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Acute effects of chlorpyryphos-ethyl and secondary treated effluents on acetylcholinesterase and butyrylcholinesterase activities in *Carcinus maenas*

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Abstract

The acute effects of commercial formulation of chlorpyrifos-ethyl (Dursban®) and the secondary treated industrial/urban effluent (STIUE) exposure on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activities in hepatopancreas and gills of Mediterranean crab *Carcinus maenas* were investigated. After 2 d of exposure to chlorpyriphos-ethyl, the AChE activity was inhibited in both organs at concentrations of 3.12 and 7.82 μg/L, whereas the BuChE was inhibited only at higher concentration 7.82 μg/L of commercial preparation Dursban®. The exposure of crabs to Dursban® (3.12 μg/L) showed a significant decrement of AChE activity at 24 and 48 h, whereas the BuChE was inhibited only after 24 h and no inhibition for both enzymes was observed after 72 h. Moreover, a significant repression of AChE activity was observed in both organs of *C. maenas* exposed to 5% of STIUE. Our experiments indicated that the measurement of AChE activity in gills and hepatopancreas of *C. maenas* would be useful biomarker of organophosphorous (OP) and of neurotoxic effects of STIUE in Tunisia.

Key words: acetylcholinesterase; butyrylcholinesterase; organophosphorous; Dursban®; secondary treated industrial/urban effluent; *Carcinus maenas*

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Introduction

Ecosystem is threatened by increasing levels of various pollutants originating from human activities, urban, agricultural and industrial discharges. This situation endangers the health of organisms. Among anthropogenic contaminants, pesticides are widely detected in freshwater and marine ecosystems. These chemicals are spread on terrestrial cultures and enter waterways from agricultural and urban run-off (Varó *et al*., 2008). The organophosphates (OP) and carbamates (Cs) are modern synthetic insecticides and are potent neurotoxic molecules (Lundebye *et al*., 1997), which are commonly used in the Mediterranean area to treat a variety of agricultural pests (Vioque-Fernández *et al*., 2007a; Banni *et al*., 2005). OP is excessively used in the treatment of phytopathologies of cereals in the north of Tunisia have threaten freshwater and marine ecosystem, especially at the raining season (Dellali *et al*., 2001; Jebali *et al*., 2007). It can produce adverse effects on non target aquatic organisms living in areas near agricultural fields. It often ends up in aquatic habitats carried up by wind, runoff, or through uncontrolled waste disposal.

Toxic action of OP on organisms is the irreversible inhibition of the acetylcholinesterase (AChE), which hydrolyzes the acetylcholine (ACH) into choline and acetic acid at the cholinergic synapses and neuromuscular junctions. OP blocks the hydrolysis of ACH, leading to an excessive accumulation of ACH and a disruption of nerve function (Pea-Llopis *et al*., 2003). In addition to AChE inhibition, OP pesticides also inhibit pseudocholinesterases like butyrylcholinesterase (BuChE) and propionylcholinesterase (PrChE), which are closely related to enzymes that hydrolyse some xenobiotics and bind to others, including OP pesticides.

BuChE seems to have no specific natural substrates and has been proposed as a scavenging enzyme for certain classes of toxic compound (Bonacci *et al*., 2006). Great variability of AChE and BuChE enzyme characteristics has also been found between different invertebrate species and different tissues of the same organism (Najimi *et al*., 1997; Mora *et al*., 1999a; Gagnaire *et al*., 2008). The investigation on ChE activity (AChE and BuChE) in different tissues of mollusc *Adamusssium colbecki* expressed ChE

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activity as gill > adductor muscle > digestive gland (Corsi *et al*., 2004). Moreover, the study of acetylcholinesterase activity in subcellular fractions of various tissues of *Mytilus edulis* demonstrated that the highest activity was found in foot mitochondrial fraction and gill microsomal fraction (Brown *et al*., 2004a).

