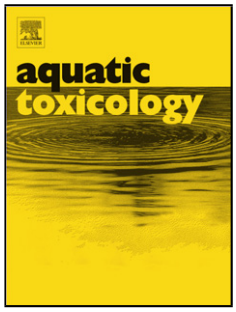

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Acetylcholinesterase of mangrove oyster *Crassostrea rhizophorae*: A highly thermostable enzyme with promising features for estuarine biomonitoring

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Highlights

- AChE from gills and viscera of *C. rhizophorae* were physicochemical and kinetically characterized;
- Parameters such as catalytic efficiency, activation energy and rate enhancements related to the enzymes were obtained;
- The enzymes were exposed to carbamate, organophosphorus and benz pesticides;
- The enzymes were highly thermostable;
- Carbaryl and carbofuran IC_{50} were below the values recommended by USEPA *Drinking Water Standards.*

Abstract

Enzyme biomarkers from several aquatic organisms have been used for assessing the exposure to contaminants at sublethal levels. Amongst them, the cholinesterases are commonly extracted from several organisms to evaluate/measure organophosphate and carbamate neurotoxic effects. Acetylcholinesterase (AChE; EC 3.1.1.7) is an enzyme of the group of serine esterases that acts on the hydrolysis of the neurotransmitter acetylcholine allowing the intermittence of the nerve impulses responsible for the neuronal communication. This enzyme is the main target for the action of some pesticides and the inhibition of its activity in bivalve mollusks may be used as biomarker due to their filter-feeding habit. In this context, the present study aimed to characterize physicochemical and kinetic parameters of the AChE extracted from gills and viscera of the oyster *Crassostrea rhizophorae* and investigate the *in vitro* effect of pesticides (dichlorvos, diazinon, chlorpyrifos, methyl-parathion, temephos, carbaryl, carbofuran, aldicarb, diflubenzuron and novaluron) in search for assessing its potential as biomarker. Specific substrates and inhibitors evidenced the predominance of AChE in both tissues. The optimum pH found for gills and viscera AChE were 8.0 and 8.5, respectively. The maximum peak of activity occurred at 70°C for gill AChE and 75°C for viscera AChE. The enzymes of both tissues presented remarkable thermostability. The Michaelis-Menten constant for both enzymes were 1.32 ± 0.20 mM for gills and 0.43 ± 0.12 mM for viscera. The V_{max} values for gills and viscera were 53.57 ± 1.72 and 27.71 ± 1.15 mU/mg, respectively. The enzymes were able to reduce the activation energy to 9.75 kcal.mol^{-1} (gills) and 11.87 kcal.mol^{-1} (viscera) obtaining rate enhancements of 3.57 x 10^5

and 1.01 x 10^4, respectively, in relation to non-catalyzed reactions. Among the pesticides under study, the carbamates carbaryl and carbofuran exerted the strongest inhibitory effects on the enzyme activity achieving important degrees of inhibition at concentrations below national and international current regulations. The first observation of the effects of benzoylurea pesticides (diflubenzuron and novaluron) on AChE from mollusks is reported here. The gills AChE of *C. rhizophorae* showed potential to be specific biomarker for the carbamate carbaryl while the viscera AChE showed it for carbofuran. According to their features, these enzymes may be proposed as promising tools for estuarine monitoring as well as biocomponent of biosensor devices.

Keywords: mangrove oyster; cholinesterase; organophosphorus; carbamate; benzoylurea; biomonitoring.

1. Introduction

A great diversity of environmental indicators (biomarkers) has been developed to detect and assess the exposure to contaminants at sublethal levels. Such biomarkers are widely used in monitoring programs (Prichard et al., 1997; Moore et al., 2004; Hyne and Maher, 2003; Wang et al., 2005; Bonacci et al., 2007; De la Torre et al., 2007; Kopecka and Pempkowiak, 2008).

Cholinesterases (ChEs) are a group of enzymes with high affinity for choline esters and are represented by acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). The first one hydrolyzes the neurotransmitter acetylcholine in the cholinergic synapses and in neuromuscular junctions clearing the respective clefts and preventing overstimulation of the receptors. The second presents a wide range of ligands with unknown substrate and is commonly regarded as a bioscavenger or detoxificant enzyme besides being involved in the metabolism of ghrelin (Chatonnet and Lockridge, 1989; Massoulié et al., 1993; Çokugras, 2003; Cousin et al., 2005; Brimijoin et al., 2016). The higher catalytic efficiency of these enzymes to hydrolyze choline esters and their high reactivity towards several inhibitors are determined by the specific functional architecture of their active site. The physiological role of these enzymes is well known in vertebrates, however there is a great diversity of ChEs in invertebrates and their function are not completely elucidated (Matos, 2012; Kozlovskaya et al. 1993). Several studies have shown great variability in ChEs in relation to their substrate preferences, tissue location and physiological role in mollusc species (Mora et al. 1999; Stefano et al. 2008; Valbonesi et al. 2003).

Biomarkers based on mollusc ChEs have been applied for organophosphate (OPs) and carbamate (CBs) pesticides exposure (Bolton-Warberg et al. 2007; Galloway et al. 2002; Solé et al. 2010; Valbonesi et al. 2003). AChE is the primary target for the action of these pesticides and the measure of the inhibition of its activity is widely used in mollusc species to assess the effects exerted by such neurotoxic compounds (Escartin and Porte, 1997; Galloway et al., 2002; Owen et al., 2002; Binelli et al., 2006; Cooper and Bidwell, 2006; Bolton-Warberg et al., 2007). The inhibition by OPs and CBs pesticides occurs due to their interaction with the steratic subsite of ChEs differing in the type of interaction – phosphorylation by OPs and carbamylation by CBs preventing the binding of substrate (Assis et al., 2011). Some benzoylurea pesticides (chitin synthesis inhibitors)

were also found to inhibit AChE (Maduenho and Martínez, 2008; Cottage and Gunning, 2004).

