

Characterization and evaluation of cholinesterase activity in the cockle *Cerastoderma glaucum*

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ABSTRACT: The aim of the present study was to partially characterize the biochemical properties of cholinesterase (ChE) activity in the tissues of the cockle *Cerastoderma glaucum*, using different substrates and sample volumes, and to evaluate ChE response following caging exposure in a fishing harbour. Comparative analysis of the Michaelis-Menten kinetics in the studied tissues of *C. glaucum* indicated that ChE activity, using acetylthiocholine as substrate, displayed a tissue-dependent response, with activity levels in the following order of magnitude: whole animal > rest of animal (adductor and retractor muscles, mantle and foot) > visceral mass > gills. The transfer of *C. glaucum* to the Téboulba fishing harbour caused a time-dependent reduction in acetylcholinesterase activity.

KEY WORDS: Biomarker · Cockle · *Cerastoderma glaucum* · Cholinesterase · ChE · Caging · Fishing harbour

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INTRODUCTION

The marine ecosystem is continuously being affected by various types of chemical pollutants. The development of sensitive ecotoxicological tools for impact assessment is therefore a priority task. Stress indices, or biomarkers, have been employed in ecotoxicological studies to assess the negative effects of contaminants in aquatic organisms, bivalves in particular, and to define environmental quality (López-Barea 1995). Among the biomarkers evaluated to date, there is a lot of interest in cholinesterase (ChE) activity as an indicator of the biological effects of exposure to neurotoxic compounds in aquatic organisms (Bocquené & Galgani 1991).

ChEs are a family of enzymes that includes acetylcholinesterase (AChE) or true cholinesterase and pseudocholinesterases (PsChE). AChE 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. PsChE seems to have no specific natural substrates and has been proposed as a scavenging enzyme for certain classes of toxic compound (Massoulié et al. 2008). In vertebrates, ChEs may be distinguished by their functional characteristics of substrate specificity and their susceptibility to diagnostic inhibitors (Sturm et al. 1999, Massoulié et al. 1993, 2008). Among selective inhibitors currently used to classify vertebrate ChEs, phenylmethylsulphonyl fluoride (PMSF) is a general inhibitor of enzymes having Ser residues in their active site, such as ChEs (Galloway et al. 2002), whereas tetra (monoisopropyl) pyrophosphor-tetramide (Iso-OMPA) and 1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51) are considered selective for butyrylthiocholine (BChE) and AChE, respectively, in vertebrates (Sturm et al. 1999). However, the classification used for vertebrates may be inappropriate for invertebrates such as bivalves (Bocquené et al. 1997). In aquatic invertebrates, classification of ChE isoforms

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has been less studied than in vertebrates. Moreover, most studies have been mainly focused on marine and freshwater species, such as molluscs (Mora et al. 1999, Corsi et al. 2007), different orders of Crustacea (Xuereb et al. 2007, Vioque-Fernández et al. 2007) and Copepoda (Monserrat & Bianchini 1998). Therefore, 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. 2009a,b). To the best of our knowledge, ChE activity in tissues of the cockle *Cerastoderma glaucum* has not been characterized to date.

Bivalve organisms such as clams have been widely used as sentinel organisms in previous field studies because of their wide geographical distribution and ability to accumulate contaminants (Banni et al. 2005, Jebali et al. 2007). In addition to their higher capacity to accumulate contaminants, bivalves can be easily caged at field sites. Using caged bivalves from a single population avoids bias related to age and reproductive status of the organisms that influence contaminant bioaccumulation and biomarker responses and allows a more accurate assessment of the real biological effects of contaminants over a predetermined exposure period (Oikari 2006). In the present study, wild *Cerastoderma glaucum* were transplanted into a fishing harbour to illustrate the kinetic responses of ChE activity using acetylthiocholine as substrate under natural conditions. The aims of the present study were to: (1) partially characterize the biochemical properties of ChE activity in the main tissues of *C. glaucum*, using different substrates and sample volumes, and (2) evaluate its response following the caging exposure in a fishing harbour.