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, polycyclic aromatic hydrocarbons (PAHs) and components of complex mixtures of contaminants (Jebali *et al*., 2006; Vioque-Fernández *et al*., 2007a; Elumalai *et al*., 2007; Bonacci *et al*., 2008). The use of this biomarker in Mediterranean crab *Carcinus maenas* for the assessment of environmental quality is lower abundant in bibliography and much less effort has been dedicated to investigate the response of ChE activity in specific tissue of this species exposed to environmental pollutant (Lundebye *et al*., 1997; Elumalai *et al*., 2007).

The shore crab *C. maenas* was selected as a "model organism" since the single-system approach has been applied frequently to this common littoral species and has provided a wealth of background information. *C. maenas*, an extremely eurythermal and euryhaline species (Hebel *et al*., 1997), is widely distributed along Mediterranean coast (Astley *et al*., 1999; Martín-Díaz *et al*., 2008). Consequently, shore crabs may be exposed to a broad range of anthropogenic contaminants, yet remain common and abundant (Pedersen *et al*., 1997). This suggests that compensatory mechanisms which allow the crab to survive natural environmental fluctuations may also confer some degree of tolerance to contaminant exposure (Hebel *et al*., 1997; Brown *et al*., 2004b). In addition, it has the capability in accumulating various pollutants including heavy metals, PAHs and PCBs (Pedersen *et al*., 1998; Orbea *et al*., 2002) and, thus, it seems to be a suitable bioindicator of environmental contamination by these agents. It has been also found to be a suitable organism for use in "*in situ*" assays (Wedderburn *et al*., 1998; Martín-Díaz *et al*., 2007).

The aim of this work was to study the acute effects of commercial formulation of chlorpyrifos-ethyl (Dursban®) and the secondary treated industrial/urban effluent (STIUE) exposure on AChE and BuChE activities in hepatopancreas and gills of Mediterranean crab *C. maenas*, to evaluate the potential of the activities as sensitive biomarkers of toxicity of these compounds in crab. Gills and hepatopanceas were chosen as sources of enzymes as their AChE activity have been extensively investigated in other marine invertebrate such as bivalves (Banni *et al*., 2005; Corsi *et al.*, 2004).

1 Materials and methods

1.1 Exposure of crabs to chlorpyrifos-ethyl (Dursban®)

Intermoult female *C. maenas* were collected at Teboulba area (30 km South of Monastir, Tunisia) which is an uncontaminated area (Jebali *et al*., 2007). Specimens were immediately transported to the laboratory in aerated buckets with seawater. Upon arrival at the laboratory, crabs were divided into 4 groups of 10 crabs and placed in plastic tank with 20 L of sea water (36.89‰ salinity) and kept at 17.2°C. Crabs were fed regularly with fish every three days. After 7 d of acclimatisation, 4 groups of crabs were exposed to chlorpyrifos-ethyl (Dursban®) solution with the dosages of 0, 1.56, 3.12, and 7.81 μg/L, respectively. After 2 d of exposure, crabs were sacrificed. Another three groups of crabs were exposed to 3.12 μg/L of chlorpyrifos-ethyl (Dursban®), and crabs were sacrificed after 24, 48 and 72 h, respectively. For each treated and relative control group, the hepatopancreas and gills were carefully removed and frozen at –80°C until analysis.

The concentrations of chlorpyriphos-ethyl (0, 1.56, 3.12, and 7.81 μg/L) were chosen as suggested in some literature, and were very lower than LC_{50} of chlorpyriphos. The LC_{50} of chlorpyriphos-ethyl for the adult crab *Eriocheir sinensis* is 460.9 μg/L (Li *et al*., 2006). Similar concentrations of chlorpyriphos to those chosen in the present work were found in freshwater river in United Kingdom (1.28–5.55 μg/L) (Maltby and Hills, 2008).

