Cholinesterase activity in the tissues of bivalves Noah’s ark shell *Arca noae* and warty venus *Venus verrucosa* (Linnaeus, 1758):

Characterisation and *in vitro* sensitivity to organophosphorous pesticide trichlorfon

Lorena Perić*, Luka Ribarić and Vedrana Nerlović
Ruder Bošković Institute, Center for Marine Research
G. Paliaga 5, 52210 Rovinj
CROATIA

* - corresponding author:

Lorena Perić
Ruder Bošković Institute, Center for Marine Research
G. Paliaga 5, 52210 Rovinj
CROATIA

e-mail: lorena.peric@cim.irb.hr
phone: 00 385 52 804 700
fax: 00 385 52 804 780
ABSTRACT

Cholinesterase (ChE) activity was investigated in gills and adductor muscle of two bivalve species: *Arca noae* and *Venus verrucosa*. The properties of ChEs were investigated using acetylcholine iodide (ASCh), butyrylcholine iodide (BSCh) and propionylcholin iodide (PrSCh) as substrates and eserine, BW254c51 and *iso*-OMPA as specific inhibitors. The highest level of ChE activity in crude tissue extracts was detected with PrSCh followed by ASCh, while values obtained with BSCh were apparently low, except in *A. noae* adductor muscle. The enzyme activity in *A. noae* gills and *V. verrucosa* gills and adductor muscle was significantly inhibited by BW254c51, but not with *iso*-OMPA. ChE activity in adductor muscle of *A. noae* was significantly reduced by both diagnostic inhibitors. The effect of organophosphorous pesticide trichlorfon on ChE activity was investigated *in vitro* in both species as well as in the gills of mussels *Mytilus galloprovincialis*. The highest sensitivity of ChE to trichlorfon was observed in *A. noae* gills and adductor muscle (IC$_{50}$ 1.6×10$^{-7}$ M and 1.1×10$^{-7}$ M, respectively), followed by *M. galloprovincialis* gills (IC$_{50}$ 1.0×10$^{-6}$ M) and *V. verrucosa* gills and adductor muscle (IC$_{50}$ 1.7×10$^{-5}$ M and 0.9×10$^{-5}$ M, respectively). The results of this study suggest the potential of ChE activity measurement in the tissues of *A. noae* as effective biomarker of OP exposure in marine environment.

Keywords: *Arca noae*, biomarker, bivalves, cholinesterase activity, trichlorfon, *Venus verrucosa*
1. INTRODUCTION

Rapidly expanding anthropogenic activity along the coastal zones and the input of wide range of chemical compounds increase the sensitivity of marine ecosystem. Among them, organophosphorous pesticides (OPs) that are widely and extensively used primarily as a protection against pest and diseases, reach the coastal systems by run-offs and leaching from agricultural fields and during treatments of infestations in aquaculture. Although OPs rapidly degrade in the water column and biota, and are considered as low persistent chemicals, concern remains for non-target organisms particularly within coastal areas characterised by poor water circulation and during periods of intensive agricultural activity (Kirby et al., 2000; Solé et al., 2000). OPs are potentially harmful for aquatic organisms due to prolonged inhibitory effect on the cholinesterase (ChE) activity (Varó et al., 2008; Coelho et al., 2011; Valbonesi et al., 2011) and capability to induce other physiological disorders (Xuereb et al., 2009; Almeida et al., 2010). Besides, accidental exposure to pesticide of edible aquatic species may pose risk for human health (Valbonesi et al., 2011).

Mammalian ChEs represent a family of enzymes that hydrolyse choline esters, and they are typically divided in two major classes: acetylcholinesterase (AChE; EC 3.1.1.7) and butryrylcholinesterase (BChE; EC 3.1.1.8), according to substrate preference and sensitivity to specific inhibitors (Massoulié et al., 1993). AChE from mammalian tissues is characterised by the preference for acetylcholine (ASCh) hydrolysis, and it is strongly inhibited by 1,5-bis(4-allyldimethyl-ammonimphenyl)penta-3-one dibromide (BW284c51). The hydrolysis of neurotransmitter acetylcholine at cholinergic synapses of central and peripheral nervous tissue is the main physiological role of AChE. BChE preferentially hydrolyse butyrylcholine (BSCh) although it can hydrolyse other choline esters as well, and it is selectively inhibited by tetraisopropyl pyrophosphoramide (iso-OMPA). The role of BChE in the organism is still not ascertained, although it seems that it actually protects AChE by metabolism of anti-cholinesterase compounds (Masson and Lockridge, 2010). ChEs from the tissues of non-mammalian species are also categorised as AChE and BChE, although they often display overlapping substrate preference and less defined susceptibility to specific inhibitors of mammalian AChE and BChE (Jung et al., 2007). Considerable differences of ChE activity
were reported not only for species belonging to the same family or genus but also for different
tissues of the same organism (reviewed by Xuereb et al., 2007).

