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Cholinesterase activity as biomarker of neurotoxicity: utility in the assessment of aquatic environment contamination *

Actividade da colinesterase como biomarcador de neurotoxicidade: avaliação da contaminação em ambientes aquáticos **

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ABSTRACT

Cholinesterase can take place in aquatic organisms under a series of environmental adverse conditions. The study of cholinesterases in these organisms can give important information about their physiological status and about environmental health. However, it is very important to know how the environmental factors such as fluctuation of physicochemical parameters associated to the presence of pollutants might affect these cholinesterase activities. We studied the response of cholinesterase activity in the caged cockle *Cerastoderma glaucum*. In addition, we evaluated the potential uses of cholinesterase activity in the common sole, which inhabit the Tunisian coast, subjected to different stress conditions, such as the exposure to different contaminants. This review summarizes the data obtained in some studies carried out in organisms from the Tunisian aquatic environment.

Keywords: Cholinesterases, Environmental stress, Biomonitoring, Aquatic Environments.

RESUMO

A colinesterase está presente nos organismos aquáticos em condições de stress ambiental. O estudo da colinesterase fornece informações acerca da condição fisiológica e saúde ambiental. Contudo é importante averiguar de que modo os factores ambientais, tais como a flutuação dos parâmetros físico-químicos, associados à presença de poluentes afectam a actividade das colinesterases. Neste trabalho são apresentadas os níveis de actividade da colinesterase em Cerastoderma glaucum. *São ainda apresentados os potenciais usos da actividade das colinesterases na solha comum que habita a costa Tunisina. Este trabalho sumariza também os resultados obtidos até agora em outros organismos estudados na Costa Tunisina.*

Palavras-Chave: *Colinesterases, Stress Ambiental, Biomonitorização, Ambientes Aquáticos.*

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1. INTRODUCTION

The initial interaction between chemicals and biological systems occurs at the cellular level. Thus, cells may be expected to react to chemical exposure. The assessment of chemicals toxicity at cellular level makes cellular responses a suitable tool for the early and sensitive detection of chemicals exposure (Monserrat *et al.*, 2007).

Chemical pollutants may pass the membrane by either special transport processes including active transport, facilitated transport, endocytosis, or passive diffusion (Helmut and Braunbeck, 1998). Having crossed the cell membrane, chemicals may exert their toxicity by different ways resulting in altering cellular structural and function as it is shown in figure 1. As a determinant of intracellular chemical toxicity, biotransformation of chemicals plays a key role, since many chemicals are not toxic, mutagenic or carcinogenic per se, but require metabolic activation to reactive species that can interact with target macromolecules in the cell (Livingstone, 1998). An example of chemicals that exert their toxicity by link to cellular molecules is the organophosphorous and carbamates pesticides that may be irreversibly linked to cholinesterase enzymes and in particular to acetylcholinesterase leading to neurotoxicity effects.

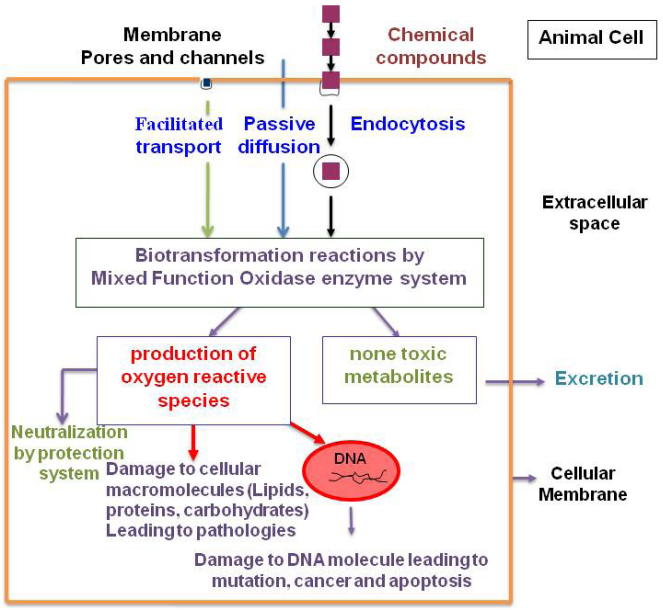


Figure1. Diagrammatic representation of the routes of uptake and possible processes in the interaction between chemicals and cells.
Figura 1. *Representação diagramática das vias de entrada e processos de interacção possíveis entre os elementos químicos e as células.*

2. CHOLINESTERASES: STRUCTURE AND FUNCTIONS

Cholinesterases (ChEs) are enzymes widely distributed in nature from microorganisms and protozoans to vertebrates, including human. They have been extensively studied since they are highly polymorphic enzymes in most species, play an important role in the transmission of nervous influx and are the specific target for most nerve agents and insecticides. The number of genes coding for ChEs varies according to species (Forget & Bocquené, 1999). There are two basic types of ChEs: acetylcholinesterases and butyrylcholinesterase (BChE) are the best known and characterized (Massoulié *et al.*, 1993). Both types of ChEs are evolutionary related, but are encoded in different genes and have their own specific features (e.g. substrate specificity, kinetic parameters, etc.) allowing for them to be discerned from one another. AChE is a specialized enzyme and its main physiological function is hydrolysis of acetylcholine (ACh), a mediator of neurotransduction in cholinergic synapses. BChE is less specific in relation to substrate specificity for hydrolysis and its physiological role remains unclear. Kinetic properties of ChEs can be determined from their amino acid composition and the conformation of their active center. Acetycholinesterases are a homo- and betero-oligomeric molecular forms, which belongs to the α/ß proteins class. Each monomer is established by 12 ß-strands surrounded with 15 α –helices (Sussman *et al.*, 1991). AChEs are two types of oligomeric forms. The first is a dimeric and tetrameric association of subunits covalently linked by disulfide bonds. These molecular forms are called globular AChEs G1, G2 and G4. The second type of AChE consists of one, two or three tetrameric of subunits associated with a collagen tail and establishes the asymmetric forms of AChE named A4, A8, and A12 (Massoulié *et al.*, 1993).

