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

3 **Characterisation and *in vitro* sensitivity to organophosphorous pesticide trichlorfon**

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20 **ABSTRACT**

21

22 Cholinesterase (ChE) activity was investigated in gills and adductor muscle of two bivalve
23 species: *Arca noae* and *Venus verrucosa*. The properties of ChEs were investigated using
24 acetylcholine iodide (ASCh), butyrylcholine iodide (BSCh) and propionylcholin iodide
25 (PrSCh) as substrates and eserine, BW254c51 and *iso*-OMPA as specific inhibitors. The
26 highest level of ChE activity in crude tissue extracts was detected with PrSCh followed by
27 ASCh, while values obtained with BSCh were apparently low, except in *A. noae* adductor
28 muscle. The enzyme activity in *A. noae* gills and *V. verrucosa* gills and adductor muscle was
29 significantly inhibited by BW254c51, but not with *iso*-OMPA. ChE activity in adductor
30 muscle of *A. noae* was significantly reduced by both diagnostic inhibitors. The effect of
31 organophosphorous pesticide trichlorfon on ChE activity was investigated *in vitro* in both
32 species as well as in the gills of mussels *Mytilus galloprovincialis*. The highest sensitivity of
33 ChE to trichlorfon was observed in *A. noae* gills and adductor muscle (IC_{50} 1.6×10^{-7} M and
34 1.1×10^{-7} M, respectively), followed by *M. galloprovincialis* gills (IC_{50} 1.0×10^{-6} M) and *V.*
35 *verrucosa* gills and adductor muscle (IC_{50} 1.7×10^{-5} M and $0.9\text{x}10^{-5}$ M, respectively). The
36 results of this study suggest the potential of ChE activity measurement in the tissues of *A.*
37 *noae* as effective biomarker of OP exposure in marine environment.

38

39 **Keywords:** *Arca noae*, biomarker, bivalves, cholinesterase activity, trichlorfon, *Venus*
40 *verrucosa*

41

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 butyrylcholinesterase (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 metabolisation 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

73 were reported not only for species belonging to the same family or genus but also for different
74 tissues of the same organism (reviewed by Xuereb et al., 2007).

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

85 This research was focused on bivalves *Arca noae* and *Venus verrucosa*, two species abundant
86 and harvested from natural population for human consumption in Atlantic Ocean and
87 Mediterranean Sea (Nordsieck, 1969). The biology and ecology of these species has been
88 extensively studied in order to determine their potential for aquaculture production (Peharda
89 et al., 2013; Popović et al., 2013). Being widely distributed in the shallow near-shore waters,
90 wildlife populations of *A. noae* and *V. verrucosa* might become subjected to the impact of
91 common environmental contaminants, consequently affecting the aquatic food chain and
92 eventually human consumption. *V. verrucosa* in particular displayed the ability to
93 bioaccumulate compounds originating from anthropogenic or natural sources (Sakellari et al.,
94 2012) but only little data on the biological effect of these potential stressors are currently
95 available in the literature (Cotou et al., 2013).

96 Considering the economical importance and ecological relevance of *A. noae* and *V. verrucosa*,
97 it is important to study the potential biological alterations rapidly induced upon exposure to
98 OPs in aquatic environment. The primary aim of the present study was to investigate the
99 substrate preference, the main properties and the basal level of ChE activity in gills and
100 adductor muscle. The sensitivity *in vitro* of ChEs to OP trichlorfon (2,2,2-trichloro-1-
101 hydroxyethyl phosphonate), was also investigated, and compared to that in *M.*
102 *galloprovincialis*, that seems to be sensitive to this particular compound (Yaqin and Hansen,
103 2010). Trichlorfon is widely applied for insects' controls and treatment of fish ectoparasites in
104 aquaculture and was found to produce decrease of ChE activity, as well as immunological,

105 biochemical, physiological and histological alterations in the tissues of various aquatic
106 organisms (Guimarães et al., 2007; Coelho et al., 2011). The obtained results provided the
107 basic information on ChE activity measurement as indicator of adverse effect of OPs in the
108 tissues of *A. noae* and *V. verrucosa*.

109

110 2. MATERIALS AND METHODS

111 *2.1 Chemicals*

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

117

118 *2.2 Sample collection and tissue preparation*

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

128

129 *2.3 Determination of enzyme activity*

130 Cholinesterase activity in the tissue homogenates was determined according to the method of
131 Ellman (1961) adapted to microtitar plates. Briefly, the reaction solution (300 µL final
132 volume) consisted of 50 µL of sample and DTNB solution (0.33 mM final). The enzymatic
133 reaction was started by the addition of 50 µL of substrate (1mM final unless indicated

134 otherwise), and the change in the absorbance as a result of TNB production (ΔA/min)
135 recorded at 405 nm for 10 minutes using microplate reader (Lab-systems, Multiscan Ascent®
136 and Ascent Software TM, 2.4. version). Each sample was determined in triplicate.
137 Spontaneous hydrolysis of substrate was determined in the absence of sample whereas the
138 reaction of thiols with DTNB was determined in the absence of substrate. The enzymatic
139 activity was expressed as nmol of hydrolysed substrate per min per mg of protein (specific
140 activity). The protein concentration in the samples was determined by the method of Lowry
141 (1951) using bovine serum albumin as standard. The samples used for analysis were
142 normalised to 1 mg/ml protein concentration.