The inhibitory effect of pesticides on the activity of AChE promotes accumulation of acetylcholine in the synaptic cleft causing cholinergic hyperstimulation (Nunes et al., 2014). Some of these pollutants do not degrade fast in environment and present high toxicity, accumulation in tissues inducing to physiological disruptions and diseases (WHO, 1986a; 1986b). Monitoring and controlling the presence of these compounds in the environment is of paramount importance since the excess of its usage has caused public health problems (Eddleston et al., 2008; Assis et al., 2010).

Several authors suggest the use of bivalve species for studies on contamination of aquatic environment (Bainy et al., 2000; Cheung et al., 2001; Gowland et al., 2002; Niyogi et al., 2001a). These organisms are selected for such purposes because they are sessile, intertidals and generally euryaline with wide distribution along the coasts and estuaries in different latitudes. As filtering organisms, they can accumulate in their tissues a large variety of compounds present in seawater (Viarengo and Canesi, 1991). In the case of estuarine and mangrove contamination in Brazil, some authors have been used the mangrove oyster *Crassostrea rhizophorae* as a sentinel organism (Nascimento, et al., 2000; Rebelo et al., 2003; Silva et al., 2001; Wallner-Kersanach et al., 2000).

The mangrove oyster *C. rhizophorae* also known as native oyster is a bivalve mollusc regarded as an excellent bioindicator of aquatic contamination due to its abundance, cosmopolitanism and rusticity. Therefore, *C. rhizophorae* can be proposed as source of ChE for monitoring studies joining the ease of collection to the high degree of specificity and fast response to changes in the target substance by these enzymes (Silva *et al*., 2004).

High variability of ChEs characteristics has been found in different mollusc species and intraspecifically in their tissues (Escartin and Porte, 1997; Basack et al., 1998; Mora et al., 1999; Talesa et al., 2001; Bonacci et al., 2004; Cunha et al., 2007). Before using ChEs from a new species as biomarker, it is important to identify, determine the properties of the enzymes and search for the best assay conditions. The different ratios between AChE/BChE contained in different tissues may interfere in the interpretation of results and comparison to other studies. Both enzymes contribute to the activity but they present diverse sensitivity to anticholinesterasic agents (Kozlovskaya et al 1993; Stefano et al 2008). Therefore, this work aimed to provide kinetic and physicochemical data about the properties and optimal assay conditions of AChEs from different tissues of

Crassostrea rhizophorae as well as to determine the effects of carbamates, organophosphorus and benzoylurea pesticides on their activities investigating the potential of these enzymes as biomarker of estuarine contamination.

2. Material and methods

2.1. Materials

Acetylthiocholine iodide, S-butyrylthiocholine iodide, propionylthiocholine iodide, tetraisopropyl pyrophosphoramide (Iso-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51), neostigmine bromide, eserine, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane, dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), temephos (97.5%), carbofuran (99.9%) and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal® (Seelze, Germany). HCl was obtained from Merck (Darmstadt, Germany). Glycine was acquired from Amersham Biosciences (Piscataway, NJ, USA). The microplate reader used was xMark™ (Bio-Rad Laboratories, Hercules, CA, USA). The centrifuges were Bio-Spin (BioAgency, São Paulo, SP, Brazil), Multifuge X1R (Thermo Fisher Scientific, Waltham, MA, USA) and Unicen MR (Herolab GmbH, Heidelberg, Germany). The Software for statistical analyzes was MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

2.2. Specimens collection and enzyme extraction

Oysters were from a fishermen depuration facility (48h depuration) in the estuarine complex of Coruripe river on the South coast of Alagoas. Eighty oysters were collected (shell length 5.389 ± 0.461 cm; visceral mass 1.90 ± 0.229 g) in dry season (summer). These specimens were transported alive at room temperature and the valves were opened to extract gills and viscera. The tissues were homogenized and diluted in 0.1 M Tris-HCl buffer pH 8.0 until reaching 20 $mg.mL^{-1}$. Then, the material was centrifuged at 10,000 x *g* for 10 min at 4 ºC and the precipitate were discarded and the supernatant were stored at -20 ºC for further assays.

2.3. Cholinesterase activity

The enzyme activity was carried out according to Ellman et al. (1961) with slight modifications by Assis et al. (2012), as follows: in microplate wells, 20 µL of the enzyme preparation was added to 200 µL of 0,25 mM DTNB (chromogenic agent, prepared in 0.5 M Tris-HCl buffer pH 8.0). Then, 20 µL of substrates acetyl- (ASCh), *S*-butyryl- (BSCh) or propionylthiocholine (PSCh) (62 mM in distilled water) were added immediately before the readings in a microplate spectrophotometer (xMark® Bio-Rad; Hercules, CA) at 405 nm in 0 and 180 s. A unity of enzyme activity (U) was defined as the amount of enzyme that hydrolyzes 1µmol of substrate per minute per mL of solution. Protein content was estimated according to Sedmak and Grossberg (1977). All assays were performed in quadruplicate.

2.4. Kinetic parameters

The Michaelis-Menten constant (k_m) and the maximum catalytic rate (V_{max}) related to the interaction between the enzyme and the substrates under study were estimated by increasing concentrations of each substrate (ASCh, BSCh and PSCh from 0.8 to 20.8 mM final concentration). The activities were fitted to the hyperbola model using the software MicroCal™ Origin® Version 8.0 (MicroCal, Northampton, MA, USA). The parameters V_{max} ratio (Relative Efficiency of Hydrolysis - REH) and k_m ratio were also used to corroborate the results for the use of specific substrates, considering k_m ratios ≥ 1 and V_{max} ratios < 1 as AChE whereas k_m ratios < 1 and V_{max} ratios ≥ 1 are considered as BChE and PChE features (Pezzementi et al., 1991; Rodríguez-Fuentes and Gold-Bouchot, 2004; Assis et al., 2014).

2.5. Selective inhibition assay

The samples were submitted to the selective inhibitors BW284c51 (AChE), Iso-OMPA (BChE), neostigmine and eserine (total ChEs) in five concentrations ranging from 0.001 to 10 mM using a 10-fold series dilution. The assays were performed in quadruplicate according to Silva and co-workers (2013), incubating in microplate 10 µL crude extract with 10 µL of inhibitor for 60 min. The continuation of the test was similar

to the section 2.3 when 200 µL of 0.25 mM DTNB was added to the incubation mixture and the reaction was started after the addition 20 µL of 62 mM acetylthiocholine. The absorbance was followed at 405 nm for 180s and the residual activity was determined considering the absence of inhibitor as 100%.