MATERIALS AND METHODS

Chemicals

Acetyl- (ASCh), propionyl- (PSCh) and butyrylthiocholine (BSCh), all in their iodide forms, and dithio-bisnitrobenzoate (DTNB) as well as all other reagents were purchased from Sigma-Aldrich.

Cockle sampling and experimental caging technique

Wild *Cerastoderma glaucum* were manually collected at Kuriat Island (Tunisia), an uncontaminated area, in September 2008. After collection, bivalves

were transported in a cold container by boat to Téboulba fishing harbour (35° 39' N, 10° 57' E), where they were exposed to ambient conditions for 1 mo. Twenty specimens of ~25 mm shell length were placed in a rectangular cage made of polyethylene netting (20 × 20 × 50 cm) and maintained at 2 m depth. After 0, 15 and 30 d, 10 bivalves were collected and immediately dissected. The tissues, i.e. gills, visceral mass and the rest of the animal (adductor and retractor muscles, mantle and foot), were carefully removed, placed in Eppendorf tubes and transported in a portable refrigerator to the laboratory. Samples were stored at –40°C until analysis. For the *C. glaucum*-specific biochemical characterization of AChE, 40 animals from the same Kuriat Island population were transported in a portable refrigerator to the laboratory. Either the whole animal, its main organs (gills and visceral mass were dissected out) or the rest of the animal were placed individually in Eppendorf tubes and frozen at –40°C until analysis.

Sample preparation

Tissues (whole animal, rest of animal, visceral mass and gills) were dissected and homogenized in ice-cold 100 mM phosphate buffer, pH 7.4 (1/4 w/v) with an Ultra-Turrax T25 at 24 000 rpm for 30 s. The homogenate was centrifuged at 9000 × *g* at 4°C for 30 min, and the supernatant was stored at –40°C until biochemical assays were performed. Protein concentration was estimated according to Bradford (1976) using bovine serum albumin as standard.

ChE activity

ChE activity was measured using the method of Ellman et al. (1961). For an assay, 40 µl of homogenate extract of each tissue group (gills, visceral mass, rest of animal and whole animal) was incubated at 25°C in a final volume of 1.2 ml containing 100 mM phosphate buffer (pH 7.5) and 50 µl of 8 mM DTNB. Measurement of enzyme activity was initiated by the addition of 75 µl of freshly prepared ASCh, PSCh or BSCh iodide solution in distilled water at 3 mM final concentration. Absorption of the 2-nitro-5-thiobenzoate anion, formed by the reaction, was then recorded at 412 nm every 60 s for 10 min. A blank without substrate was used to evaluate the reaction of thiols with DTNB, and another without sample was used to estimate the rate of spontaneous substrate hydrolysis.

ASCh was chosen as the routine substrate, as it is most commonly used for measuring ChE activity in invertebrate extracts and is considered the most specific for AChE (EC.3.1.1.7; Massoulié et al. 1993). For the determination of the kinetic constants of AChE activity, assays with ASCh concentrations ranging from 0.04 to 10 mM were investigated in each of the tissue groups of *Cerastoderma glaucum*. The effect of sample volume (1 to 40 μ l) was investigated to optimize the AChE assay conditions in *C. glaucum*.

Statistical analysis

Statistical analyses were performed using SPSS software. Significant differences between means were determined using one-way ANOVA followed by Duncan's test. Significant correlations between caging time and AChE responses were examined using Pearson correlation analysis.

In order to calculate the kinetic parameters of AChE, such as the Michaelis-Menten constant (K_m), the maximum substrate hydrolysis velocity, and the catalytic efficiency (v_{max}/K_m), we used GraphPad Prism v. 4 for Windows (GraphPad Software).

RESULTS

Substrate affinity of ChE in tissue groups

Preliminary screening to determine ChE substrate affinities in extracts of whole animal, rest of animal, visceral mass and gills was carried out at 3 mM fixed concentration. As shown in Fig. 1, for each tissue group, there were significant differences in ChE activity among the 3 substrates. In whole animals, the highest ChE activity was obtained with ASCh. In the other 3 tissue groups, ChE activity was highest using PSCh, representing 46, 55 and 49% of the total ChE activity in the rest of animal, visceral mass and gills, respectively.