2.1. Cholinesterases of vertebrates: acetylcholinesterase and pseudocholinesterases

Vertebrates possess two cholinesterases, acetylcholinesterase and butyrylcholinesterase. The first is synthesized in hematopoiesis, occurs in the brain, endplate of skeletal muscle and erythrocyte membrane, and its main function is to regulate neuronal communication by hydrolyzing the ubiquitous neurotransmitter acetylcholine in synaptic cleft (Quinn, 1987; Silman & Sussman, 2005). The second is synthesized in liver and is present in plasma, smooth muscle, pancreas, adipocytes, skin, brain and heart (Çokugras, 2003). Although its physiological function is not well defined, BChE is pointed as one of the main detoxifying enzymes able to hydrolyze or scavenge a broad range of xenobiotic compounds like cocaine, heroin, anaesthetics, and pesticides (Soreq & Zakut, 1990; Taylor, 1991; Çokugras, 2003; Nicolet *et al.*, 2003). Some studies hypothesized that one of the functions of BChE is to protect AChE against anticholinesterasic agents (Whitaker, 1980; Whitaker, 1986). Pezzementi & Chatonnet (2010) reported that ChEs emerged from a family of proteins with adhesion properties. Both play other roles in the neuronal tissue, particularly in neuronal differentiation and development, cell growth, adhesion and signaling. In addition, AChE participates even in hematopoietic differentiation (Chatonnet & Lockridge, 1989; Taylor, 1991; Johnson and Moore, 2000; Silman & Sussman, 2005). Moreover, AChE and BChE are different concerning several other aspects: while AChE has an *in vivo* half-life of 120 days, BChE lasts 7-12 days. AChE is inhibited by substrate excess and BChE is activated by substrate excess

(Lopez-Carillo & Lopez-Cervantes, 1993; Çokugras, 2003). AChE is selectively inhibited by propidium, DDM, caffeine, Nu1250, 62c47 and BW284c51 while BChE is selectively inhibited by percaine, isopestox, ethopropazine, Iso-OMPA, bambuterol and haloxon (Chatonnet & Lockridge, 1989; Harel *et al.*, 1992; Kovarik *et al.*, 2003). BChE has a larger space in its active site, which can hydrolize or be inhibited by a range of compounds. AChE has a more specific active site (Çokugras, 2003).

Some of these features are governed by crucial differences in the structure of the enzymes such as: 1) the difference in size of active site can be explained by six aromatic residues lining the active site of AChE that are missing in BChE; 2) two of these (Phe-288 and Phe-290) are replaced by leucine and valine, respectively, in BChE. This feature prevents the entrance of butyrylcholine in the AChE active site; 3) peripheral site specific-ligands such as propidium does not inhibit BChE because the residue Trp-279, which is part of the peripheral anionic site located at the entrance of the active site gorge in AChE, is absent in BChE (Harel *et al.*, 1992). According to Rosenberry (1975), AChE is more sensitive to the size of the acyl group than to the alcohol moiety (whether charged or neutral) of the substrate, while for BChE the opposite is observed. Both are inhibited by 50 µM of physostigmine (eserine), which is a condition that affords to discriminate cholinesterases (ChEs) from other esterases (Augustinsson, 1963).

The class of AChEs is more homogeneous in terms of their primary structure than the class of BChEs (Rosenberry, 1975). Despite of these differences, the amino acid sequence identity between AChE and BChE from vertebrates ranges from 53 to 60%, even in evolutionarily distant species (Chatonnet & Lockridge, 1989; Taylor, 1991). In addition, a study promoted the replacement of only two amino acids by site-directed mutagenesis in AChE for it to develop BChE activity (Harel *et al.*, 1992). Both enzymes present the active site within a deep and narrow gorge, approximately in the middle of its globular structure, which apparently could disturb the substrate traffic. However, in fact this structure follows a rational organization which entraps substrate and transports it to the active site through the arrangement of amino acids lining the gorge. All this occurs very efficiently (Quinn, 1987; Tõugo, 2001).

2.2. Acetylcholinesterases of invertebrates

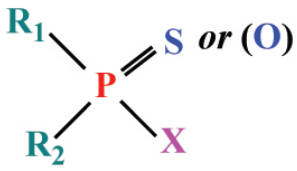
Only globular forms of AChE are present in invertebrates (Massoulié *et al.*, 1993; Talesa *et al.*, 1993, 1996; Pezzementi *et al.*, 1989; Sanders *et al.*, 1996). Such enzymes consist of monomers (G1), dimers (G2) and tetramers (G4) of catalytic subunits, without the asymmetric collagenic-tailed forms typical of vertebrates. Generally, most AChEs described in invertebrates display less defined substrate specificity and a marked variability in the kinetic behavior compared to vertebrates (Talesa *et al.*, 1994). In particular, a dimeric AChE linked to the cell membrane by a phosphatidylinositol (PtdIns) anchor is always expressed in invertebrates. Such an enzyme is thought to fulfil a key role in cholinergic transmission, while the function of similar AChE forms is so far unknown in vertebrates (Romani *et al.*, 2006).

In invertebrates, the genetics of AChEs differ in the various phyla and sometimes within the same phylum: the AChE subunits are encoded by one, two or several genes (Talesa *et al.*, 1999, 2002; Romani *et al.*, 2006), thus giving a complex polymorphism of the enzyme.