2.6. Physicochemical parameters

Optimum pH was determined assaying enzyme activity as described in section 2.2 in a pH range from 4.0 to 9.5 using the following buffers: citrate-phosphate (4.0 - 7.5), Tris-HCl (7.2 – 8.7) and NaOH-Glycine (9.0 – 9.5). Optimum temperature and thermostability of ChEs were determined as described in 2.3 at temperatures ranging from 25 to 100ºC with 5ºC intervals. In the thermostability measurements, samples were incubated for 30 min in a water bath and after 15 min of equilibration with room temperature (25°C), the activity was assayed.

2.7. Estimation of catalytic efficiency of the enzymes

The amount of enzyme of the extracts (Et) was estimated by an *Electrophorus electricus* AChE standard curve according to Assis and co-workers (2014) with some modifications. Briefly, using the curve equation, the activities of the extracts were converted from absorbance to $\mu g.mL^{-1}$ and after converted to $mol.L^{-1}$ using the molecular weight of *E. electricus* AChE tetramer (266 kDa - as determined by Assis et al., 2014) as standard. With this variable (Et), in addition to Michaelis-Menten constant (k_m) and maximum catalytic rate (V_{max}), it was possible to estimate the turnover number (k_{cat}) and catalytic efficiencies (k_{cat}/k_m and V_{max}/k_m) of the enzyme extracts.

2.8. Activation energy (AE) and rate enhancement estimation

To estimate the decrease of activation energy (AE) produced by the AChEs of *C. rhizophorae* in the hydrolysis of the substrate ASCh, the assays were performed under increasing temperatures up to the optimum temperature using Arrhenius plot from 25 to 70 ºC. Then, the rate enhancement produced by the enzymes relatively to the non-enzymatic (spontaneous) reaction was estimated using the equation I and replacing AE (in non-enzymatic conditions) by the Arrhenius data of blanks (Assis et al., 2014):

$$k = \left(\frac{\mathbf{k}T}{h}\right) x\, e^{-\frac{AE}{R}} \quad \text{(I)}$$

Where *k* is the rate constant, **k** is the Boltzmann constant, *T* corresponds to the absolute temperature, *h* represents the Planck constant, AE is the activation energy, and *R* is the gas constant.

2.9. Effect of pesticides

The extracts were incubated for 60 min at 25°C with five organophosphates (dichlorvos, diazinon, chlorpyrifos, methyl-parathion and temephos), three carbamates (carbaryl, carbofuran and aldicarb) and two benzoylureas (diflubenzuron and novaluron). The insecticides were firstly dissolved in dimethyl sulfoxide (DMSO) and then diluted in distilled water reaching five concentrations ranging from 0.001 to 10 mM (0.00025 to 2.5 mM for novaluron) and using a 10-fold series dilution as well as in the section 2.5. The incubation was performed according to Assis et al (2012) and the residual activity was determined considering the absence of pesticides as 100%. These data were fitted to the best non-linear regression models: exponential decay and polynomial ($\rho < 0.05$) using the software Microcal™ Origin® 8.0.

2.10. Estimation of IC_{50}, IC_{20} e Ki

The concentrations capable of inhibiting enzyme activity in 50% and 20% (IC_{50} and IC_{20}, respectively) were estimated for each inhibitor (specific inhibitors and pesticides). These data were necessary to calculate the inhibition constant (k_i), using the equation II (Cheng and Prusoff, 1973):

$$k_i = \frac{IC_{50}}{1 + \left(\frac{[S]}{k_m}\right)} \quad \text{(II)}$$

Where [S] corresponds to the concentration of substrate.

2.11. Statistical analyzes

All assays, excepting optimum temperature and thermal stability were carried out at room temperature (25ºC). Residual activity percentage was plotted versus *ln* of inhibitor concentration in mM. For the effect of pesticides, data were expressed as mean ± SD of four replicates from three homogenates and were statistically analyzed by linear and non-linear regression fitted to exponential decay, sigmoidal or polynomial models using Microcal™ Origin® version 8.0. For selective inhibitors, one-way analysis of variance (ANOVA) followed by Tukey's test, when indicated, were used for mean comparison and differences were reported as statistically significant when $p < 0.05$.

3. Results

3.1. Kinetic parameters of specific substrates

The kinetic parameters maximum hydrolysis rate (V_{max}) and the Michaelis-Menten constant (k_m) were analyzed using the substrates ASCh, BSCh and PSCh. The V_{max} relative to the hydrolysis of ASCh found in gills and viscera were 53.57 ± 1.72 and 27.71 ± 1.15 mU/mg, respectively. The k_m values were 1.32 ± 0.20 mM for gills and 0.43 ± 0.12 mM for viscera. The substrate PSCh was more efficiently hydrolyzed by both tissues than BSCh. BChE-like activity was only found in viscera. These values are compared to those reported in literature for several mollusks species (Table 1). In this table are shown the parameters V_{max} ratio and k_m ratio used in the characterization of BChE-like and PChE-like activities. The k_m ratios of BChE-like (viscera) and PChE-like (gills) activities were > 1 whereas their respective V_{max} ratios were < 1 which classify them as AChE. The PChE-like activity of viscera showed atypical features (k_m ratio < 1 and V_{max} ratio < 1).

3.2. Selective inhibitors

The ChE activity in the viscera of *C. rhizophorae* was strongly inhibited in presence of the BChE specific inhibitor Iso-OMPA whereas in gills the activity only significantly changed after 0.1 mM during the exposure to this inhibitor (Figure 1A and 1B). However, both tissues were inhibited by the AChE specific inhibitor BW284c51

confirming the presence of AChE in gills and viscera (Figure 1C and 1D). Significant inhibition of the ChE activity in both tissues was also observed in presence the total ChEs inhibitors neostigmine (Figure 1E and 1F) and eserine (Figure 1G and 1H). The values of the concentration that inhibits 20% of enzyme activity (IC_{20}) and median inhibitory concentration (IC_{50}) related to these inhibitors are present in Table 2 and showed a trend where eserine was the strongest inhibitor followed by BW284c51 and neostigmine. Iso-OMPA was the weakest one, mainly for gills enzyme (which presented no BChE-like activity).