Characterization of AChE activity versus ASCh

Effects of substrate concentration

The effects of substrate concentrations ranging from 0.04 to 10 mM on AChE activity versus ASCh were assayed in whole animal, rest of animal, visceral mass and gill extracts of *Cerastoderma glaucum* (Fig. 2). In all tissue groups, AChE activity

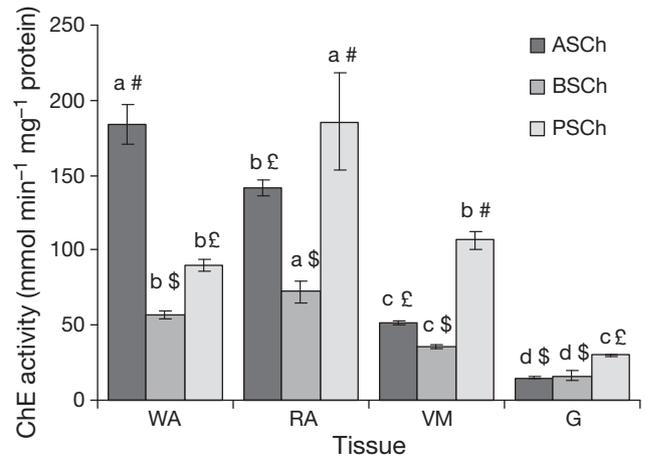


Fig. 1. Substrate specificity of cholinesterase (ChE) activity in the tissues of *Cerastoderma glaucum*. Data are means \pm SD of 4 determinations. Different lowercase letters indicate a significant difference between tissues. Significant differences between ChE activity in the same tissue are shown with different symbols. Substrates used were acetyl- (ASCh), butyryl- (BSCh) and propionylthiocholine (PSCh) iodide. WA: whole animal; RA: rest of animal (adductor and retractor muscles, mantle and foot); VM: visceral mass; G: gills

depended on substrate concentrations. A slight decrease in activity was observed at high ASCh concentrations in whole animal, rest of animal and visceral mass. A maximum AChE activity of approx. 185, 152, 57 and 20 $\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$ were measured in the whole animal, rest of animal, visceral mass and gill, respectively, using 3 mM ASCh at pH 7.5. This concentration was used for subsequent analysis (data not shown) as it was not rate limiting over the 10 min time period chosen.

The Michaelis-Menten kinetic parameters (K_m , v_{max} and v_{max}/K_m) are reported in Table 1. The K_m value was higher in gills than in the other tissue groups; this low affinity was in agreement with the low v_{max}/K_m value. AChE had a higher affinity towards ASCh as a substrate in the whole animal; this higher affinity was in agreement with the higher activity and v_{max}/K_m value.

Effects of supernatant sample volume

To optimize sample volumes, increasing volumes of extracts from whole animal, rest of animal, visceral mass and gills were tested and the results are reported in Fig. 3. Assay conditions were as follows: 3 mM substrate concentration, pH 7.5, 25°C. In each organ, AChE activity showed reaction rates directly proportional to the sample volume in the range