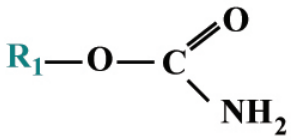
3. CHOLINESTERASE INHIBITORS AND MECHANISM OF ENZYME INHIBITION

A wide range of contaminants including organophosphorus and carbamates pesticides, heavy metals, and polycyclic aromatic hydrocarbons may affect ChE (Jebali *et al.*, 2006; Elumalai *et al.*, 2007; Bonacci *et al.*, 2008). The best known inhibitors of ChE are those belonging to the carbamate and organophosphate classes. Organophosphorus esters (OPs) and carbamates form two important classes of agrochemicals. Most of the commercially relevant bioactives OPs have sulfur direct attached to the central pentavalent phosphorus.

Organophosphorus pesticides have the following general chemical structure:



where the R-groups may be alkoxy, alkyl, aryl, or amide. The X-group is generally a carboxylating, cyanide, thiocyanate, phosphate, halide, phenoxy, or thiophenoxy group Carbamate insecticides have the following structures:

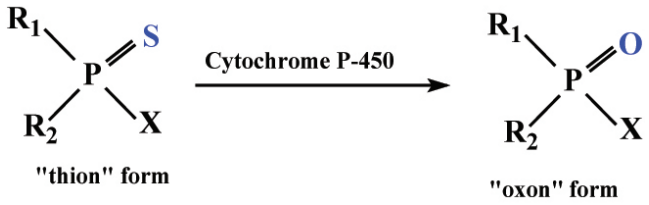


Carbamates and organophosphates generally have low environmental persistence, especially when compared with organochlorine pesticides. Their degradation is often accelerated by an increase in temperature or alkalinity (Kanazawajun 1975; Solé *et al.*, 2000). Although organophosphorus and carbamate pesticides are chemically different, exposure to either of these groups of pesticides usually results in the inhibition of cholinesterase enzyme (ChE) activity.

Acetylcholine (ACh), the neurotransmitter secreted by cholinergic postganglionic neurons, allows for transmission of nerve impulses across the synapse. Acetylcholinesterase (AChE; Enzyme Commission Number 3.1.1.7) plays an important role in neurotransmission in both vertebrates and invertebrates, being responsible for the hydrolysis of acetylcholine into choline and acetic acid at the cholinergic synapses and neuromuscular junctions (Penã-Lopis *et al.*, 2003). Acetylcholinesterase or *true cholinesterase* is found in high concentration in the nervous system. Pseudocholinesterase (Butyrylcholinesterase (BChE; peopionylcholinesterase (PChE)) is found in muscle

at variable proportions of both aquatic vertebrate and invertebrate; although its physiological function has not been well defined, it is useful as an indicator of organophosphate and carbamate exposure (Massoulié *et al.*, 1993).

The figure 2 shows schematically the mechanisms of action of organophosphorus and carbamates on acetylcholinesterase. The molecular activity of anticholinesterase OPs associated with the inhibition mechanism consists in a nucleophilic attack of the serinic oxygen of cholinesterase active site to the phosphorus atom of OPs. Cleavage of the acidic OP leaving group enables formation of a covalent P-O (serine) bond, thus inhibiting enzymatic hydrolysis of the natural substrate acetylcholinesterase, which would proceed via a reaction between serine OH and the electrophilic carbonylcarbon of the neurotransmitter. Phosphorothionates (ester containing the thiol group P=S) are less effective as AChE inhibitors than their oxon metabolites (with P=S being replaced by O=P), which are formed in vivo by cytochrome P-450 oxidation (Mastrantonio *et al.*, 2008):



Phosphorothionates are less reactive to hydrolysis and more lipophilic than phosphates, which makes them more efficient in reaching critical endogenous sites in sufficiently effective concentrations, with cytochrome P-450 oxidation to oxon metabolites forming a major toxification step in vivo. Organophosphorus chemicals phosphorylate acetylcholinesterase in an irreversible reaction that inhibits the activity of cholinesterase to hydrolyze the neurotransmitter at the nerve synapse. The accumulation of acetylcholine results in a continuous nerve firing and eventual failure of nerve impulse propagation. Respiratory paralysis is generally the immediate cause of death (Massoulié *et al.*, 1993).

One of the main targets of carbamates is acetylcholinesterase activity, as organophosphorus, but less reactive (See Fig. 2), since in this case the complex carbamyl-enzyme is hydrolysable with release of CO_2, methylamine and the regenerated enzyme. The same kinds of troubles neurohormonal as organophosphorus by O'Brien (1976), but the degree of inhibition is reduced in proportion to rate of the enzyme regeneration. In addition to anticholinesterase pesticides, some organic and metallic compounds such as polyaromatic hydrocarbons and metals have the potential to inhibit cholinesterase enzyme (ChE) activity, but the mechanisms of inhibition is not fully known (Jebali *et al.*, 2006). Some in vivo studies have reported inhibitory effects (but sometimes activating effects) of metals on fish brain AChE activity (Jebali *et al.*, 2006), but the concentrations used in these tests