3.3. Physicochemical parameters

Physicochemical parameters of enzyme functioning are shown in Table 3. The optimum pH found for gills and viscera AChE of *C. rizhophorae* was 8.0 and 8.5, respectively (Figure 2A). The maximum enzyme activity was found to be at 70ºC in gills and 75ºC in visceral tissue (Figure 2B). The AChE in gills and viscera showed to be highly thermostable maintaining increasing activity at least up to 100ºC after 30 min incubation and 15 min (Figure 2C).

3.4. Catalytic efficiency of the enzyme, activation energy and rate enhancement

Catalytic performance parameters such as V_{max}/k_m ratio, k_{cat}, k_{cat}/k_m, rate enhancement and activation energy are presented in Table 3. The V_{max}/k_m ratio values of gills and viscera were in the same order of magnitude (10^{-2}) to those reported by Monserrat et al. (2002) for *C. rhizophorae* and were lower than those of *C. gigas*. It can also be observed that the turnover number (k_{cat}) of gills AChE is 3-fold higher than its counterpart from viscera while catalytic efficiencies (k_{cat}/k_m) from both enzymes are very close to each other. Gills AChE managed to reduce activation energy of the hydrolytic reaction to a value 1.22-fold lower than viscera AChE and its rate enhancement reached one order of magnitude above the last one.

3.5. Effects of pesticides and estimation of IC_{50}, IC_{20} and Ki

All of the pesticides under study exerted significant inhibitory effect on AChE of gills and viscera of *Crassostrea rhizophorae*, excepting the organophosphorus temephos

and the benzoylureas diflubenzuron and novaluron (Figures 3-5). The values of the IC_{50} for gills and viscera AChE showed inhibitory power in the following decreasing orders: carbaryl > diazinon > aldicarb > dichlorvos > methyl-parathion > chlorpyrifos (gills) and carbofuran > dichlorvos > carbaryl > methyl-parathion > aldicarb (viscera). Table 4 also shows the IC_{20} and the inhibition constant (k_i) related to these pesticides.

4. Discussion

Data of the literature reported that high variability of cholinesterases features have been found in different mollusks species and different tissues in the same organism (Talesa et al., 2001; Bonacci et al., 2004; Cunha et al., 2007). Investigations carried out by Monserrat et al., (2002) pointed to the presence of only AChE (two isoforms - types S9 and TX S9) extracted with and without detergent (Triton X-100) in gills of *Crassostrea rhizophorae*. Similar results were found for gills (types A and B) of the Pacific oyster *C. gigas*, showing that type A ChE is poorly sensitive to Iso-OMPA exposure while type B is insensitive to this BChE inhibitor (Bocquené et al., 1997). In the present study, it was decided not to use Triton X-100 in the AChE extraction due to the aims of the work that included the evaluation of enzyme susceptibility to pesticides. According to Rosenfeld et al. (2001), Triton X-100 presents a kinetic interaction with organophosphorus compounds that can hinder the results.

V_{max} ratio (relative efficiency of hydrolysis - REH) and k_m ratio are parameters used to add information to the characterization of BChE-like and PChE-like activities based on the ability of AChE to hydrolyze the specific substrates of these other ChEs (BSCh and PSCh) and based on the divergence between AChE and BChE towards higher concentrations of substrates: AChE undergo inhibition by excess of substrate while BChE does not show inhibition (Pezzementi et al., 1991; Çokugras, 2003; Rodríguez-Fuentes and Gold-Bouchot, 2004). In the present study, AChE and PChE-like activities were observed in gills of *C. rhizophorae* and according to k_m ratio and V_{max} ratio analyzes, both activities can be ascribed to AChE (PChE-like activity in this tissue presented k_m ratio > 1 and V_{max} ratio < 1). In viscera, AChE, BChE-like and PChE-like activities were observed. The k_m ratios and V_{max} ratios of them suggested that BChE-like activity could be attributed to AChE (k_m ratio > 1 and V_{max} ratio < 1) whereas PChE-like activity could be an atypical ChE activity (k_m ratio < 1 and V_{max} ratio < 1 in viscera). In literature, it can

be seen that k_m ratios and V_{max} ratios would point BChE-like activity as AChE in *Cerastoderma edule* (Nilin, 2012) and would ascribe PChE-like activity to BChE in *Cerastoderma edule* (Nilin, 2012) and to an atypical ChE in *Corbicula fluminea* (Mora et al., 1999). Here, the use of specific inhibitors corroborated the presence of only AChE in gills and AChE associated with some atypical ChE activity in viscera of *C. rhizophorae*, since the gills enzyme was poorly inhibited by Iso-OMPA whereas this inhibitor exerted a strong effect in viscera. Enzymes from gills and viscera were similarly inhibited by BW284c51 reinforcing the presence of AChE in both tissues. Moreover, AChE from viscera presented higher affinity for acetylthiocholine than that from gills as seen by the k_m values of the present work for *C. rhizophorae* in Table 1 suggesting (together with their different susceptibility to selective inhibitors) that these enzymes may be different isoforms.

The maximum hydrolytic activities of the enzymes from gills and viscera were observed in pH 8.0 and 8.5, respectively. This pH range is similar to the values found for *Crassostrea gigas* and *Mytilus edulis* (Bocquené et al., 1990) shown in Table 1. These findings are also close to that found for AChE in several works with fish such as *Parachromis managuensis* (Araújo et al., 2016), *Cichla ocellaris* (Silva et al., 2013) and *Colossoma macropomum* which were around 8.0 (Assis et al., 2010).