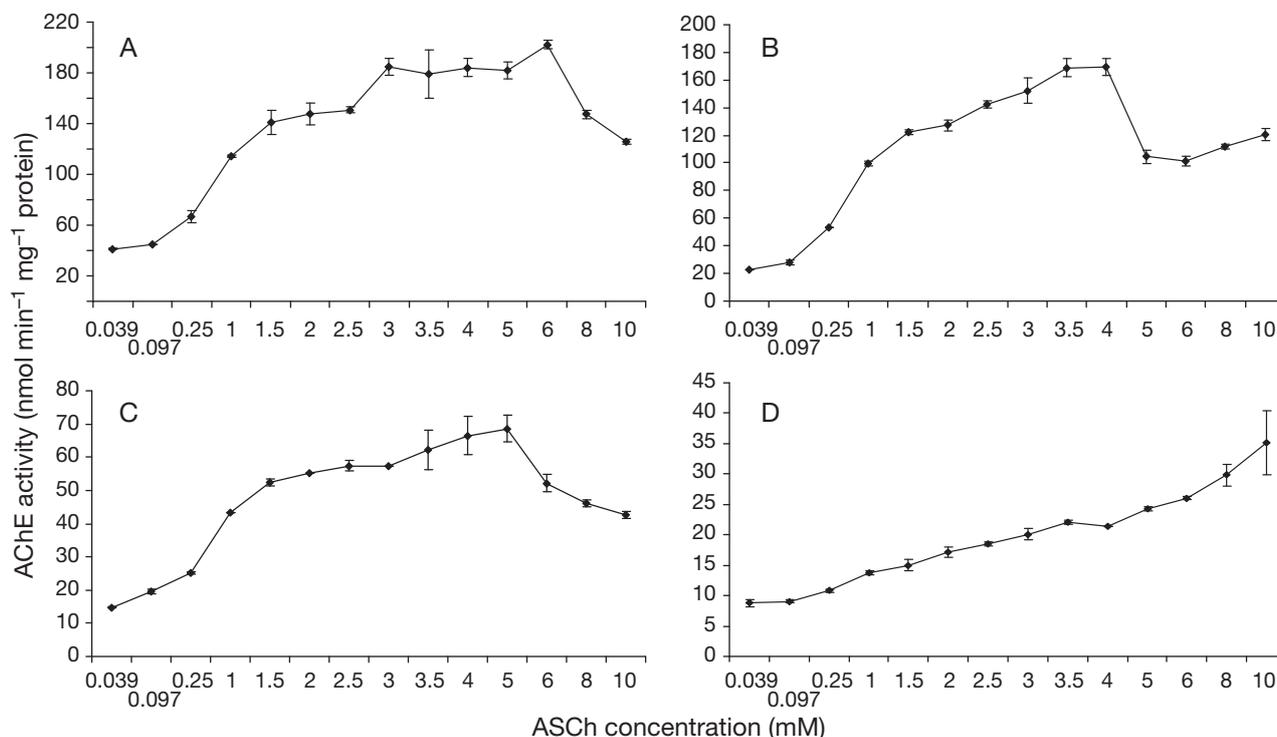


Fig. 2. Effects of substrate concentration on acetylcholinesterase (AChE) activity using acetylthiocholine (ASCh) as a substrate in tissues of the cockle *Cerastoderma glaucum*: (A) whole animal, (B) rest of animal (adductor and retractor muscles, mantle and foot), (C) visceral mass and (D) gills. Data are means \pm SD of triplicate determinations

tested. A volume of 40 μ l was therefore selected for all subsequent analyses.

Field evaluation of AChE response in caged *Cerastoderma glaucum*

To validate the use of AChE activity in biomonitoring the Tunisian coast, *Cerastoderma glaucum* individuals were transferred to the Téboulba fishing harbour for 30 d and AChE activity was evaluated in 2 tissue groups: the gills and the rest of the animal (adductor and retractor muscles, mantle and foot). Gills are considered an important route for uptake, bioconcentration and excretion of toxicants, and a prime target of contaminants because of the wide surface area in contact with the external medium and reduced distance between the internal and external medium. In caged cockles, gill AChE activity showed a time-dependent decrease and the Pearson coefficient was -0.90 ($p < 0.01$). Activity loss was approximately 15 and 68% after 15 and 30 d exposure, respectively (Fig. 4). In the rest of the animal, AChE activity decreased by approximately 30 and 24% after 15 and 30 d, respectively, and the Pearson coefficient was -0.55 ($p < 0.05$).

