% in digestive gland and eyes respectively) of Cu-exposure, decreased to become critical at 7d and the activity was estimated at 45.5% and 30.96% of control in digestive gland and eyes, respectively. In addition to the observed AChE activity increase in Cu-treated crab, the exposure to mixture of Cd and Cu caused pronounced increases after 0.5d and 1d. Compared to control, I observed a significant increase of AChE activity in muscle at short exposure times (0.5d and 1 d) and then a high decrease at long exposure times (43.77% and 55.82% of AChE activity were lost after 2 and 7 days, respectively). In digestive gland, an inhibition of AChE activity was observed throughout the period of contamination which reaches its maximum after 7 days (75.03%). In eyes, a critical decrease of activity was observed at the early time of the exposure (0.5d; 70.39%). The mixture of Cd and Cu causes a rapid decline in AChE activity at 0.5d which is maintained throughout the entire exposure. The transfer of contaminated crabs into the clean sea water for one week allowed to recuperate the totally lost activity in muscle of Cd and Cu-treated crab. In eyes, AChE activity was partially recovered of Cd- and Cu treated crabs. In mixture (Cd+Cu) treated crab, a partial AChE activity was recuperated in selected organs. Thus, 44.72%, 16.41% and 19.98% were recuperated in digestive gland, muscle and eyes organs, respectively.

Histopatogical lesions of crabs: We examined the digestive gland of crabs after 7d of exposure to metals and CPF and after 7 days of recovery. The digestive gland of control group (Figs. 3A, B, C, D, Q, R, S, T) showed the typical organization of glandular tubular structure as normally. It is composed of branched tubules and of different types of epithelial cells lining the tubules. The cells are Embryonic (E) cells, Fibrillar (F) or “dark” cells, Restzellen (R) or “light” cells and Blasenzellen (B) or extrusion cells (Fig. 3B). The lumen (LU) of each tubule was found to have a ‘star’ like appearance (Figs. 3B, D, H, Q). The interstitial sinuses between tubules are normal (Fig. 3A). The digestive gland exposed to metals (Cd, Cu and mixture) and CPF exhibited an abnormal lumen (ALU) (Figs. 3F, N, V) and haemocytic infiltration (HI) in the interstitial sinus (IS) (Figs. 3N, R, U). Other pathological lesions were also observed in crabs exposed to contaminants in laboratory. In fact, we observed necrotic tubules (NT) (Fig. 3E) of the digestive gland containing tissue debris (TD) in the lumen (Fig. 3J). The thickened basal laminae (TBL) (Figs. 3I, U) and the walling off of the tubules by haemocytes (HC) around the thickened basal laminae (Fig. 3N) were abundant in crabs exposed to mixture (Cd+Cu). The coagulation (CO) in the thickened basal laminae was observed only in digestive gland of crabs exposed to mixture (Cd+Cu) (Fig. 3M). We observed also the presence of four types of cells (B, E, F and R) in crabs exposed to metals and CPF. It was noted that the number of B cells in crabs exposed to Cd were less than that those of digestive gland of control crabs and the B cells are vacuolated in crabs exposed to Cu (Table 1). However, we exhibited the presence of reserve inclusion (RI) in control group and those exposed to metals and CPF (Figs. 3A, E, I, M, U).

We noted the intensity of each histopathological alteration in digestive gland of crabs after 7 days of exposure to contamination in Table 1. The results revealed that abnormal lumen (ALU), haemocytic infiltration in the interstitial sinus (HI) and thickened basal laminae (TBL) were important histopathological lesions of the crabs exposed to metals and CPF. The study of the histology of crabs after 7 days of recovery showed that crabs return to their normal structure. It was noted the absence of alterations in digestive gland of this group (Figs. 3G, H, K, L, O, P, W, X).

Discussion

The measurement of the biological effects of toxicants have become of major importance for the assessment of the quality of the environment. The use of biochemical markers has been proposed as sensitive "early warning" tools for biological measurement effect (Van der Oost et al., 2003). The inhibition of acetylcholinesterase (AChE) activity has been used widely as a biomarker of exposure to organophosphorous pesticides (OPs).

Sensitivity to pollutants: *in vivo* exposure to priorities pollutants: Crabs have often been proposed as possible bioindicators of marine pollution, but very few data regarding the effect of priorities pollutants such as heavy metals and organophosphorous on ChE activity in selected tissues are available. The present study is focused on enzyme sensitivity to exposure *in vivo* to pollutants, with the aim of validating ChE in tissues of *C. maenas* as a useful tool for monitoring environmental quality and exposure to pollutants in marine environments.