Fast and reliable detection of ChE inhibition as early biomarker of OP exposure has become
increasingly important for preservation of natural aquatic habitats and consumers safety
(Valbonesi et al, 2011; Sumith et al., 2012). Mussels (Mytilus sp.) are most commonly
employed bivalves for monitoring of exposure to OP compounds in marine environment
(Perić and Petrović, 2011). Besides, properties of ChEs and sensitivity to OPs have been
investigated in bivalves that have different lifestyle and habitats (Valbonesi et al., 2003;
Bonacci et al., 2008), to evaluate their potential as biomarker of exposure to OPs, particularly
within coastal areas not inhabited by mussels. Among them, clams Ruditapes philippinarum
and Cerastoderma glaucum were successfully employed in field research for assessment of
OP impact on environmental health (Matozzo et al., 2005; Jebali et al., 2011).

This research was focused on bivalves Arca noae and Venus verrucosa, two species abundant
and harvested from natural population for human consumption in Atlantic Ocean and
Mediterranean Sea (Nordsieck, 1969). The biology and ecology of these species has been
extensively studied in order to determine their potential for aquaculture production (Peharda
et al., 2013; Popović et al., 2013). Being widely distributed in the shallow near-shore waters,
wildlife populations of A. noae and V. verrucosa might become subjected to the impact of
common environmental contaminants, consequently affecting the aquatic food chain and
eventually human consumption. V. verrucosa in particular displayed the ability to
bioaccumulate compounds originating from anthropogenic or natural sources (Sakellari et al.,
2012) but only little data on the biological effect of these potential stressors are currently
available in the literature (Cotou et al., 2013).

Considering the economical importance and ecological relevance of A. noae and V. verrucosa,
it is important to study the potential biological alterations rapidly induced upon exposure to
OPs in aquatic environment. The primary aim of the present study was to investigate the
substrate preference, the main properties and the basal level of ChE activity in gills and
adductor muscle. The sensitivity in vitro of ChEs to OP trichlorfon (2,2,2-trichloro-1-
hydroxyethyl phosphonate), was also investigated, and compared to that in M. galloprovincialis, that seems to be sensitive to this particular compound (Yaqin and Hansen,
2010). Trichlorfon is widely applied for insects’ controls and treatment of fish ectoparasites in
aquaculture and was found to produce decrease of ChE activity, as well as immunological,
biochemical, physiological and histological alterations in the tissues of various aquatic organisms (Guimarães et al., 2007; Coelho et al., 2011). The obtained results provided the basic information on ChE activity measurement as indicator of adverse effect of OPs in the tissues of *A. noae* and *V. verrucosa*.

### 2. MATERIALS AND METHODS

#### 2.1 Chemicals

Acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh), propionylthiocholine iodide (PrSCh), eserine (physostigmin), BW254c51, *iso*-OMPA, trichlorfon (PESTANAL®, analytical standard, 97% purity, CAS-No 52-68-6), 5,5′-dithio-bis(-2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA) were purchased from Sigma-Aldrich. All other reagents were of analytical grade.

#### 2.2 Sample collection and tissue preparation

Specimens of *A. noae* and *V. verrucosa* of similar shell length (55-60 and 48-52 mm, respectively) and weight (20-25 and 35-42 g, respectively) were collected by SCUBA diving from shoreline sites away from known local sources of contamination, on west coast of istrian peninsula, north – eastern Adriatic, Croatia. The animals were immediately dissected, the tissues frozen in liquid nitrogen and stored at −80°C until analyses. The tissue was homogenised with a 1:4 (w/v) ratio of homogenisation buffer (0.1 Tris-HCl buffer pH 7.4) and centrifuged at 10000 g for 30 min at +4°C. The supernatant was collected and used for subsequent analysis. The sample homogenates were prepared from the pool of tissues extracted from five animals (five pools were used for analysis).