AChE from both tissues showed to work efficiently at high temperatures and to be highly thermostable. Bocquené and co-workers (1990) also observed high activity of AChE from *Mytilus edulis* above 48°C and increasing. The high optimum temperatures of the functioning of *C. rhizophorae* and *Mytilus edulis* AChE may be ascribed to the fact that these organisms are from warm environments and these enzymes may present a structure with smaller number or buried (in its globular structure) disulfide bonds (McPhee-Quigley et al., 1986). Several studies indicate that repetitive exposure to the air followed by resubmersion in salt water caused by the tidal cycles can be responsible for large variations in physiology of these organisms (Steffani and Branchi, 2003). The oysters remain adhered to mangrove roots (while mussels remain adhered in rocks and reefs) and alternate exposure to wind and sunlight and to warm water with pH variations and high/low oxygen concentrations. In such situations these organisms endure large variations of temperature justifying their high thermostability. This high stability of *C. rhizophorae* AChEs towards pH and temperature variations may be very useful for biomonitoring of estuarine warm waters and for industrial applications of these biomolecules such as biocomponents of biosensors.

V_{max}/k_m is the rate constant that influences the rate of catalytic turnover at subsaturating conditions ([s] < k_m) and demonstrates the efficiency of the reaction taking into account the affinity of the enzyme for a given substrate in a known concentration. Some alterations of enzyme kinetics such as inhibitions not always correlated with V_{max} and k_m are, sometimes, correlated with V_{max}/k_m (Radika and Northrop, 1984). The efficiency is directly proportional to the value of this parameter and here indicate that the gills enzyme is slightly more efficient than that from viscera.

Commercial AChE from *Electrophorus electricus* was used (AChE constituted by three tetramers linked to a collagenQ-like tail) as standard in the enzyme quantity (Et) estimation of AChE from *C. rhizophorae* tissues because it becomes compatible due to the protease treatment used to release the collagen tail from the tetramers and separate them (Chatonnet and Lockridge, 1989; Assis et al., 2014). The estimates of k_{cat} and k_{cat}/k_m were possible using this methodology. The values of k_{cat} corroborates the slight higher efficiency of gills AChE in relation to the viscera isoform and their values are approximately one order of magnitude lower than those described for *E. electricus* (1.2 x 10^4 s^{-1}) and other works using computational simulations (1.6 x 10^4 and 1.4 x 10^4 s^{-1}) (Augustinsson, 1971; Fuxreiter and Warshel, 1998; Fersht, 1999).

The other catalytic efficiency parameter k_{cat}/k_m describes the rate of product formation under physiological conditions at unknown substrate concentrations. The values of k_{cat}/k_m estimated here are very similar to 10^7 $M^{-1}.s^{-1}$described by Assis et al. (2014) for brain AChE of *Arapaima gigas*, *Colossoma macropomum*, *Oreochromis niloticus* and *Rachycentron canadum*, and described by Miller and Wolfenden (2002) and lower than 10^9 $M^{-1}.s^{-1}$ found by Nolte et al. (1980) using *E. electricus*. These differences in k_{cat}/k_m values can be ascribed to the rate of catalysis by AChE which is governed by the ability of the substrate to diffuse in the medium being restricted by the medium viscosity.

Activation energies (AE) estimated for the hydrolysis of acetylthiocholine by the enzymes from gills and viscera were decreased by 43.8% and 31.6%, respectively, which means some orders of magnitude in the final rates of reactions. In the present work, the AE value of gills AChE is similar to 9.0 $kcal.mol^{-1}$ obtained from *Heterorhabditis bacteriophora* AChE hydrolytic action by Mohamed et al. (2007). The AChE from viscera decreased AE similarly to *O. niloticus* brain AChE (11.0 $kcal.mol^{-1}$) in relation to the non-catalyzed reaction (17.36 $kcal.mol^{-1}$ as described by Assis et al., 2014). The rate enhancement obtained by these enzymes were 10^5 (gills) and 10^4 (viscera) and were lower

than values from literature such as 10^8 (Tõugu, 2001) which resulted from the non-catalyzed reaction by Wright (1968) and catalyzed reaction by Nolte et al. (1980) using *E. electricus* AChE. The values were also lower than AChE rate enhancements found by Fuxreiter and Warshel (1998) in computational simulations (ranging from 10^7 to 10^{11}). Again, these differences could be ascribed to the effect exerted by the substrate limit of diffusion on the AChE rate of hydrolysis due to experimental differences between assay mediums (buffered extract *versus* purified enzyme/computational simulated medium) and, again, to the fact that small differences in k_{cat}/k_m values implies in really larger differences in rate enhancement values.

The parameters IC_{20} (concentration that inhibits the enzyme activity in 20%) and IC_{50} (median inhibitory concentration) are meaningful in relation to anticholinesterasic compounds since an inhibition of 20% is the lower limit from which the presence of these substances is detected whereas 50% marks the appearance of signs and symptoms and 90% of inhibition means death (FAO, 2007). According to IC_{50} values, the order of susceptibility of *C. rhizophorae* gills AChE to the pesticides was carbaryl > diazinon > aldicarb > carbofuran > dichlorvos > methyl-parathion > chlorpyrifos, whereas for the visceral enzyme this order was: carbofuran > dichlorvos > carbaryl > methyl-parathion > aldicarb.

The organophosphates chlorpyrifos, diazinon and methyl-parathion, in some cases, presents similar pattern of inhibition. In most of the cases, they exert moderate to highly toxic effects whereas the organophosphate temephos does not reach 50% of inhibition in the concentration range of several studies (Assis et al., 2012; Araújo et al., 2016; Silva et al., 2013). Here, diazinon was the second most inhibitory pesticide for gills AChE of *C. rhizophorae* while methyl-parathion moderately inhibited both enzymes and chlorpyrifos did not affect strongly any of the tissues AChEs. Exposure assay (*in vivo* - 96h) to chlorpyrifos with *Crassostrea corteziensis* showed inhibition of AChE activity without mortality of oysters and valve closure after 12h (Benitez-Trinidad et al., 2014). Some reports suggest that these oysters are able to isolate themselves from a contaminated environment by closing their valves reducing the filtration rates in contact with toxic compounds and, therefore, decreasing the exposure time (Kramer et al, 1989; Mersch et al, 1993; Wrisberg et al., 1992).