The exposure to 4.8 µg/l of CPF caused a significant decrease of AChE activity in main organs of crabs (muscle and eyes). Similary, after 96 hrs, muscle ChE activity was significantly inhibited by 49% when fish (*Acanthochromis polyacanthus*) were exposed to 10 µg/L of CPF (Botté et al., 2012). Varó et al. (2003) showed an important inhibition (76%) in nervous system in the fish (*Dicentrarchus labrax*) exposed to 1 mg/l of dichlorvus after 96 hrs. Very strong AChE inhibition was observed in the digestive gland of blue mussel (*Mytilus trossulus*) exposed for 2 days to 100 µg/L of dichlorvos (Kopecka-Pilarczyk, 2010). In addition, the transfer of contaminated crabs to the clean sea water for a short period (7 days) allowed to a rapid recovery of the AChE activity. In fact, recovery is defined as a significant increase in the AChE activity that occurs following cessation of exposure to anti-cholinesterase agents (Kumar et al., 2010). Several studies with shrimp, crab and lobster species have shown that AChE inhibition in the animals still occurred days after exposure had ended (McHenery et al., 1991; Abdullah et al., 1994). Recovery from the effects of OP compounds is both chemical and species specific (Zinkl et al., 1991). De la Torre et al. (2002) noted that the inhibition of fish brain AChE can be detected soon after the beginning of "*in field*" exposure to OPs but the time required for recovery of basal values after transfer fishes to unpolluted media can take several weeks. Similarly, Kumar et al. (2010) showed that recovery of AChE activity following reduction in exposure to OPs has been found to be a process that takes time, depending on factors such as the type of insecticide, tested species and the extent of depression of AChE. Indeed, organophosphorus compounds are generally irreversible inhibitors because the dephosphorylation rate of the bound enzyme proceeds at an insignificant rate. Therefore, the inhibitory effects of OP exposure may be long lasting, with recovery depending on new enzyme synthesis (Habig and DiGiulio, 1991). When levels are depressed more, a greater amount of enzyme must be produced. Some OPs, including chlorpyrifos, can also be metabolically altered to more active AChE inhibitors as an oxon analogue (Kumar et al., 2010).

Regarding effects of the heavy metals such as Cd, Cu and their mixture on AChE activity, it was dependent of investigated tissue and time of exposure. Indeed, Cd is a heavy metal commonly used in environmental studies because it is highly toxic (Lane and Morel, 2000), widely distributed in the environment and can adversely affect the organisms at relatively low exposure concentrations (Waisberg et al., 2003; Banni et al., 2009). The neurotoxicity effect of Cd illustrated in many *in-field* and *in-vivo* studies used invertebrate organisms as bioindicators (Roméo

et al., 2006). The negative Cd-effect with time of exposure on ChE activity in all tissues was clear. Similary, Jebali et al. (2006) showed inhibition of AChE in fish *Seriola dumerilli* after exposition of 100 and 250 μg/Kg of Cd.

The copper is a trace element that plays a fundamental role in the biochemistry of organisms, including aquatic organisms that can take it up directly from water (Grosell et al., 2003). However, it can become toxic at high concentrations (Alquezar et al., 2008; Vieira et al., 2009). In several aquatic animals, AChE and other cholinesterases (ChE) have been found to be inhibited by copper *in vivo* conditions (Roméo et al., 2006; Elumalai et al., 2007). In fact, AChE activity in *Hexaplex trunculus* is decreased by exposure to copper in the digestive gland after 2d (Roméo et al., 2006). However, no significant effect on AChE was observed in the brain of seabream *Sparus aurata* fingerlings exposed for 1 day to sublethal concentrations of copper sulphate (Varó et al., 2007; Frasco et al., 2008) and increases of AChE activity were also reported in specimens of *S. auratus* exposed to sublethal copper concentrations for 20 days (Romani et al., 2003). In the present study the results of *in vivo* exposure to Cu were much more difficult to interpret, as significant enhancements of AChE activity were detected in muscle tissues, at the early time exposure (2d) and dramatic repression at 7d. The study of Romani et al. (2003) showed that the copper has been found to induce AChE activity and catalytic efficiency in white muscle and brain tissues of *S. aurata* fishes exposed to Cu without accumulation of metals. These results suggest an increase of free Cu aliquot into the cells, likely due to mechanisms of metal homeostasis. Similarly, Cunha et al. (2007) showed an increase of AChE activity in the gastropod *Nucella lapillus* exposed to 44 mg/l Cu, an increase which may be due to direct interaction of metal with the enzyme.

The exposure to Cu+Cd led to a high increases in AChE activity (23.88±1.75 nmol/min/mg protein in muscle) at 0.5d, then decrease time-dependent. This results support that Cu is an essential metal and may allow a competitive effect with Cd on AChE activity and thus, the enhancement AChE activity by Cu exposure may cover or/ prevent the negative effect of Cd.

To sum up, our results clearly show that AChE activity in different tissues of *C. maenas* tends to have different patterns in response to *in vivo* exposure to Cd, Cu and Cu+Cd. In eyes tissue, the AChE activity was highly affected by Cd, Cu, and more critical affected by their mixture. This may be of particular concern for eyes, the primary organs of sensory and in contact with external environment, where the highest susceptibility was observed. As regard sensitivity of investigated tissues to heavy metals, eyes are the most sensitive tissue where the risk of neurotoxicity is therefore higher.