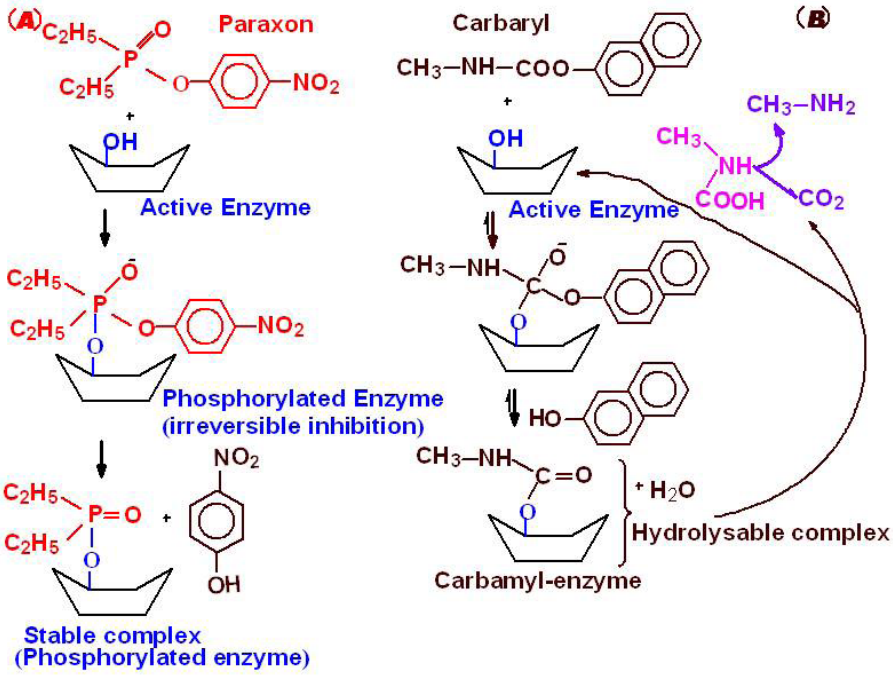


Figure 2. Mechanisms of action of organophosphorus (A) and carbamate (B) on acetylcholinesterase enzyme.
Figura 2. *Mecanismos de acção dos organofosforados (A) e carbamatos (B) na acetilcolinesterase.*

are usually far removed from the concentrations measured in situ. As with all enzymes, cholinesterases conformation can be modified by the presence of metals, so there are no specific effects of these types of pollutants on cholinesterases (Bocquené *et al*., 1997).

4. CHOLINESTERASE AS A BIOMARKER OF NEUROTOXICITY: UTILITY IN THE ASSESSMENT OF AQUATIC ENVIRONMENT

ChE activity has been widely studied and employed as a biomarker in aquatic invertebrate and vertebrate species to detect exposure to chemicals in natural ecosystem (Dellai *et al*., 2001; Roméo *et al*., 2003; Lavado *et al*., 2006). The potential use of ChE activity in sentinel species for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during the recent years. In fact, we measured the cholinesterase activity in organs from endemic species collected from different sites in Tunisia. Indeed, C*yprinus* carpio is widely used as sentinel organism in biomonitoring programs and toxicity bioassays (Van der Oost *et al*., 1998; Chuiko, 2000; De la Torre *et al*., 2002). We measured the AChE and BChE in brain and muscle of common carp *Cyprinus carpio* sampled from three Tunisian dam lakes submitted to various adverse environmental conditions. We found a high inhibition of AChE activity in both organs, since the BChE was significantly reduced only in muscle (Tlili *et al*., 2010a). Ozmen *et al*. (2007) showed that there is a strong relationship between AChE inhibition in the brain of *C. carpio* collected from Sariyar Dam Lake (Turkey) and the organochlorine pesticides and their residues in water, sediments, and *C. carpio* adipose tissues. In other in field investigations using AChE as neurotoxicity biomarker, Lavado *et al*. (2006) reported that AChE was strongly inhibited in the muscle of *C. carpio* sampled from some stations of Ebro River (Spain) highly polluted by organophosphorous, carbamates, and heavy metals. The measurement of cholinesterase in the brain or muscles, involves the sacrifice of the animals to provide information on both exposure and toxic effects on animals. The serum cholinesterase determination, achievable by non-invasive methods, does not, in general reliably assess the toxic effects associated with exposure (Bocquené *et al*., 1997). Regarding the tissue distribution, we found in the following rank of AChE activity in *Solea solea* fish: brain > gills> kidney>liver (Table 1) (Jebali *et al*., 2012). The reasons for these differences could be due to innervations levels between the different tissues. Accordingly, different tissues did not respond in the same way to both exposure and effects of pollutants. *S. solea* is a sentinel fish for environmental studies because, as a flatfish, it lives in a restricted range. Thus, if there is evidence for exposure of *S. solea* to chemical contaminants, it can be used to determine where the exposure occurred (Ribecco *et al*. 2012). In recent years, it has also been selected as sentinel infield pollution monitoring and laboratory toxicity experiments (Davoodi & Claireaux 2007; Trisciani *et al*. 2011; Palermo *et al*. 2012). Recent field study, showed a significant inhibition of brain AChE in *S. solea* fish collected from polluted sites, whereas small changes were observed in gills, liver and kidney AChE (Table 1) (Jebali *et al*., 2012). This inhibition is due to the polluted sites. In fact, Fathallah *et al*. (2012) clearly showed that the seawater of Monastir bay contained high concentrations of metals expressed in ppm: Cd = 7.24, Pb = 19.5, Cu = 15.7, Ni = 3.8, Zn = 48.7, Hg = 18.1. Indeed, a geochemical study at Monastir bay by Dahmane (2000) showed that the levels of heavy metals such as copper (> 150 ppm), lead (> 150 ppm) , zinc (<75 ppm) , cadmium (50 - 100 ppm) and manganese (<70 ppm) in sea water at the site of Khniss are

Table 1. Acetylcholinesterase (AChE) activity in liver, gills and kidney of fishes (*S. solea*) collected from five sites of Tunisian coast. All results are expressed as mean ± SD; n=10. a: Significant difference with reference site (hergla) (P<0.05). Control = Hergla; S1 = Sidi Abdelhamid; S2 = Khniss; S3 = Sayada; S4 = Teboulba. Data are from Jebali *et al*., 2013c.