1.2 Exposure of crabs to the secondary treated sewage effluent

The secondary treated industrial/urban effluent (STIUE), which would be directly discharged into the receiving marine water, was collected from large sewage treatment plan in Sousse (Centre of Tunisia). Twenty litters of STIUE were collected one day before the experiment and stored at 4°C.

Group of 10 crabs was exposed to 5% of STIUE for 2 d. Relative control groups were kept in clean sea water. At the end of experiment, the hepatopancreas and gills of the treated and control crabs were carefully removed and frozen at –80°C until analysis.

1.3 Cholinesterase activity

Tissues were weighed and homogenised in ice-cold 100 mmol/L phosphate buffer, pH 7.4 (1/3, *W/V*) with an Ultra-Turrax. The homogenate was centrifuged at 9000 ×*g* at 4°C for 30 min, and the supernatant stored at –80°C until biochemical measurement.

Enzyme activity was measured at room temperature in duplicates according to the colorimetric method by Ellman *et al*. (1961). In a typical assay, 1050 μL of 0.1 mol/L phosphate buffer, 50 μL of 0.008 mol/L dithiobisnitrobenzoate (DTNB), 50 μL of supernatant (S_9) and 50 μL of 0.045 mol/L substrate (ACH; BuCH) were successively added. 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)).

The sample protein concentration was estimated according to the method described by Bradford *et al*. (1976) using bovine serum albumin as standard.

1.4 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.

2 Results

Figure 1 reports the AChE and BuChE activities in hepatopancreas and gills of *C. maenas* exposed to commercial formulation of chlorpyrifos-ethyl (Dursban®) at sublethal concentrations under experimental conditions. In both organs, a significant AChE inhibition was exhibited at 3.12 and 7.81 μg/L of Dursban®, while BuChE was inhibited only at the highest concentration of 7.81 μg/L. The inhibition of AChE activity in treated crabs with 3.12 and 7.81 μg/L of Dursban® was respectively estimated at 36.19% and 40.83% in gills; 42.78% and 43.62% in hepatopancreas. BuChE was also reduced by 69.17% in gills and 30.40% in hepatopancreas at 7.81 μg/L.

The exposure of *C. maenas* to Dursban® (3.12 μg/L) led to the inhibition of ChE activities (AChE and BuChE). The AChE activity significant decreased after 24 and 48 h exposure, and the inhibition represent 41.03% and 47.20% in hepatopancreas and 36.08% and 27.79% in gills compared to the control (Fig. 2). The BuChE activity was significantly inhibited after 24 h exposure in studied organs. The inhibitions of BuChE represent respectively 40.94% and 73.34% in hepatopancreas and gills, respectively, compared to the control (100%) (Fig. 2).

In order to assess the neurotoxicity potential of STIUE, a preliminary study under controlled conditions was conducted. Crabs exposed to 5% of STIUE displayed only a significant decrease of AChE activity in hepatopancreas and gills (Fig. 3).

The BuChE activity shows no significant difference between relative control and treated crabs. The physico-chemical parameters of marine water were controlled (Table 1). Light diminution of the physico-chemical parameters was observed after crab exposure to the 5% of STIUE solution at the end of experiment.

3 Discussion

The ChE activities were investigated in different tissues of various molluscs invertebrate exposed to various OP pesticides (Mora *et al*., 1999b; Galloway *et al*., 2002;

Table 1 Physico-chemical parameters of marine water measured at the end of contamination experiment

	pH	Temperature (°C)	Dissolved oxygen (ppmv)	Salinity (‰)
Control	8.51	17.20	4.70	36.89
Treated crab	8.34	16.10	4.52	35.42