During the depuration period, the AChE activity was totally recuperated in muscle tissues of Cu or Cd-treated crab and partially in the mixture (Cd+Cu) treated crab. This increase of AChE activity can be explained by the biochemical and physiological mechanisms such as increases of metallothionein synthesis, storage in lysosomes compartment and excretion of metals absorbed and distributed in their tissues and synthesized new molecules of AChE (Serafim and Bebianno, 2009), so these animals are found in their normal physiological state. Furthers studies focused on the kinetic uptake of metals and metallothionein responses in a longer depuration period of *C. maenas* are needed to confirm the advanced hypothesis. Others studies on bivalve, showed that a short period of depuration no longer than 10 days was sufficient for the animal to return to its normal physiological state after exposure to Cu and this depend in concentration exposure (Gnassia-Barelli et al., 1995; Serafim and Bebianno, 2009).

Histopatogical lesions of crabs: Studies on crustacean health status have focused on the response of individual organ systems to laboratory exposure to a range of contaminants (Victor, 1993, 1994; Soegianto et al., 1999 a, b; Bhavan and Geraldine, 2000). The relative ease at which a holistic assessment of health can be made using histopathology and the suitability of these species as environmental sentinels provide support for the inclusion of crustaceans as indicators

of aquatic environmental health. In fact, the exposure to a noxious chemical, such as pesticide, would be reflected in alterations in the structure of the tubules and epithelial cells. The essential metals, like copper, can also produce toxic effects when the metal intake is excessively elevated (Wagner and Boman, 2004; Turkmen and Turkmen, 2005). Cadmium, non-essential metal, known by its purely toxic effect on the marine organism produces a general state of stress in contaminated organisms (Olabarrieta et al., 2001; Geffard, 2001). The crustacean digestive gland is a sensitive organ and liable to injury by pesticides and other water-born pollutants (Baticados and Tendencia, 1991; Bhavan and Geraldine, 2000). The digestive gland is essentially composed of branched tubules and of different types of epithelial cells (E-cells, R-cells, F-cells and B-cells) lining the tubules (Ben Khedher et al., 2014). Several such structural alterations were noted in the digestive gland tubules of test crabs that had been exposed to CPF, Cd, Cu and Cd+Cu after 168 hrs in the present study. Abnormal lumen, hemocytic infiltration in the interstitial sinuses and necrosis lesions were observed in crabs exposed to contaminants of this study. Similar observations have been made in the freshwater prawn *Macrobrachium malcolmsonii* exposed to endosulfan. The thickened basal laminae (TBL), another aspect of alterations, were also observed in crabs contaminated by CPF and metals in the present study. This thickening might have been due to production of collagenous fibers and melanin or due to the coagulation and walling off by hemocytes, as noted in *Penaeus monodon* due to aflatoxin toxicosis (Bautista et al., 1994); this thickening might also represent a defensive reaction against the toxicity of insecticides, a phenomenon noted in the tiger prawn *P. monodon* infected with *Vibrio harveyi* (Jiravanichpaisal and Miyazaki, 1994). Other distinct pathological alterations were also observed in crabs after 168 hrs of exposition to CPF and metals. We observed the walling off of the tubules by haemocytes around the thickened basal laminae and the coagulation in the thickened basal laminae (observed only in crabs exposed to Cd+Cu). These results were in accordance with those noted in digestive gland of *C. maenas* collected from Bizerta lagoon (Ben Khedher et al., 2014). We showed also the presence of reserve inclusion (RI) in control and exposed *C. maenas* collected in September. In fact, Ben Khedher et al. (2014) observed the presence of RI cells both in control and polluted sites only in *C. maenas* collected in summer (July). The function of RI cells are likely associated with the synthesis and storage of haemocyanin and other products such as glycogen; these reserves are utilized during stressful periods such as during moulting, disease or hibernation and during normal reproduction (Johnson, 1980; Stentiford and Feist, 2005; Ben Khedher et al., 2014). In the present study, we showed that after 7 days of recovery, crabs are not altered. In fact, we noted the absence of alterations in digestive gland of *C. maenas.*

Conclusions

This work has clearly shown the ChEs activities in muscle and eyes tissues of the Mediterranean crab *C. maenas*. The AChE activity in different tissues (digestive gland, muscle and eyes) of *C. maenas* was relatively sensitive to the exposure of chlorpyrifos-ethyl and tends to have different patterns in response to exposure to Cd, Cu and Cu+Cd mixture. The present results showed that depuration experiments allow to recover totally or partially the lost activity in treated crabs depending in selected tissue and contaminant exposure (metals or organophosphorous compounds). Further studies focused on the kinetic uptake of metals and ChEs activities responses in an increasing depuration period of *C. maenas* needs to be carried out to investigate the neurotoxicity effects of these compounds. To conclude, the exposure of *C. maenas* to metals and CPF allowed observing the histopathological alterations in digestive gland. It is imperative that contamination of the aquatic environment by metals and CPF (pesticide) should be prevented.

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