Tabela 1. *Actividade da acetilcolinesterase em fígado, brânquias e rins de peixes (S. solea) colhidos em cinco locais da costa Tunisina. Todos os resultados estão expressos como médias ± SD; n=10. a: Diferenças significativas para o sítio de referência (hergla) (P<0.05). Controle = Hergla; S1 = Sidi Abdelhamid; S2 = Khniss; S3 = Sayada; S4 = Teboulba. Dados retirados de Jebali et al. (2013c).*

	Tissues			
Sites	Brain	Liver	Gills	Kidney
Control	96.23± 14.06	1.98±0.52	33.09±11.12	5.77±1.42
S1	66.1±16.93[a]	1.62±0.51	23.32±8.53	4.47±2.15
S2	104.18±13.76	2.54±0.49	31.91±5.75	4.32±0.47
S3	86.2±24.12	3.04±0.49[a]	34.52±12.87	8.45±2.83
S4	80.19±18.04	1.93±0.6	20.22±4.77	9.75±4.28[*]

higher than those recorded by Goldberg *et al*. (1970) and Martin (1976) in the unpolluted sea and suggested that the Khniss site is highly polluted by heavy metals originating from contributions by the drain Khniss, fishing activities and it receives several domestic wastes from the surrounding areas and industrial activities in the region site (Kessabi *et al*., 2013; Zrafi *et al*., 2013). In Khniss, the levels of aromatic hydrocarbons are ranging from 1-14 mg/l (7-33 % of total hydrocarbons) in seawater and from 2280-7700 µg/g (5-9% of total hydrocarbons) in sediments (Zrafi *et al*., 2013). Similarly, it has been shown by a chemical study in surface sediments at Khniss and Sayada that concentrations of cadmium (1-6 ppm), zinc (75-100 ppm), copper (15-30 ppm) and lead (45-100 ppm) are very high compared to levels reported in other Tunisian sites (Gulf of Tunis) (Sahnoun, 2000; Rezgui, 2007). Teboulba is influenced by the treated and none treated municipal wastewater from limitroph agglomerations and fishing harbor (Jebali *et al*., 2011). At this site, PAH levels in seawater range from 4-22 mg/l (3-31% of total hydrocarbons) in sea water and from 93-1050 mg/g in superficial sediments. This charge of PAHs would be petroleum from the fishing and port.

The levels of total hydrocarbons in sediments of Sidi Abdelhamid vary between 30 and 70 mg/kg. This slight contamination is caused mainly by fishing boats and pleasure craft Sabbagh, 2011). Solé *et al*. (2012) reported that *S. senegalensis* AChE was dominant in brain (53–65 %), followed by kidney (11– 13 %), liver (4–7 %) and gills (4 %). Koenig *et al*. (2013) showed that *S. Solea* AChE was inhibited in brain after exposure to chlorpyriphos. These results could indicate the highest sensitivity of the brain in comparison with the other organs. The differences in the sensitivity of the tissues may be related to the pollutants' difficulty in reaching the sites where AChE is located: e.g. it may be easier to reach the synapses in the brain than the neuromuscular junctions in the gills. So far, these findings point out the convenience of focusing on fish brain during future monitoring.

The use of ChE activity as an environmental biomarker requires a careful characterization of the enzymes present in a given species and tissue to minimize possible erroneous interpretation of the results (Vioque-Fernández *et al*., 2007). ChE activities have been investigated in tissues of the cockle *Cerastoderma glaucum*. The basal levels of enzyme activities, substrate specificity and tissue distribution were characterized and the kinetic response under natural conditions was evaluated using a caging approach. The results of the preliminary screening of ChE activity in whole animal, rest of animal (adductor and retractor muscles, mantle and foot), visceral mass and gills of *Cerastoderma glaucum* (Table 2) show that the highest ChE activity was obtained using ASCh and PSCh, whereas the hydrolysis of BSCh was noticeably low (Jebali *et al*., 2011).

In view of future national biomonitoring programmes, we illustrate the different sensitivity and kinetic response of AChE activity after caging exposure of *C. glaucum* at fishing harbour. In fact, we know that Over 450 ships of different size are based in this harbour (Jebali *et al*., 2013a), thus receiving contaminants from ship traffic and waste from fish industry, that represent a key pollutant source for the central Tunisian littoral (Banni *et al*., 2005; Jebali *et al*., 2007; 2011). Therefore, the pollutants responsible for the inhibition of AChE are the Polycyclic aromatic hydrocarbons (PAH) and other pro-oxidant chemicals, present also in Téboulba fishing harbor (Jebali *et al*., 2013a). *Cerastoderma glaucum* caged at Teboulba fishing harbour for 4 week experienced a critical alteration in gill AChE activity compared with the tissue activity of the rest of the animal (Table 3). The control *Cerastoderma glaucum* was collected from Kuriat. This site is characterized by the absence of any source of pollution (Jebali *et al*., 2011; Ben-Khedher *et al*., 2013). In the gills, AChE activity decreased in a time-dependent pattern, losing approx. 15 and 68% activity after 2 and 4 week of transplantation, respectively, whereas in muscle tissue (rest of animal) the loss of AChE was approximately 24% at the end of the 4 week caging period (Jebali *et al*., 2011) Consequently, different profile responses of AChE activity were noted and the gill seems to be more sensitive to the effect of harbour pollutants than the muscle. In fact, few data exist about the measurement of ChE activity in *Cerastoderma glaucum* (Machreki-Ajmi *et al*., 2008), but this does not prevent the use of this species as an interesting bioindicator in other ecotoxicological studies worldwide (Matozzo *et al*., 2007; González-Wangüermert *et al*., 2009; Borówka *et al*., 2012).