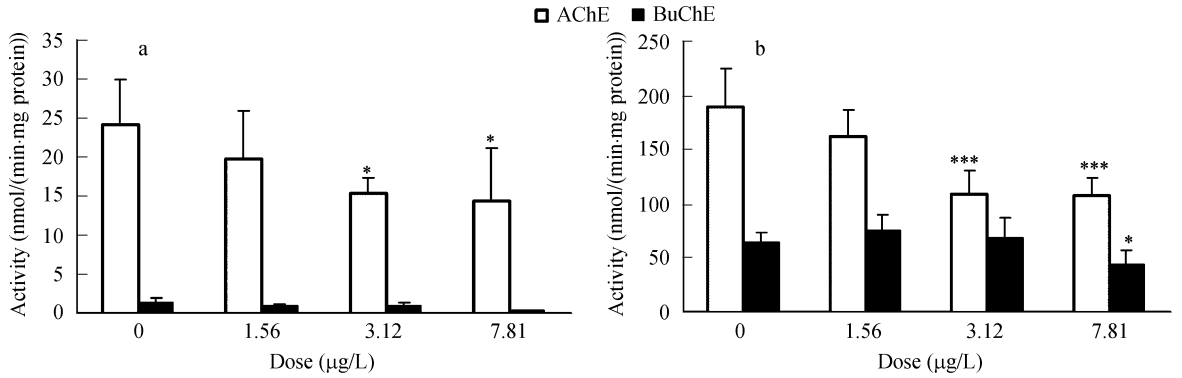

Fig. 1 *In vivo* effect of commercialized chlorpyrifos-ethyl on acetylcholinesrase (AchE) and butyrylcholinesterase (BuChE) activities in the gills (a) and hepatopancreas (b) of *Carcinus maenas* after 48 h exposure. All results are expressed as mean ± SD. Statistical differences between different treatments and relative controls were made at 0.05 confidence level. Statistical significance of the results is indicated as * $p < 0.05$ and *** $p < 0.001$.

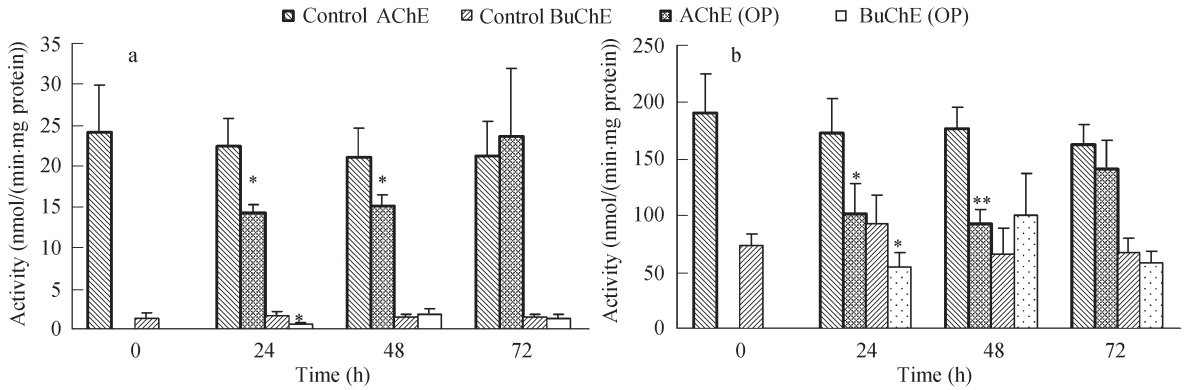

Fig. 2 AchE and BuChE activities in the gills (a) and hepatopancreas (b) of *C. maenas* exposed to chlorpyrifos-ethyl for 24, 48 and 72 h. All results are expressed as mean ± SD. Statistical differences between different treatments and relative controls were made at 0.05 confidence level. Statistical significance of the results is indicated as * $p < 0.05$ and ** $p < 0.01$.