#### 2.3 Determination of enzyme activity

Cholinesterase activity in the tissue homogenates was determined according to the method of Ellman (1961) adapted to microtitrar plates. Briefly, the reaction solution (300 µL final volume) consisted of 50 µL of sample and DTNB solution (0.33 mM final). The enzymatic reaction was started by the addition of 50 µL of substrate (1mM final unless indicated...
otherwise), and the change in the absorbance as a result of TNB production ($\Delta A$/min) recorded at 405 nm for 10 minutes using microplate reader (Lab-systems, Multiscan Ascent® and Ascent Software TM, 2.4. version). Each sample was determined in triplicate. Spontaneous hydrolysis of substrate was determined in the absence of sample whereas the reaction of thiols with DTNB was determined in the absence of substrate. The enzymatic activity was expressed as nmol of hydrolysed substrate per min per mg of protein (specific activity). The protein concentration in the samples was determined by the method of Lowry (1951) using bovine serum albumin as standard. The samples used for analysis were normalised to 1 mg/ml protein concentration.

2.4 Substrate preference and in vitro effect of specific inhibitors on ChE activity

Acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh) and propionylthiocholine iodide (PrSCh) were used as substrates for determination of ChE activity in the gills and adductor muscle homogenate. Substrate concentration ranged from 0.005 to 5 mM.

The sensitivity of tissue ChE was examined by incubation of samples with eserine (total inhibitor of ChE), BW284c51 (selective inhibitor of AChE activity) and iso-OMPA (selective inhibitor of BChE activity). The appropriate volume of inhibitor solution was added to sample homogenate to achieve the final concentrations of $10^{-12}$-10$^{-4}$ M eserine, $10^{-9}$-10$^{-3}$ BW284c51, $10^{-8}$-10$^{-3}$ iso-OMPA and $10^{-9}$-10$^{-3}$ trichlorfon. Since stock solution of eserine was prepared in ethanol and iso-OMPA in methanol, the control samples containing only solvents were also included in the test at final concentrations of 0.1 % (ethanol) and 0.05% methanol (iso-OMPA). Stock solutions of BW284c51 and trichlorfon were prepared in ultra-pure water.

All incubation reaction were performed at 23°C, for 30 min, and remaining ChE activity was determined after addition of DTNB and substrate to 50 µL of sample (reaction mixture), as described in subsection 2.3.

2.5 Data analysis

All the results are presented as means ± S.D. (standard deviation). The IC$_{50}$ values were evaluated by probit analysis (Finney, 1971) using data on percentage of ChE inhibition relative to control, obtained for each concentration of inhibitors. Statistical comparison of
experimental data was performed by non parametric Kruskal-Wallis test. When significant, it was followed by the Mann-Whitney test. A value of p<0.05 was considered significant.

3. RESULTS

3.1 Substrate preference

In order to determine the substrate preference of ChE in the gills and adductor muscle of *A. noae* and *V. verrucosa*, three thiocholine esters were assayed: ASCh, BSCh and PrSCh.

The results show that gills and adductor muscle ChE of both species were capable of hydrolysing all three thiocholine esters, with the highest values obtained with PrSCh followed by ASCh, with exception of ChE from *V. verrucosa* gills that hydrolysed both substrates almost at the same rate (Fig. 1). Activity of ChE obtained with PrSCh was significantly higher than with ASCh in *A. noae* gills and *V. verrucosa* adductor muscle, for substrate concentration above 0.2 mM and 0.025 mM, respectively. For *A. noae* adductor muscle, significant difference between ChE activity with PrSCh and ASCh as substrate was found for concentrations of 0.2 and 0.5 mM. The highest ChE activity was achieved in the gills of *A. noae* (9.2 nmol min\(^{-1}\) mg prot\(^{-1}\)) and *V. verrucosa* adductor muscle (10.1 nmol min\(^{-1}\) mg prot\(^{-1}\)), with 0.5 and 1 mM PrSCh as substrate, respectively. The hydrolysis of BSCh was markedly lower than ASCh and PrSCh particularly in *A. noae* gills and *V. verrucosa* gills and adductor muscle. In these tissues, ChE activity exceeded 1 nmol min\(^{-1}\) mg prot\(^{-1}\) only at the highest substrate concentration (5 mM). Mild ChE activity with BSCh was detected in *A. noae* adductor muscle.