Amongst the interaction between *C. rhizophora* AChEs and the pesticides under study, the carbamate carbaryl exerted the strongest inhibitory effect on gills AChE whereas the carbamate carbofuran and the organophosphate dichlorvos were the strongest

inhibitors for viscera AChE. Carbamates are direct active site inhibitors that do not need biotransformation to achieve all of their toxic potential (Tham et al., 2009). Although is well accepted that carbamates are direct inhibitors of AChE, some of them undergo biotransformation becoming stronger inhibitors due to their sulfoxide derivatives. Aldicarb is one of these carbamates, after formation of aldicarb-sulfoxide (Perkins and Schlenk, 2000). However, aldicarb did not act as strong as carbaryl and carbofuran in the present experimental conditions.

The IC_{20} and IC_{50} values related to some carbamate pesticides under study interacting with *C. rhizophorae* AChE are below the tolerance limits recommended by national and international regulations. The Resolution nº 357/2005 of CONAMA (Brazilian National Council of Environment) recommends a maximum concentration limit of 70.0 $\mu g.L^{-1}$ ($\approx$ 0.35 μM) of carbaryl for waters *class 3* ("waters for domestic supply after conventional treatment, for livestock and for irrigation forage") which is 3.5 and 2.7-fold above the IC_{20} and IC_{50} values related to this pesticide action on the *C. rhizophorae* gills AChE. The *USEPA National Primary Drinking Water Standards* (2017) provides a maximum limit of 40 $\mu g.L^{-1}$ ($\approx$ 0.18 μM) for the carbamate carbofuran. This value is slightly above the IC_{20} value for this pesticide on AChE from viscera of *C. rhizophorae*. The high degree of inhibition caused by carbaryl and carbofuran found here point these AChEs as strong candidates to be specific biomarkers of such compounds.

Benzoylurea pesticides can inhibit AChE from arthropods and fish (Maduenho and Martínez, 2008; Cottage and Gunning, 2004). According to a personal communication quoted by Cottage and Gunning (2004) the benzoylurea novaluron may disrupt AChE activity by "methods such steric hindrance, dynamically inhibiting the widening of the gorge, by changing the opening behavior of alternative passages and disrupting the active site conformation". In the present study, novaluron and diflubenzuron inhibited, although weakly, the AChE activity in both tissues of *C. rhizophorae*. This is the first report about inhibition of cholinesterases from mollusks by benzoylurea pesticides.

These results showed not only the suitability of *C. rhizophorae* AChEs as biomarkers of pesticides but evidenced deleterious effects exerted by such compounds on this organism due to important degree of inhibition caused by low concentrations that are under the limits present in current regulations. It is worth mentioning that sublethal exposure may change several physiological, biochemical and morphological processes in the life cycle of these mollusks affecting their population development.

5. Conclusion

Specific substrates and inhibitors evidenced the predominance of AChE in gills and viscera of *C. rhizophorae.* The enzymes showed high thermostability which may be very important for environmental (as biocomponent of biosensors, for example) applications of these biomolecules. Amongst the pesticides under study the carbamates carbaryl and carbofuran were the strongest inhibitors making these enzymes as potential specific biomarkers for these carbamate pesticides. In addition, the benzoylurea pesticides diflubenzuron and novaluron were found to inhibit *C. rhizophorae* AChEs. This is the first report of benzoylurea effects on mollusks cholinesterases.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Figures

Figure 1. ChE activity of *Crassostrea rhizophorae* in presence of increasing concentrations (0-10 mM) of selective inhibitors: Iso-OMPA (A - gills; B - viscera); BW284c51 (C - gills; D - viscera); neostigmine (E - gills; F - viscera); eserine (G - gills; H - viscera). Data are expressed as mean ± SD of four replicates from three homogenates.

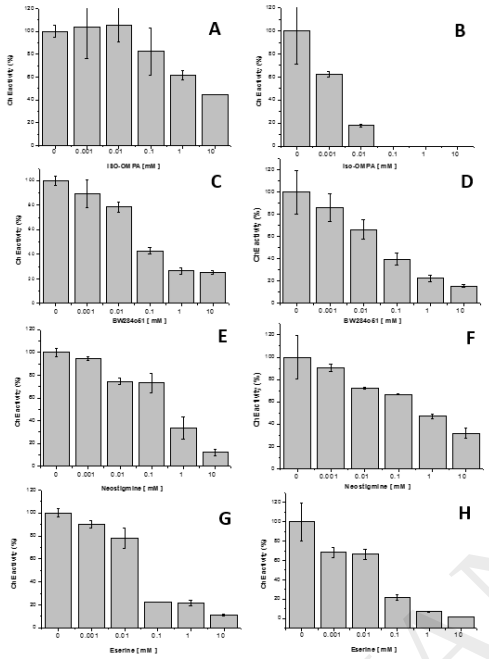


Figure 1. A - Effect of pH on the activity of *C.rhizophorae* AChE (gills and viscera); B - Effect of temperature on the *C.rhizophorae* AChE activity (gills and viscera); C - Thermostability of *C.rhizophorae* AChE activity (gills and viscera). Data are expressed as mean ± SD of four replicates from three homogenates.

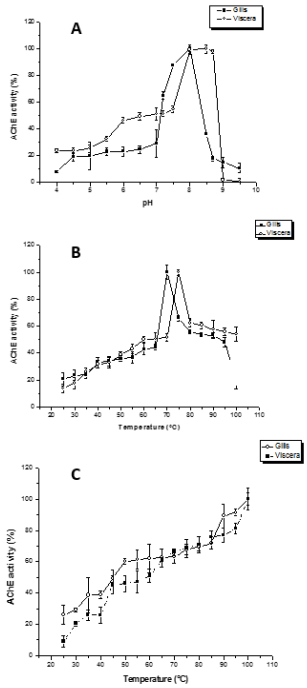


Figure 3. Effect of carbamates pesticides on the activity of AChE in *Crassostrea rhizophorae*: carbaryl (A- gills; B - viscera); carbofuran (C - gills; D - viscera); aldicarb (E - gills; F - viscera). Data are expressed as mean ± SD of four replicates from three homogenates.