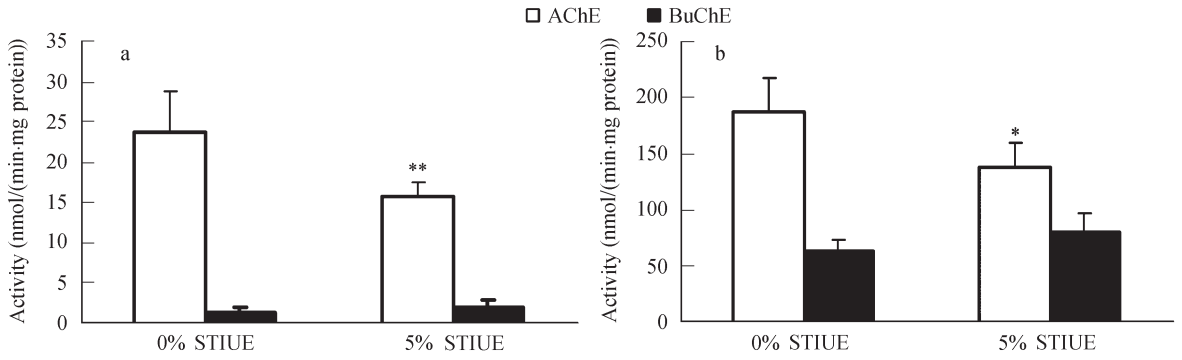

Fig. 3 AchE and BuChE activities in gills (a) and hepatopancreas (b) of *C. maenas* following exposure to 5% of STIUE for 48 h. All results are expressed as mean ± SD. Statistical differences between different treatments and relative controls were made at 0.05 confidence level. Statistical significance of the results is indicated as * $p < 0.05$ and ** $p < 0.01$.

Valbonesi *et al*., 2003; Corsi *et al*., 2004). The validations of ChE activities under natural condition "in field" were also well studied (Romeo *et al*., 2003; Banni *et al*., 2005). However, few studies were interested in ChE activities in target tissues of crustacean and in particular in marine crab *C. maenas*.

The use of AChE inhibition as biomarker in crab *C. maenas* was evaluated after *in vivo* exposure to dimethoate (Lundbaye *et al*., 1997). More recently, AChE was assayed in *C. maenas* eye and its sensitivity to heavy metals such as zinc and mercury was established (Elumalai *et al*., 2007). In aquatic organisms, the combined assay of AChE, BuChE and ChE activities was proposed as more useful indicator of organophosphorous/carbamate exposure than of a single esterase (Galloway *et al*., 2002; Vioque-Fernández *et al*., 2007b).

The present work demonstrated that the AChE and BuChE activities are higher in hepatopancreas than that in gills of *C. maenas*. The AChE and BuChE activities were 8 and 47 folders higher in hepatopancreas than in gills of uncontaminated crabs. Hepatopancreas is confirmed to be useful tissue for ChE activities in invertebrate (Corsi *et al*., 2004).

ChE show a distinct tissue distribution and wide substrate specificity in different organisms. Dissimilar organ allocation of AChE was found in bivalves, prevailing in *Mytilus galloprovincialis* digestive gland and gills, and *Perna perna* digestive gland and muscle (Najimi *et al*., 1997). In addition to the gills and hepatopancreas, the hemolymph is appropriate for testing AChE activity as non destructive biomarker in *C. maenas* (Lundbaye *et al*., 1997; Fossi *et al*., 2000). For both gills and hepatopancreas, the AChE activity was higher than BuChE activity. This result indicated that enzyme activity measured in two tissues under our experiment conditions showed a preference for acetylthiocholine as substrate over butyrythiocholine.

Anti-cholinesterases constitue a major portion of modern synthetic insecticides, among which members of the organophosphate and carbamate classes are included. The exposure of crab to lower range of sub-lethal concentrations of Dursban® (1.56–7.82 μg/L) caused a high inhibition of AChE in gills (36.19%–40.83%) ($p < 0.05$) and hepatopancreas (42.78%–43.62%) ($p < 0.001$). This results suggested that hepatopancreas is more sensitive than gills to Dursban® exposure. Hepatopancreas seemed to be the most susceptible to Dursban® and the AChE inhibition was well appreciated when the enzyme was abundant. The difference in ChE sensitivity between tissues with different tested chemicals has been reported for marine bivalves (Escartin and Porte, 1997; Najimi *et al*., 1997; Galloway *et al*., 2002; Valbonesi *et al*., 2003).