In general, the increase of ChE activity was most pronounced between 0.005 and 0.01 mM concentration of all three substrates, and the highest activities were achieved between 1 and 5 mM. As an exception, *A. noae* gills displayed asymmetric bell-shaped curve of ChE activity, with an apparent decrease at concentrations above 0.5 mM of either ASCh or PrSCh.

For subsequent inhibition experiments, ChE activity was determined using 1 mM substrate concentrations. Exceptionally, ChE activity in *A. noae* gills with ASCh and PrSCh was determined using 0.2 mM and 0.5 mM concentrations of substrates, respectively.
3.2 Effects of eserine, BW284c51 and iso-OMPA

Significant inhibition of ChE activity was detected at $10^{-9}$-$10^{-4}$ M and $10^{-6}$-$10^{-4}$ M concentration of eserine in *A. noae* and *V. verrucosa* tissues, respectively (Fig. 2). Almost complete inhibition (to below 10% of control) of ChE activity was detected at $10^{-4}$ M eserine. The values of IC$_{50}$ for ChE inhibition by eserine are given in Table 1, together with the literature data for other bivalve species. The IC$_{50}$ values were by three orders of magnitude lower for *A. noae* than for *V. verrucosa* tissues.

The ChE activity measured with all substrates was significantly inhibited with BW284c51 in a concentration dependent manner in *A. noae* and *V. verrucosa* gills and adductor muscle (Fig. 3). Residual ChE activity for ASCh and PrSCh at the highest concentration of inhibitor tested ($10^{-3}$ M) was below 10% of control, except in *V. verrucosa* gills (11% and 17% of control with ASCh and PrSCh, respectively). The values for residual ChE activity at highest concentration of inhibitor with BSCh as substrate were 24% and 10% (*A. noae* gills and adductor muscle, respectively) and 44% and 27% of control (*V. verrucosa* gills and adductor muscle, respectively). The IC$_{50}$ values for BW254c51 for *A. noae* gills were $2.2\times10^{-5}$ M (95% CI: 1.7 – $2.8\times10^{-5}$), $7.8\times10^{-5}$ M (95% CI: 1.8 – $13.8\times10^{-5}$), and $2.0\times10^{-5}$ M (95% CI: 1.4 – $2.6\times10^{-5}$) for ASCh, BSCh and PrSCh, respectively. For *A. noae* adductor muscle, the IC$_{50}$ values were $7.7\times10^{-6}$ M (95% CI: 6.0 – $9.5\times10^{-6}$), $1.6\times10^{-5}$ M (95% CI: 1.1 – $2.1\times10^{-5}$) and $1.7\times10^{-5}$ M (95% CI: 1.3 – $2.1\times10^{-5}$) for ASCh, BSCh and PrSCh, respectively. The IC$_{50}$ values for *V. verrucosa* gills were $2.3\times10^{-7}$ M (95% CI: 1.2 – $3.5\times10^{-7}$), and $2.2\times10^{-7}$ M (95% CI: 0.5 – $3.9\times10^{-7}$) for ASCh and PrSCh, respectively (IC$_{50}$ could not be determined with BSCh). For *V. verrucosa* adductor muscle the IC$_{50}$ values were $1.7\times10^{-7}$ M (95% CI: 1.0 – $2.3\times10^{-7}$), $3.2\times10^{-6}$ M (95% CI: 0.9 – $5.5\times10^{-6}$) and $1.7\times10^{-7}$ M (95% CI: 0.8 – $2.5\times10^{-7}$) for ASCh, BSCh and PrSCh, respectively. Significant effect of *iso-OMPA* was observed only in *A. noae* adductor muscle, with up to 60% (PrSCh and ASCh) and 80% (BSCh) reduction with respect to control (Fig. 4). The values of IC$_{50}$ for *iso-OMPA* were $1.5\times10^{-4}$ M (95% CI: 0.6 – $2.4\times10^{-4}$), $1.6\times10^{-4}$ M (95% CI: 0.8 – $2.4\times10^{-4}$) and $1.7\times10^{-4}$ M (95% CI: 0.3 – $1.8\times10^{-4}$), for ASCh, BSCh and PrSCh, respectively. In the gills of both bivalves, ChE activity with BSCh as substrate decreased for 15% in comparison to control at highest concentration of *iso-OMPA* ($10^{-3}$ M), but the effect was not significant.
3.3 In vitro exposure to trichlorfon