Table 1. Maximal velocity (v_{\max}) and the Michaelis-Menten constant (K_m) of acetylcholinesterase in different tissue groups of *Cerastoderma glaucum*. v_{\max}/K_m is the catalytic efficiency. Results are expressed as the means \pm SD of triplicate determinations. Rest of animal: adductor and retractor muscles, mantle and foot

Organ	v_{\max} (nmol min^{-1} mg^{-1} protein)	K_m (mM)	v_{\max}/K_m
Whole animal	179.1 ± 11.21	0.34 ± 0.14	526.76
Rest of animal	141.6 ± 10.92	0.29 ± 0.15	488.27
Visceral mass	58.82 ± 3.34	0.21 ± 0.09	280.09
Gills	36.48 ± 5.36	2.06 ± 0.87	17.7

DISCUSSION

Several studies have demonstrated the effectiveness of using measurement of ChE activity as a biomarker of exposure to neurotoxic compounds in bivalves, such as clams and mussels (Najimi et al. 1997, Dellali et al. 2001, Banni et al. 2005). Nevertheless, few data exist about the measurement of ChE activity in *Cerastoderma glaucum* (Machreki-Ajmi et al. 2008). There is also a general lack of knowledge of ChE activities in cockle species, regarding either

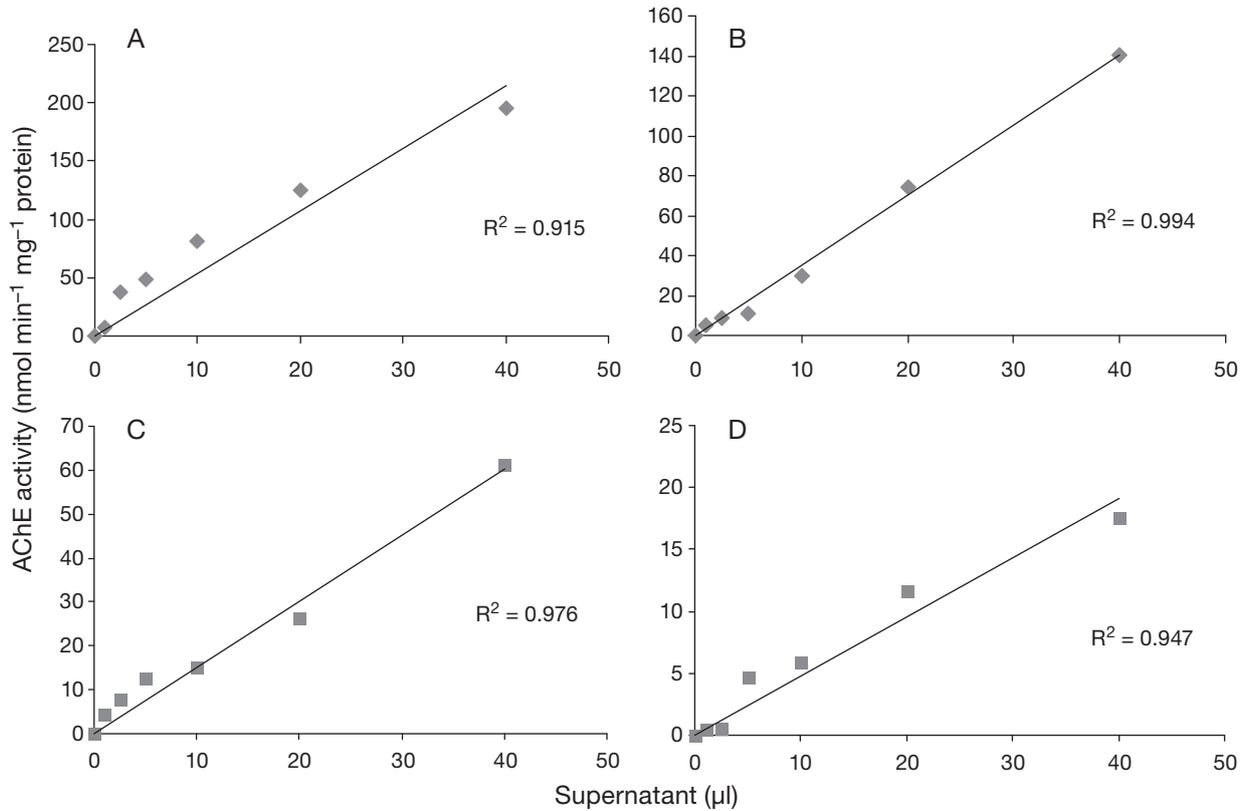


Fig. 3. Effects of variations in supernatant volume on acetylcholinesterase (AChE) activity using acetylthiocholine as substrate in (A) whole animal, (B) rest of animal (adductor and retractor muscles, mantle and foot), (C) visceral mass and (D) gills of the cockle *Cerastoderma glaucum*. Data are means ± SD of duplicate determinations