In literature, chlorpyrifos has already been shown as a powerful AChE inhibitor in invertebrates. Chlorpyrifos decreased AChE activity after 2–8 h contact in *Euvola* (*Pecten*) *ziczac* at 0.1, 1, and 10 ng/L (Owen *et al*., 2002) and in *Corbicula fluminea* at 1.4 and 2.8 μmol/L (Cooper and Bidwell, 2006). Chlorpyrifos also decreased *in vitro* AChE activity in *Mytilus edulis* (Galloway *et al*., 2002); *Adamussium colbecki* (18.9%–71.4% inhibition at 0.1–1 μmol/L) and in *in vivo* exposures in the midge, *Chironomius riparius* (Callaghan *et al*., 2001) and in *Potamopyrgus antipodarum* (Gagnaire *et al*., 2008).

Difference in sensibility of AChE and BuChE to increasing concentrations of Dursban® was observed. In contrast of AChE inhibition at 3.12 and 7.82 μg/L of Dursban®, BuChE was inhibited in both tissues of crab only at 7.82 μg/L substrates. In contrast, the *in vitro* incubation of gills ChE with chlorpyrifos in the range of 10^{-4}–10^{-10} mol/L led to concentration-dependent inhibition of ChE activity and a complete inhibition of AChE at 60 μmol/L and BuChE at 15 μmol/L. As consequence, BuChE was more sensitive to the increasing concentration of chlorpyrifos (Corsi *et al*., 2004).

In our study, crabs exposed to 3.12 μg/L of Dursban® display a higher repression of BuChE activity at 24 h whereas the AChE activity was maintained inhibited in hepatopancreas and gills for 24 and 48 h but not after 72 h. The lack of AChE inhibition after 3 d in *C. maenas* indicated the total detoxification of chloropyryphos-ethyl after this period and the recuperation of AChE and BuChE activities, probably due to de novo synthesis. Few studies have investigated the recovery of AChE and BuChE activities in pre-exposed crabs. Gagnaire *et al*. (2008) noted a time-dependance of the effect of 5 μg/L chloropyryphos on *P. antipodarum* AChE and the inhibition 40% after 24 h and 80% after 168 h with respect to the relative control were detected. At very low dosage of Dursban® (0.1, 1, and 10 ng/L) AChE was significantly inhibited

in hemolymphe of contaminated tropical scallop (*Euvola ziczac*) for 2 and 8 h (Owen *et al*., 2002). In previous works, we demonstrate that the exposure of fish (*Seriola dumerilli*) to sublethal dosage of 4 and 6 mg/kg of malathion could cause a higher repression of brain AChE after 2 and 7 d but no significant effect was observed after 13 d (Jebali *et al*., 2006). As consequence, the effects of single sublethal dosage of malathion on brain AChE were transient and largely reversible.

It has been known that AChE activity is more sensitive for OP and Cs pesticides than other contaminants, but the inhibition of this enzyme have been also used to indicate the exposure and/or effects of other contaminants in invertebrate such as heavy metals and polycyclic aromatics hydrocarbons (PAHs). Some heavy metals and PAHs are probably part of STIUE. The current work demonstrated that the exposure of crabs to 5% of STIUE caused a significant inhibition of AChE in both organs hepatopancreas and gills. The higher inhibition of AChE probably related to the presence of chemicals such as naphthalene and benzo(a)pyren and heavy metals in STIUE (Gravato and Santos, 2003).

4 Conclusions

Taking into account the inhibition of AChE and BuChE activities in hepatopancreas and gills by Dursban® and STIUE, it can be concluded that the AChE activity is promising biomarker of exposure and effects of OP and STIUE in field studies.

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