Since the highest ChE activity in both bivalve tissues was obtained with PrSCh, this substrate was used for further experiments with pesticide inhibition in vitro. For comparison purpose, the ChE activity was also measured in Mytilus galloprovincialis gills, with ASCh as substrate. The results presented in Fig 5. show a typical dose response curves, with significant inhibition of ChE activity starting at $10^{-7}$, $10^{-6}$ and $10^{-5}$ M trichlorfon for A. noae, M. galloprovincialis and V. verrucosa tissues, respectively. The IC$_{50}$ values for ChE inhibition by trichlorfon were lower for A. noae gills ($1.6 \times 10^{-7}$ M; 95% CI: 0.7 – $2.4 \times 10^{-7}$) and adductor muscle ($1.1 \times 10^{-7}$ M; 95% CI: 0.6 – $1.5 \times 10^{-7}$) than for V. verrucosa gills ($1.7 \times 10^{-5}$ M; 95% CI: $1.1 \times 10^{-5}$ – $2.2 \times 10^{-5}$) and adductor muscle ($0.9 \times 10^{-5}$ M; 95% CI: 0.6 – $1.2 \times 10^{-5}$) and M. galloprovincialis gills ($1.0 \times 10^{-6}$ M; 95% CI: 0.8 – $1.3 \times 10^{-6}$).

4. DISCUSSION

ChEs from invertebrate species typically display a variety of biochemical properties, tissue-specific distribution and activity. Substrate preference of ChE for ASCh is commonly found among invertebrate species, such as crustaceans (Xuereb et al., 2007), gastropods (Gagnaire et al., 2008) and bivalves (Lau and Wang, 2003; Valbonesi et al., 2003; Brown et al., 2004; Yaqin, 2010). In contrast, A. noae and V. verrucosa ChEs displayed preference for PrSCh over ASCh and particularly BSCh. Similar results were reported for the whole tissue homogenate of bivalves Corbicula fulminea (Mora et al., 1999) and Cerastoderma edule (Nilin et al., 2012) as well as for some annelid species (Rault et al., 2007; Hannam et al., 2008). Besides, ChE might show a tissue specific preference for either ASCh or PrSCh such as in two Anodonta species (Corsi et al., 2007), Adamussium colbecki (Romani et al., 2006) and Cerastoderma glaucum (Jebali et al., 2011).

In general, most prominent ChE activity has been almost regularly detected in the gills tissue, with the highest values reported for green mussel Perna viridis [137 nmol min$^{-1}$ mg prot$^{-1}$ (Yaqin, 2010)] and P. perna [above 200 nmol min$^{-1}$ mg prot$^{-1}$ (Lau and Wang, 2003)]. However, ChE activity in the tissues of bivalves most commonly range between 2-30 nmol
min⁻¹ mg prot⁻¹ (Mora et al., 1999; Valbonesi et al., 2003; Matozzo et al., 2005; Lehtonen et al., 2006; Corsi et al., 2007; Bonacci et al., 2008, Nilin et al., 2012). In this study, ChE activity in A. noae gills reached values slightly below 10 nmol min⁻¹ mg prot⁻¹. ChE activity was particularly low in V. verrucosa gills, displaying values below those reported by Cotou et al (2013) for samples along the Greek coast. The observed variations in the level of ChE activity among bivalves possibly reflect differences in the structure of gills’ cilia that are responsible for filtration and feeding. In fact, Corsi et al (2007) recently inferred that higher ChE activity in the gills of Mytilus sp. with respect to Anodonta sp. could be related to more developed ciliary epithelium of mussels. On the other hand, A. noae and V. verrucosa exhibit different lifestyle and behaviour, the former being attached to the hard-bottom surface and the latter burrowed in soft sediment. Thus, markedly higher ChE activity level in the gills of A. noae with respect to V. verrucosa could be explained by different routes and extent of exposure of these two bivalves to bioavailable pollutants (Lehtonen et al., 2006).