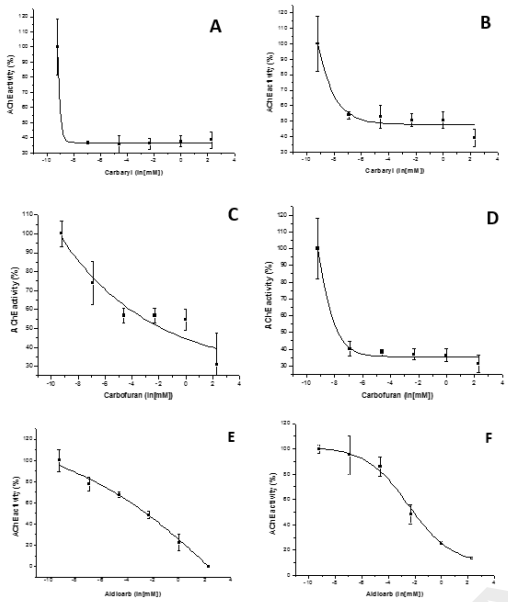


Figure 4. Effect of organophosphorus pesticides on the activity of AChE in *Crassostrea rhizophorae*: dichlorvos (A - gills; B - viscera);diazinon (C - gills; D - viscera); chlorpyrifos (E - gills; F - viscera); methyl-parathion (G – gills; H – viscera); temephos (I – gills; J - viscera). Data are expressed as mean ± SD of four replicates from three homogenates.

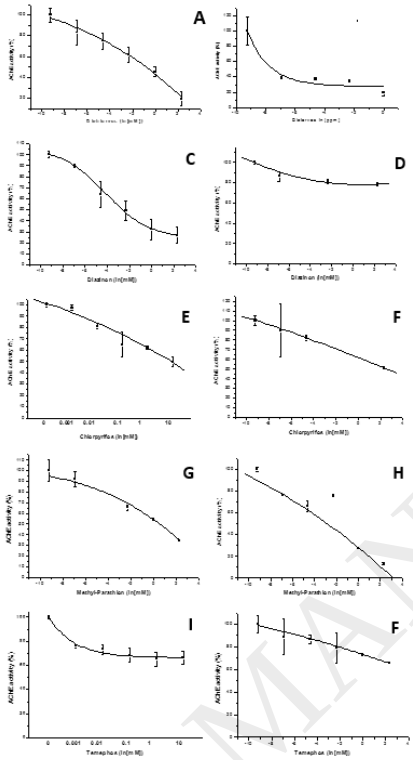
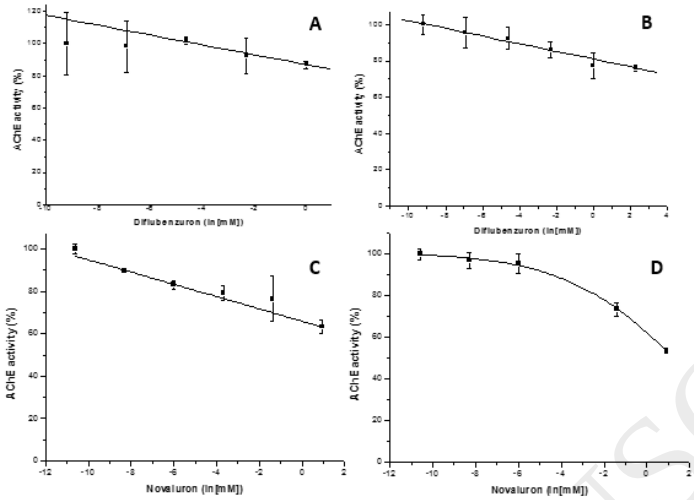


Figure 5. Effect of benzoylurea pesticides on the activity of AChE in *Crassostrea rhizophorae*: diflubenzuron (A - gills; B - viscera); novaluron (C - gills; D - viscera). Data are expressed as mean ± SD of four replicates from three homogenates.



Tables

Table 1. Kinetic parameters (k_m and V_{max}), k_m ratios and V_{max} ratios of ChE specific substrates activities in different tissues of some mollusks species

Species (substrate)	k_m (mM)	k_m ratio	V_{max} (mU/mg)	V_{max} ratio	Tissue	Reference
Crassostrea rhizophorae (ASCh) A	0.43 ± 0.12	-	27.71 ± 1.15	-	viscera	Present work
Crassostrea rhizophorae (BSCh) B	0.79 ± 0.31	1.83	5.90 ± 0.52	0.21	viscera	Present work
Crassostrea rhizophorae (PSCh) P	0.30 ± 0.23	0.69	11.50 ± 1.15	0.41	viscera	Present work
Crassostrea rhizophorae (ASCh) A	1.32 ± 0.20	-	53.57 ± 1.72	-	gills	Present work
Crassostrea rhizophorae (BSCh) B	NA	-	NA	-	gills	Present work
Crassostrea rhizophorae (PSCh) P	1.46 ± 1.20	1.11	10.12 ± 4.39	0.19	gills	Present work
Crassostrea rhizophorae (ASCh) A	0.046 ± 0.003	-	1.92 ± 0.04	-	gills S9	Monserrat et al., 2002
Crassostrea rhizophorae (ASCh) A	0.059 ± 0.008	-	5.84 ± 0.04	-	gills TX S9	Monserrat et al., 2002
Crassostrea rhizophorae (ASCh) A	3.08 ± 0.60	-	1,628 ± 1,071	-	adductor muscle	Domingos et al., 2007
Crassostrea gigas (ASCh) A	0.078	-	23.18	-	gills*	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	0.018	-	4.92	-	gills**	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	18.78		hepatopancreas	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	6.71		hepatopancreas	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	4.05		mantle	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	20.25		mantle	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	2.16		labial palps	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	10.83		labial palps	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	4.60		striated adductor	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	2.30		striated adductor	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	3.50		smooth adductor	Bocquené et al., 1997
Crassostrea gigas (ASCh) A	-	-	2.69		smooth adductor	Bocquené et al., 1997
Cerastoderma edule (ASCh) A	5.57 ± 2.68	-	2,540 ± 480	-	pool***	Nilin, 2012
Cerastoderma edule (BSCh) B	6.64 ± 4.70	1.19	1,970 ± 400	0.77	pool***	Nilin, 2012
Cerastoderma edule (PSCh) P	0.94 ± 0.13	0.17	4,570 ± 150	1.80	pool***	Nilin, 2012
Adamussium colbecki (ASCh)A	-	-	2,290 ± 130	-	adductor muscle	Bonacci et al., 2006
Adamussium colbecki (BSCh) B	-	-	600.0 ± 50	0.26	adductor muscle	Bonacci et al., 2006
Adamussium colbecki (PSCh) P	-	-	1,570 ± 140	0.68	adductor muscle	Bonacci et al., 2006
Ostrea edulis (ASCh) A	0.09 ± 0.01	-	4.82 ± 0.29	-	gills	Valbonesi et al., 2003
Mytilus galloprovincialis (ASCh) A	0.08 ± 0.01	-	18.36 ± 0.56	-	gills	Valbonesi et al., 2003
Mytilus galloprovincialis (ASCh) A	0.03	-	≈ 2.7	-	whole body	Mora et al., 1999
Corbicula fluminea (ASCh) A	≈ 0.3	-	≈ 5.5	-	whole body	Mora et al., 1999
Corbicula fluminea (PSCh) P	0.07	2.33	≈ 6.0	1.10	whole body	Mora et al., 1999