AChE can change significantly in Tunisian bivalve species in response to different stress conditions. In *Donax trunculus*, the enzymatic activity can change seasonally, mostly according to reproduction cycles, water temperature and food availability and pollution level (Tlili *et al*., 2010b).

Table 2. Substrate specificity of cholinesterase (ChE) activity in the tissues of *Cerastoderma glaucum*. Data are means ±SD of 4 determinations. Different lowercase letters indicate a significant difference between tissues. Significant differences between ChE activity in the same tissue are shown with Capital letters. Substrates used were acetyl- (ASCh), butyryl- (BSCh) and pro pio nylthiocholine (PSCh) iodide. WA: whole animal; RA: rest of animal (adductor and retractor mucsles, mantle and foot); VM: visceral mass; G: gills. Data are from Jebali *et al*., 2011.

Tabela 2. *Especificidade de substrato para a actividade de colinesterase nos tecidos de* Cerastoderma glaucum *(Médias ± SD de 4 determinações). As letras minúsculas indicam diferenças significativas entre tecidos. As diferenças significativas dentro do mesmo tecido estão representadas por letras maiúsculas. Os substratos usados foram a acetyl- (ASCh), butyryl- (BSCh) e pro pio nylthiocholine (PSCh) iodide. WA: todo o animal; RA: restos do animal (músculos adutor e retrator, manto e pé); VM: massa visceral; G: brânquias. Dados retirados de Jebali et al. (2011).*

	Cholinesterase activity		
Organe	ASCh	BSCh	PSCh
WA	$183.78\pm12.8^{a,A}$	$57.12\pm2.91^{b,B}$	$90.02\pm4.51^{b,C}$
RA	$141.53\pm5.25^{b,B}$	$72.10\pm6.77^{a,C}$	$185.34\pm32.35^{a,A}$
VM	$51.65\pm1.15^{c,C}$	$35.71\pm0.79^{c,C}$	$106.56\pm6.41^{b,B}$
G	$15.21\pm0.95^{d,D}$	$16.23\pm2.99^{d,D}$	$29.83\pm0.83^{c,C}$

Table 3. Acetylcholinesterase (AChE) activity measured in gills and rest of animal (adductor and retractor muscles, mantle and foot) of the cockle *Cerastoderma glaucum* in cages in the Téboulba fishing harbour. Data are means ± SD. [a]: Significant difference with control group (P<0.05). Data are from Jebali *et al.*, 2011.
Tabela 3. *Actividade da acetilcolinesterase em brânquias e restos do animal (músculos adutor e retrator, manto e pé)* Cerastoderma glaucum *(Porto de pesca Téboulba). Todos os resultados estão expressos como médias ± SD. a: Diferenças significativas com o grupo controle (P<0.05). Dados retirados de Jebali et al. (2011).*

	AChE	
Caging time (day)	Gills	Rest of animal (adductor and retractor muscles, mantle and foot)
0	15.21±0.95	141.53±5.25
15	12.98±2.39	99.75±24.4[a]
30	4.9±0.93[a]	107.81±23.13