their biochemical properties or their responses to pollutants in natural ecosystems. Nevertheless, such information is essential if these biomarkers are to be successfully used in biomonitoring programs for pollutant risk assessment. To our knowledge, this is the first time that ChE activities have been investigated

in tissues of the cockle *C. 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.

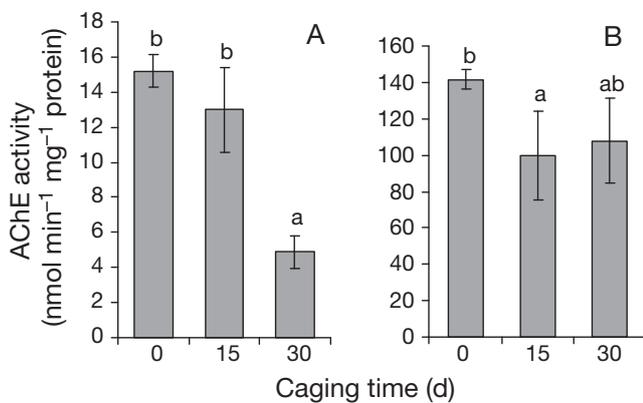


Fig. 4. Acetylcholinesterase (AChE) activity measured in (A) gills and (B) rest of animal (adductor and retractor muscles, mantle and foot) of the cockle *Cerastoderma glaucum* in cages in the Téboulba fishing harbour. Data are means ± SD. Different lowercase letters indicate a significant difference between groups

Characterization of ChE activity in tissue groups

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* (Fig. 1) show that the highest ChE activity was obtained using ASCh and PSCh, whereas the hydrolysis of BSCh was noticeably lower. These results agree with those obtained by Mora et al. (1999), who found PSCh and ASCh to be the most effective substrates for ChE in whole-body homogenates of the bivalve *Corbicula fluminea*. Similar results have also been reported for esterase activities by Bonacci et al. (2004), who found the following rank of substrate affinity in gill homogenates of *Adamussium colbecki*: PSCh > BSCh = ASCh.

The results of the present study clearly confirm the presence of ChE activity in the main tissues of the

cockle *Cerastoderma glaucum* and indicate that the enzyme activities using ASCh, PSCh and BSCh as substrates were tissue dependent. The results are in line with numerous studies on bivalves from different marine areas, although great variability of ChE basal levels in tissues, and variability in substrate specificity, have been recorded between species (Mora et al. 1999, Corsi et al. 2007). Several studies, showing a different ranking of substrate preference for esterase activities in bivalves such as *Ostrea edulis* (Valbonesi et al. 2003), clam *Corbicula fluminea* (Mora et al. 1999) and in other mollusc species (Bonacci et al. 2008), are in agreement with the well-accepted point of view that a high variability of esterase specificity to substrate often occurs both among different invertebrate species and within different tissues of the same organism (Gagnaire et al. 2008, Corsi et al. 2007, Valbonesi et al. 2003). In the present study, the ChE versus ASCh activity levels in the rest of the animal (adductor and retractor muscles, mantle and foot) are of the same order of magnitude as those found by Machreki-Ajmi et al. (2008) in rest of animal (foot, mantle, adductor muscles and siphons) of *C. glaucum*.

Effects of substrate concentration and sample volume

Regarding optimization of substrate concentrations, esterase activity with AChE in the whole animal, rest of animal and visceral mass showed a clear concentration-dependent pattern from 0.04 to 3 mM, and inhibition by excess substrate was observed at higher concentrations, depending on the selected tissue. A characteristic of the AChE enzyme is substrate inhibition (Massoulié & Toutant 1988). For each selected tissue, the highest rates of hydrolysis were detected at the substrate concentration of 3 mM.