Opening and closure of bivalve shell valves by contraction and relaxation of adductor muscle is under neural control (Funabara et al., 2005). Nevertheless, the ChE activities in bivalves’ adductor muscle vary considerably, from below 2 nmol min⁻¹ mg prot⁻¹ in Mytilus (Mora et al., 1999; Brown et al., 2004) to above 50 nmol min⁻¹ mg prot⁻¹ found in cockle Cerastoderma edule (Nilin et al., 2012) and invasive and indigenous species of unionid clams Anodonta (Corsi et al., 2007). In the latter study, it was suggested that elevated ChE activity found in adductor muscle and other tissues of invasive Anodonta wodiana could be important for its adaptation capability in the new environment. Levels of adductor muscle ChE activity in A. noae and V. verrucosa that were observed in the current study with ASCh and particularly PrSCh as substrate, were either within the range or notably higher with respect to those recorded in the gills, thus underlying further the importance of adductor muscle ChE activity of these two bivalves for regulation of valve function (Corsi et al., 2007).

To evaluate the contribution of non-specific esterases, the crude tissue homogenate was incubated with eserine, a general inhibitor of ChE activity. Significant ChE inhibition by eserine found in gills and adductor muscle of A. noae and V. verrucosa, was also reported previously for gills of M. galloprovincialis (Valbonesi et al., 2003; Brown et al., 2004) and european oysters Ostrea edulis (Valbonesi et al., 2003) and suggest only negligible contribution of non-specific esterase activity.
The sensitivity *in vitro* of ChE to eserine could be used to anticipate general sensitivity to OPs and the utility of an organism as indicator of exposure to OP in environment (Tortelli et al., 2006; Gagnaire et al., 2008). When taking into account the IC$_{50}$ values reported for other bivalve species (Table 1), the sensitivity to eserine could be ranked in decreasing order: *A. noae* > *M. galloprovincialis* > *O. edulis* > *Perna perna* > *V. verrucosa* > *Crassostrea rhizophorae*. Although it is necessary to consider variations in sample processing and measurement conditions between different laboratories, these results suggest the highest sensitivity of *A. noae* and rather low sensitivity of *V. verrucosa* ChEs to inhibition by eserine in comparison to other bivalve species. In general, a wide range of IC$_{50}$ values for *in vitro* inhibition of ChE is regularly observed, preventing the determination of the most and least sensitive animal taxa. However, the IC$_{50}$ of eserine obtained for *A. noae* tissues is among the lowest values observed so far for freshwater, marine and estuarine vertebrate and invertebrate species (Xuereb et al., 2007; Gagnaire et al., 2008; Valbonesi et al., 2011). Thus, *A. noae* could be regarded as highly susceptible to OPs.

ChE activity investigated among fish and invertebrate species has been very often attributed to AChE (Rault et al., 2007; Varó et al., 2008; Yaqin, 2010; Valbonesi et al., 2011) as distinguished by preferential hydrolysis of ASCh over BSCh and PrSCh, BW284c51 sensitivity and *iso*-OMPA resistance that represent typical features of “true” mammalian AChE (Massoulié et al., 1993).

The results of current investigation indicate the presence of ChE form in the gills of *A. noae* and gills and adductor muscle of *V. verrucosa*, sensitive to BW284c51 and resistant to *iso*-OMPA, but displaying preference for PrSCh over ASCh and BSCh. Thus, it could be classified as an intermediate PChE/AChE-like form, similar to that found in snail *Potamopyrgus antipodarum* (Gagnaire et al., 2008). In these tissues, weak activity was recorded with BSCh as substrate, but it is not likely than it might be related to a mammalian-like BChE form, considering its discernible sensitivity to BW284c51, and only weak and insignificant inhibition by *iso*-OMPA.