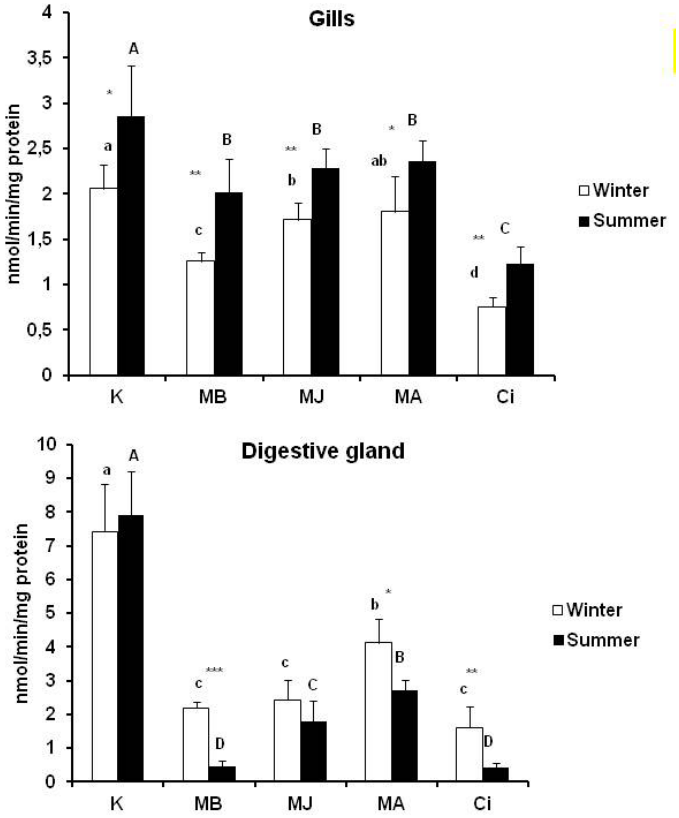


Figure 3. Responses of acetylcholinesterase (AChE) activities in the gills and digestive gland of crabs collected from the five sites in February and July. S1 Kuriat (control: absence of known pollution sources), S2 Menzel Bourguiba (high metal pollution), S3 Menzel Jemil (conchyliculture practice and industrial imput), S4 Menzel Abdelrahmen (urban effluent) and S5 Cimentery (Industrial imput and urban discharge). All results are expressed as mean ± SD; n06. Letters a, b, c, and d indicate significant differences between sites. *p<0.05, **p<0.01, and ***p<0.001 indicate significant differences between seasons, respectively. Data are from Ben-Khedher *et al.*, 2013.
Figura 3. *Actividade da acetilcolinesterase nas brânquias e glândulas digestivas de caranguejos colhidos nos cinco locais em Fevereiro e Julho. S1 Kuriat (controle: ausência de fontes poluidoras), S2 Menzel Bourguiba (elevada poluição por metais), S3 Menzel Jemil (entradas industriais e maricultura), S4 Menzel Abdelrahmen (efluentes urbanos) e S5 Cimentery (entradas industriais e descargas urbanas). Todos os resultados expressos como médias ± SD; n06. Letras a, b, c, e d indicam diferenças significativas entre locais. *p<0.05, **p<0.01, e ***p<0.001 indicam diferenças significativas entre estações do ano. Dados retirados de Ben-Khedher et al., 2013.*

The marked inhibition observed in AChE activities in bivalves from polluted sites (Gulf of Tunis, Tunisia), during the warm period could be explained by the negative effect of temperature as recently reported in other marine species crabs (Ben Khedher *et al.*, 2013). Moreover, the effect of temperature on AChE activity in *D. trunculus* from the polluted site appeared to act synergistically with the effect of anthropogenic inputs by comparison with the reference site (Tlili *et al.*, 2010b). In order to use AChE inhibition as a pollution biomarker, it is necessary that the change due to contamination exceeds the natural variability (Bocquené *et al.*, 1997). Indeed, in the case of *in situ* biomonitoring, it is difficult to separate the contribution of abiotic factors and the chemical pollutants in the response of the AChE activity. However, the contribution of abiotic factors is low compared with the effect of chemical contaminants. Also, the use of control can clearly show the effects of chemical pollutants on AChE activity. Ben Khedher *et al.* (2013) showed the inhibition of AChE activity in *Carcinus maenas* collected from four contaminated sites from Bizerta lagoon and from Kuriat as control site in February and July and those in both organs (gills and digestive gland) (Fig 3). In fact, this inhibition is due to the presence of contaminants (metals and hydrocarbons) in water and sediments in these sites (Table 4). Positive correlations were recorded between the AChE in gills and chemical parameters in water (Cd, r = 0.925, p<0.01; Cu, r = 0.821, p<0.01; Mn, r = 0.895, p<0.01; Ni, r = 0.846, p<0.01) and in sediment (Cd, r = 0.688, p<0.05; Fe, r = 0.653, p<0.05; Pb, r = 0.912, p<0.01; Mn, r = 0.685, p<0.05; Ni, r = 0.688, p<0.05; hydrocarbons, r = 0.730, p<0.05).

In general, 20% or greater depression in AChE activity in several species invertebrates can be an indicator of exposure to pesticides (Day and Scott 1990). Moreover, we investigated the effect of OP (chlorpyrifos-ethyl: CPF) at sublethal concentrations and after 3 days of exposure under experimental conditions on AChE and BuChE in target tissues of crab *Carcinus maenas* (Ghedira *et al.*, 2011). This work demonstrated that 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 experimental conditions showed a preference for acetylthiocholine as substrate over butyrythiocholine. The results showed also that the exposure of *C. maenas* to OP led to the inhibition of ChE activities (AChE and BuChE).

Table 4. Concentration ranges of dissolved metals (Al, Cd, Co, Cu, Fe, Pb, Mn, Ni, Zn, and Cr) and hydrocarbons in water and sediments collected at sampling sites in two seasons. S1: Kuriat, S2: Menzel Bourguiba, S3: Menzel Jemil, S4: Menzel Abdelrahmen and S5: Cimentery. Data from Ben Khedher *et al.*, 2013.

Tabela 4. *Concentrações dos metais dissolvidos (Al, Cd, Co, Cu, Fe, Pb, Mn, Ni, Zn, and Cr) e hidrocarbonetos na água e sedimentos colhidos nos locais de amostragem em duas estações do ano. S1: Kuriat, S2: Menzel Bourguiba, S3: Menzel Jemil, S4: Menzel Abdelrahmen e S5: Cimentery. Dados retirados de Ben Khedher et al. (2013).*