Differences were observed between ChE and ASCh activity in the gills, which displayed a more regular pattern of responses in the concentration range of 0.04 to 3.0 mM than the other tissue groups, with the highest concentration eliciting the highest activities. Results similar to those of our study have previously been reported in molluscs (Bonacci et al. 2004, 2009).

Comparative analysis of the kinetic parameters (v_{max} , K_m and v_{max}/K_m) in tissues of *Cerastoderma glaucum* indicated that ChE versus ASCh activity was tissue-dependent and their level was following order: whole animal > rest of animal > visceral mass > gills.

More detailed characterization of ChE activity using ASCh as a substrate in the tissue groups of

Cerastoderma glaucum was carried out by investigation of the effects of sample volume (Fig. 3). In each tissue, the reaction rate was linear and directly proportional to the sample volume in the range tested, in accordance with results reported by Bonacci et al. (2004) in the bivalve mollusc *Adamussium colbecki*.

In situ study: AChE response in caged cockles

Cerastoderma glaucum is considered a key species in the food web on the Tunisian coast, and is used to assess environmental health, particularly where mussels are rare. Furthermore, the tissue composition of bivalves reflects the variability of heavy metal levels in environmental matrices (Machreki-Ajmi & Hamza-Chaffai 2006). In view of future national bio-monitoring programmes, we illustrate the different sensitivity and kinetic response of AChE activity after caging exposure of *C. glaucum* at Téboulba fishing harbour. The use of caged bivalves is a useful strategy for monitoring marine pollution through the analysis of biomarker responses (Damiens et al. 2007). Transplanting bivalves from a reference site to a polluted area can reduce the influence of genetic and/or population differences, seasonal variability or adaptive phenomena, all of which can attenuate the capability to discriminate between different levels of environmental disturbance. The use of caged organisms provides a time-integrated assessment of environmental quality, and reveals the early biological effects induced by accumulated pollutants (Gorbi et al. 2008).

Cerastoderma glaucum caged at Téboulba fishing harbour for 4 wk experienced a critical alteration in gill AChE activity compared with the tissue activity of the rest of the animal (Fig. 4). In the gills, AChE activity decreased in a time-dependent pattern, losing approx. 15 and 68% activity after 2 and 4 wk of transplantation, respectively, whereas in muscle tissue (rest of animal) the loss of AChE was approximately 24% at the end of the 4 wk caging period. 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. Similar results were obtained by Roméo et al. (2003), who noted a significant reduction in the AChE activity of mussels transplanted at harbour entrance in Cannes, France, attributed to the heavy metal concentration in mussel tissues. Téboulba harbour is a large fishing harbour built in 1970 and located in the center of the Tunisian littoral area. It is an economically important facility due to a variety of

fishing activities and contributed 30% of the national fish captures in the last 5 yr. Because over 450 ships of varying size are based in Téboulba harbour, it receives contaminants released from ship traffic and waste from the fishing industry. Thus, the harbour represents a key potential pollutant source for the central Tunisian littoral (Jebali et al. 2007), and thus the higher concentrations of heavy metals, oils and fuels in the harbour environment may be responsible for the kinetic reduction of *C. glaucum* AChE activity.

CONCLUSIONS

We have demonstrated the presence of ChE activity in tissue groups of the cockle *Cerastoderma glaucum* (whole animal, rest of animal [adductor and retractor muscles, mantle and foot], visceral mass and gills). The present study emphasizes the importance of standardizing the optimal conditions for measuring AChE activity in different tissues. Moreover, results showed that AChE activity diminished in a time-dependent pattern in a caging exposure of *C. glaucum*, highlighting the potential of such ecotoxicological tools for the assessment of the marine quality of the Tunisian and Mediterranean ecosystems. Further ecotoxicological characterization studies focused on the kinetic uptake of chemical pollutants (PAHs and heavy metals) and AChE activity response of *C. glaucum* during increasing periods of caging/depuration will be carried out in the future.

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