ChE in adductor muscle of *A. noae* displayed preference for PrSCh over ASCh, but the hydrolysing activity was sensitive to both diagnostic inhibitors nearly at the same rate. Interestingly, mild ChE activity was also noticeable on BSCh as substrate, and furthermore, it was sensitive to *iso*-OMPA. However, this ChE form shows little similarity with mammalian BChE, since the activity with BSCh was sensitive also to BW284c51.
In this work, ChE activity in the tissues of *A. noae* and *V. verrucosa* was analysed following *in vitro* exposure to trichlorfon, an OP compound widely used in aquaculture, and a well-known inhibitor of ChE activity in fish and invertebrates (Guimarães et al., 2007; Feng et al., 2008; Coelho et al., 2011). The sensitivity to trichlorfon of ChE in *A. noae* gills and adductor muscle was by one order of magnitude higher than in gills of *M. galloprovincialis*, previously shown to be significantly reduced upon *in vivo* treatment with this compound (Yaqin and Hansen, 2010). Besides, ChE activity in the tissues of *V. verrucosa* was considerably less reduced than in the other two bivalves. This reflects higher susceptibility to trichlorfon of ChEs from gills and adductor muscle of *A. noae* than from gills of *M. galloprovincialis* and in particular gills and adductor muscle of *V. verrucosa*.

5. CONCLUSION

At this point, it could be hypothesised that the conspicuous inhibitory effect of trichlorfon on ChE that coincides with the inhibition caused by eserine indicates high susceptibility of *A. noae* to anti-ChE compounds. Conversely, lower sensitivity of ChE in *V. verrucosa* tissues to both trichlorfon and eserine, indicate generally higher resistance of enzyme to ChE-inhibiting chemicals. Consequently, the measurement of ChE activity in the tissues of *A. noae* has the potential as very sensitive biomarker of OP exposure whereas *V. verrucosa* seems to be less useful for this purpose. However, due to variation of physicochemical properties and half-life that was well illustrated in the literature (Kopecka-Pilarczyk, 2010), other commonly used OPs should be examined for their ChE-inhibiting capacity, to provide sufficient information on the suitability of *A. noae* as bioindicator of OP contamination. Besides, since intake routes, bioaccumulation, biotransformation and elimination capacity modulate the OP toxicity (Xuereb et al., 2007; Hannam et al., 2008), it is necessary to perform *in vivo* exposure experiments as a prerequisite for more realistic picture of the OPs effect on ChE activity. In addition, future studies should be focused on the influence of biotic and abiotic factors, to support the utility of ChE activity measurement in natural populations of *A. noae* as concomitant early warning signal of OP contamination in marine environment.

References:


Table 1.
Comparison of IC50 of eserine for in vitro inhibition of ChE from bivalve species

<table>
<thead>
<tr>
<th>Species</th>
<th>tissue</th>
<th>IC50 (M) *</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arca noae</td>
<td>gills</td>
<td>1.6×10^{-9} (1.3-1.9×10^{-9})</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td>adductor muscle</td>
<td>1.3×10^{-9} (0.7-1.8×10^{-9})</td>
<td>this work</td>
</tr>
<tr>
<td>Mytilus galloprovincialis</td>
<td>gills</td>
<td>2.1×10^{-8}</td>
<td>Valbonesi et al., 2003</td>
</tr>
<tr>
<td>Ostrea edulis</td>
<td>gills</td>
<td>1.0×10^{-7}</td>
<td>Valbonesi et al., 2003</td>
</tr>
<tr>
<td>Perna perna</td>
<td>gills</td>
<td>4.6×10^{-6} (4.0-5.1×10^{-6})*</td>
<td>Monserrat et al., 2002</td>
</tr>
<tr>
<td>Venus verrucosa</td>
<td>gills</td>
<td>2.1×10^{-6} (1.1-3.1×10^{-6})</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td>adductor muscle</td>
<td>1.1×10^{-6} (0.8-1.5×10^{-6})</td>
<td>this work</td>
</tr>
<tr>
<td>Crassostrea rhizophorae</td>
<td>gills</td>
<td>1.0×10^{-6} (0.6-1.2×10^{-6})*</td>
<td>Monserrat et al., 2002</td>
</tr>
</tbody>
</table>

IC50 – Concentration of eserine that inhibits 50% of ChE activity after 30 min of in vitro incubation

(*) - duration of incubation reaction was not reported
Figure captions:

Fig. 1. ChE activity in gills and adductor muscle from *Arca noae* and *Venus verrucosa* with ASCh (○), BSCh (△) and PrSCh (□) as substrates. Results represent the mean of five pooled samples (N=5). Values for ChE activity labeled with the same lowercase letters are not significantly different (p>0.05).