ASCh – acetylthiocholine; BSCh – S-butyrylthiocholine; PSCh – propionylthiocholine; k_m ratio and V_{max} ratio = B/A or P/A; NA – no activity; S9 – fraction from sucrose gradient; TX S9 - fraction from sucrose gradient with triton X-100; *AChE-A; **AChE-B; ***pool of the tissues: muscle, foot, mantle, gills and digestive gland; ≈ approximated values observed in graphs axes.

Table 4. Values of IC_{20} and IC_{50} estimated for *Crassostrea rhizophorae* ChE in the presence of selective inhibitors.

Inhibitor	Gills			Viscera		
	IC_{20} (mM)	IC_{50} (mM)	k_i (μM)	IC_{20} (mM)	IC_{50} (mM)	k_i (μM)
BW284c51	0.0068	0.0612	1.28×10^{-3}	0.004	0.047	3.24×10^{-4}
Iso-OMPA	0.10	0.65	13.6	0.50	3.0	2.07×10^{-2}
Neostigmine	0.0052	0.45	1.08×10^{-4}	0.004	0.62	4.27×10^{-3}
Eserine	0.0055	0.06	1.15×10^{-4}	0.0005	0.04	2.76×10^{-4}

Table 3. Physicochemical properties, catalytic efficiency and thermodynamic parameters of ChEs* from several mollusks.

Species	Tissue	pH	T (ºC)	V_{max}/k_m[a] ($mL.min^{-1}.mg^{-1}$)	kcat (s^{-1})	$kcat/k_m$ ($M^{-1}.s^{-1}$)	AE[b] ($kcal.mol^{-1}$)	Rate Enhancement[b]	Reference
Crassostrea rhizophorae	gills	8.0	70	4.05×10^{-2}	1.23×10^{3}	9.37×10^{5}	9.75	3.57×10^{5}	Present work
Crassostrea rhizophorae	viscera	8.5	75	6.40×10^{-2}	0.40×10^{3}	9.42×10^{5}	11.87	1.01×10^{4}	Present work
Crassostrea rhizophorae	gills[c]	-	-	4.17×10^{-2}	-	-	-	-	Monserrat et al., 2002
Crassostrea rhizophorae	gills[d]	-	-	9.90×10^{-2}	-	-	-	-	Monserrat et al., 2002
Crassostrea gigas	gills[e]	-	-	2.97×10^{-1}	-	-	-	-	Bocquené et al., 1997
Crassostrea gigas	gills[f]	-	-	2.73×10^{-1}	-	-	-	-	Bocquené et al., 1997
Crassostrea gigas	gills	8.5	-	-	-	-	-	-	Bocquené et al., 1990
Mytilus edulis	gills	8.5	> 48	-	-	-	-	-	Bocquené et al., 1990
Mytilus galloprovincialis	gills	-	-	2.40×10^{-1}	-	-	-	-	Valbonesi et al., 2003
Ostrea edulis	gills	-	-	5.16×10^{-2}	-	-	-	-	Valbonesi et al., 2003

AE – activation energy; *using substrate ASCh; [a]according to Valbonesi et al., (2003); [b]according to Assis et al., (2014); [c]S9 – fraction from sucrose gradient; [d]TX S9 - fraction from sucrose gradient with triton X-100; [e]AChE-A; [f]AChE-B.

Table 2. Estimates of IC_{20}, IC_{50} and k_i of *Crassostrea rhizophorae* AChE in the presence of organophosphorus, carbamate and benzoylurea pesticides.

Pesticide	**Gills**			**Viscera**		
	IC_{20} (mM)	IC_{50} (mM)	k_i (μM)	IC_{20} (mM)	IC_{50} (mM)	ki (μM)
aldicarb	0.0015	0.075	1.56	0.012	0.12	9.0×10^{-3}
carbofuran	0.0006	0.17	3.54	0.0001	0.0004	2.76×10^{-3}
carbaryl	0.0001	0.00013	2.71×10^{-3}	0.0002	0.0035	2.41×10^{-2}
dichlorvos	0.004	0.43	8.96	0.00017	0.0005	3.45×10^{-3}
diazinon	0.0035	0.062	1.29	0.11	-	-
chlorpyrifos	0.023	7.55	157	0.015	-	-
methyl-parathion	0.011	1.6	33.4	0.0006	0.055	3.79×10^{-1}
temephos	0.0015	-	-	0.113	-	-
diflubenzuron	-	-	-	1.35	-	-
novaluron	0.008	-	-	0.091	-	-