	Winter					**Summer**				
	Water (mg/l)									
	S1	**S2**	**S3**	**S4**	**S5**	**S1**	**S2**	**S3**	**S4**	**S5**
Al	0	0,397	0,718	0,42	0,735	< 0,05	0,344	0,687	0,374	3,294
Cd	< 0,005	0,204	0,111	< 0,05	0,311	< 0,005	0,202	0,104	< 0,05	0,297
Co	< 0,05	< 0,05	< 0,05	< 0,05	< 0,05	< 0,05	< 0,05	< 0,05	< 0,05	< 0,05
Cu	< 0,05	0,258	0,208	< 0,05	0,268	< 0,05	0,281	0,208	< 0,05	0,286
Fe	< 0,05	132	0,492	0,295	0,251	< 0,05	131	0,489	0,273	1,65
Pb	< 0,05	0,225	< 0,05	< 0,05	0,057	< 0,05	0,247	< 0,05	< 0,05	0,039
Mn	< 0,05	0,953	< 0,05	< 0,05	0,603	< 0,05	0,926	< 0,05	< 0,05	0,774
Ni	< 0,05	0,112	< 0,05	< 0,05	0,081	< 0,05	0,083	< 0,05	< 0,05	0,061
Zn	< 0,05	4,638	0,084	< 0,05	1,191	0,068	4,778	0,084	< 0,05	1,497
Cr	< 0,05	0,243	< 0,05	< 0,05	< 0,05	< 0,05	0,139	< 0,05	< 0,05	< 0,05
	Sediments (mg/Kg dry matter)									
	S1	**S2**	**S3**	**S4**	**S5**	**S1**	**S2**	**S3**	**S4**	**S5**
Cd	< 0,6	< 0,6	< 0,6	< 0,6	1,625	< 0,6	< 0,6	< 0,6	< 0,6	1,53
Co	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
Cu	< 5	< 5	36,7	18,55	29,56	< 5	14,51	51,13	< 5	30,51
Fe	183,83	40151	7728	1235,9	12963	192,63	43787	2361,8	321,85	12103
Pb	< 5	92,44	24,9	13,02	80,34	< 5	94,36	23,71	< 5	81,42
Mn	9,95	299,2	119,69	23,33	138,1	10,49	386,9	58,19	< 5	139,8
Ni	< 5	5,086	11,4	< 5	16,657	< 5	6,613	< 5	< 5	14,794
Zn	< 5	1164,75	484,75	22,02	190,95	< 5	1241,68	38,09	< 5	179,65
Cr	< 5	18,45	81,09	< 5	35,14	< 5	22,41	7,23	< 5	32,65
	Hydrocarbons									
	S1	**S2**	**S3**	**S4**	**S5**	**S1**	**S2**	**S3**	**S4**	**S5**
Water (mg/l)	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1
Sediments (mg/Kg dry matter)	< 1,7	12,5	< 1,7	3,7	120	< 1,7	6,1	< 1,7	< 1,7	93

AChE in bivalve from the Tunisian coast can also change in response to pollutant exposure. Metals such as Ni, under acute exposure, are able to decrease AChE activity in dose-dependant in *M. galloprovincialis* (Attig *et al.*, 2010). Interestingly, AChE activity inhibition was correlated with the Ni uptake in the digestive gland of mussels exposed to both the tested concentrations (2.5 and 13 mM Ni). In another study, *R. decussatus* clams exposed continuously for 14 day to treated municipal effluents had seriously altered the AChE in gills and digestive gland. Interestingly, AChE activity inhibition was correlated with the Zn, Cu and Cd accumulation in both organs (digestive gland and gills) of treated clams (Kamel *et al.*, 2012).

Furthermore, pesticides, carbamates and heavy metals are known by their capacity to inhibit *in vitro* or *in vivo*, AChE activity (Galloway *et al.*, 2004; Banni *et al.*, 2005), especially in fish (Monserrat *et al.*, 2002. Sturn *et al.*, 2000; Kirby *et al.*, 2000). In fact, 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 OP. AChE inhibition was observed in the fishes *Seriola dumerilli* exposed under laboratory conditions to Cd (Jebali *et al.*, 2006). Indeed, the neurotoxicity effect of Cd illustrated in many *in-field* and *in-vivo* studies using aquatic organisms as bioindicators (Roméo *et al.*, 2006). Jebali *et al.* (2013b) investigated the effects of Ni and CPF on AChE activity in sea bass (*Dicentrarchus labrax*) following 1, 3 and 7 days exposure (Table 5). CPF have a higher significant repression on brain AChE at day1 and then its return to the physiological activity at day 3 and 7. The lack of AChE inhibition after 3 and 7 days in *D. labrax* seen in this work would indicate the total detoxification of CPF after this period and the recuperation of AChE activity, probably due to the novo synthesis (Table 5). In the contrary of no effect of Ni on AChE activity, the mixture Ni and CPF, highly enhanced this activity in the late exposure time (day 3 and 7), consequently displaying neurotoxicity to the sea bass, which was inconsistent with the previous studies which show that metals or metals associated with pesticide may exert toxicity on the AChE activity (Scheil & Köhler, 2009; Wang & Wang, 2009)

5. CONCLUSION

The use of ChE activity in species cultivated for human consumption such as bivalves and fishes is of great importance for the assessment of the aquatic environment contamination. The results presented and discussed here indicate that different environmental factors, like the exposure to different classes of contaminants, can cause substantial changes in cholinesterase activity. The characterization and the evaluation of the AChE activity of these aquatic organisms may be useful for biomonitoring of other natural ecosystems.

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Table 5. Brain acetylcholinesterase (AChE) in juvenile sea bass (Dicentrarchus labrax) exposed to 500 µg/kg of Ni; 10 mg/kg of CPF and their mixture (Ni+CPF) for 1, 3, and 7 days. Two control groups of fishes were considered; 9% NaCl injected fishes and DMSO injected fishes. Data are means ± SD (n = 10). Significant difference between treated and relative controls was indicated as *, p<0.05; ** p<0.01. Data from Jebali *et al.*, 2013b.

Tabela 5. *Colinesterase cerebral em Dicentrarchus labrax juvenil exposto a 500 µg/kg de Ni; 10 mg/kg de CPF e a sua mistura (Ni+CPF) para 1, 3, e 7 dias. Foram considerados dois grupos controle; peixes injectados com 9% NaCl e peixes injectados com DMSO. Resultados expressos como médias ± SD (n = 10). Diferenças significativas entre controles indicadas como *, p<0.05; ** p<0.01. Dados retirados de Jebali et al. (2013b).*

	Time exposure (Days)		
Group	1	3	7
[NaCl]	35.271±3.313	34.451±3.132	31.715±5.092
[DMSO]	39.671±7.965	36.942±2.476	33.366±6.616
[Ni]	43.037±3.336	43.560±7.575	39.218±2.537
[CPF]	24.840±6.160*	29.140±1.616	30.735±5.920
[Ni+CPF]	42.282±7.539	61.655±15.994**	42.441±7.817*

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