Fig. 2. ChE inhibition in *Arca noae* gills (○) and adductor muscle (□) and *Venus verrucosa* gills (△) and adductor muscle (◊) by eserine with ASCh as substrate. Results represent the mean ± S.D. of five pooled samples (N=5). Control values of ChE activity were 4.8 ± 1.2 nmol min⁻¹ mg prot⁻¹ for *A. noae* gills, 5.3 ± 1.5 nmol min⁻¹ mg prot⁻¹ for *A. noae* adductor muscle, 2.5 ± 0.3 nmol min⁻¹ mg prot⁻¹ for *V. verrucosa* gills and 6.8 ± 0.4 nmol min⁻¹ mg prot⁻¹ for *V. verrucosa* adductor muscle. Filled symbols indicate inhibitor concentrations at which the remaining activity was significantly different from the respective control (p<0.05).

Fig. 3. ChE inhibition in gills and adductor muscle of *Arca noae* and *Venus verrucosa* by BW284c51, with ASCh (○), BSCh (△) and PCTC (□) as substrate. Control values for ChE activity were 5.6 ± 1.4, 0.8 ± 0.3 and 7.7 ± 1.7 nmol min⁻¹ mg prot⁻¹ for *A. noae* gills, 5.1 ± 1.2, 1.9 ± 0.3 and 7.2 ± 2.2 nmol min⁻¹ mg prot⁻¹ for *A. noae* adductor muscle, 2.4 ± 0.5, 0.3 ± 0.1 and 1.8 ± 0.4 nmol min⁻¹ mg prot⁻¹ for *V. verrucosa* gills and 6.9 ± 1.6, 0.8 ± 0.3 and 8.2 ± 2.4 nmol min⁻¹ mg prot⁻¹ for *V. verrucosa* adductor muscle, with ASCh, BSCh and PrSCh as substrate, respectively. Results represent the mean ± S.D. of five pooled samples (N=5). Filled symbols indicate inhibitor concentration at which the remaining activity was significantly different from the respective control (p<0.05).

Fig. 4. ChE inhibition in gills and adductor muscle of *Arca noae* and *Venus verrucosa* by iso-OMPA, with ASCh (○), BSCh (△) and PrSCh (□) as substrate. Control values for ChE activity were 5.7 ± 1.8, 0.4 ± 0.1 and 8.7 ± 1.3 nmol min⁻¹ mg prot⁻¹ for *A. noae* gills, 4.9 ± 0.9, 3 ± 0.9 and 6.4 ± 2.5 nmol min⁻¹ mg prot⁻¹ for *A. noae* adductor muscle, 2.3 ± 0.3, 0.4 ± 0.1 and 1.9 ± 0.2 nmol min⁻¹ mg prot⁻¹ for *V. verrucosa* gills, 5.9 ± 1.4, 1 ± 0.4 and 7.6 ± 1.4 nmol min⁻¹ mg prot⁻¹ for *V. verrucosa* adductor muscle, with ASCh, BSCh and PrSCh as substrate, respectively. Results represent the mean ± S.D. of five pooled samples (N=5). Filled symbols indicate inhibitor concentration at which the remaining activity was significantly different from the respective control (p<0.05).

Fig. 5. ChE inhibition in *Arca noae* gills (○) and adductor muscle (□), *Venus verrucosa* gills (△) and adductor (◊) and *Mytilus galloprovincialis* gills (◇) by trichlorfon. Control values of ChE activity were 7.8 ± 1.5 nmol min⁻¹ mg prot⁻¹ (*A. noae* gills), 6.8 ± 1 nmol min⁻¹ mg prot⁻¹ (*A. noae* adductor muscle), 2.1 ± 0.4 nmol min⁻¹ mg prot⁻¹ (*V. verrucosa* gills), 9.4 ± 3.2 nmol min⁻¹ mg prot⁻¹ (*V. verrucosa* adductor muscle) and 13.3 ± 3.3 nmol min⁻¹ mg prot⁻¹ (*M. galloprovincialis* gills). Results represent the mean ± S.D. of five pooled samples (N=5). Filled symbols indicate inhibitor concentrations at which the remaining activity was significantly different from the respective control (p<0.05).
Arca noae

Venus verrucosa

substrate (mM)

ChE activity (nmol min⁻¹ mg protein⁻¹)
Fig 2
Arca noae

Venus verrucosa

log (BW284c51) M
Fig 4