143

144 *2.4 Substrate preference and in vitro effect of specific inhibitors on ChE activity*

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

148 The sensitivity of tissue ChE was examined by incubation of samples with eserine (total
149 inhibitor of ChE), BW284c51 (selective inhibitor of AChE activity) and *iso*-OMPA (selective
150 inhibitor of BChE activity). The appropriate volume of inhibitor solution was added to sample
151 homogenate to achieve the final concentrations of 10^{-12}-10^{-4} M eserine, 10^{-9}-10^{-3} BW284c51,
152 10^{-8}-10^{-3} *iso*-OMPA and 10^{-9}-10^{-3} trichlorfon. Since stock solution of eserine was prepared in
153 ethanol and *iso*-OMPA in methanol, the control samples containing only solvents were also
154 included in the test at final concentrations of 0.1 % (ethanol) and 0.05% methanol (*iso*-
155 OMPA). Stock solutions of BW284c51 and trichlorfon were prepared in ultra-pure water.

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

159

160 *2.5 Data analysis*

161 All the results are presented as means ± S.D. (standard deviation). The IC_{50} values were
162 evaluated by probit analysis (Finney, 1971) using data on percentage of ChE inhibition
163 relative to control, obtained for each concentration of inhibitors. Statistical comparison of

164 experimental data was performed by non parametric Kruskal-Wallis test. When significant, it
165 was followed by the Mann-Whitney test. A value of $p<0.05$ was considered significant.

166

167 **3. RESULTS**

168

169 *3.1 Substrate preference*

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

172 The results show that gills and adductor muscle ChE of both species were capable of
173 hydrolysing all three thiocholine esters, with the highest values obtained with PrSCh followed
174 by ASCh, with exception of ChE from *V. verrucosa* gills that hydrolysed both substrates
175 almost at the same rate (Fig. 1). Activity of ChE obtained with PrSCh was significantly higher
176 than with ASCh in *A. noae* gills and *V. verrucosa* adductor muscle, for substrate concentration
177 above 0.2 mM and 0.025 mM, respectively. For *A. noae* adductor muscle, significant
178 difference between ChE activity with PrSCh and ASCh as substrate was found for
179 concentrations of 0.2 and 0.5 mM. The highest ChE activity was achieved in the gills of *A.*
180 *noae* (9.2 nmol min^{-1} mg $prot^{-1}$) and *V. verrucosa* adductor muscle (10.1 nmol min^{-1} mg $prot^{-}$
181 ^{1}), with 0.5 and 1 mM PrSCh as substrate, respectively. The hydrolysis of BSCh was
182 markedly lower than ASCh and PrSCh particularly in *A. noae* gills and *V. verrucosa* gills and
183 adductor muscle. In these tissues, ChE activity exceeded 1 nmol min^{-1} mg $prot^{-1}$ only at the
184 highest substrate concentration (5 mM). Mild ChE activity with BSCh was detected in *A.*
185 *noae* adductor muscle.

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

190 For subsequent inhibition experiments, ChE activity was determined using 1 mM substrate
191 concentrations. Exceptionally, ChE activity in *A. noae* gills with ASCh and PrSCh was
192 determined using 0.2 mM and 0.5 mM concentrations of substrates, respectively.

193

194 *3.2 Effects of eserine, BW284c51 and iso-OMPA*

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

201 The ChE activity measured with all substrates was significantly inhibited with BW284c51 in a
202 concentration dependent manner in *A. noae* and *V. verrucosa* gills and adductor muscle (Fig.
203 3). Residual ChE activity for ASCh and PrSCh at the highest concentration of inhibitor tested
204 (10^{-3} M) was below 10% of control, except in *V.verrucosa* gills (11% and 17% of control with
205 ASCh and PrSCh, respectively). The values for residual ChE activity at highest concentration
206 of inhibitor with BSCh as substrate were 24% and 10% (*A. noae* gills and adductor muscle,
207 respectively) and 44% and 27% of control (*V. verrucosa* gills and adductor muscle,
208 respectively). The IC_{50} values for BW254c51 for *A. noae* gills were 2.2×10^{-5} M (95% CI: 1.7
209 – 2.8×10^{-5}), 7.8×10^{-5} M (95% CI: 1.8 – 13.8×10^{-5}), and 2.0×10^{-5} M (95% CI: 1.4 – 2.6×10^{-5})
210 for ASCh, BSCh and PrSCh, respectively. For *A. noae* adductor muscle, the IC_{50} values were
211 7.7×10^{-6} M (95% CI: 6.0 – 9.5×10^{-6}), 1.6×10^{-5} M (95% CI: 1.1 – 2.1×10^{-5}) and 1.7×10^{-5} M
212 (95% CI: 1.3 – 2.1×10^{-5}) for ASCh, BSCh and PrSCh, respectively. The IC_{50} values for *V.*
213 *verrucosa* gills were 2.3×10^{-7} M (95% CI: 1.2 – 3.5×10^{-7}), and 2.2×10^{-7} M (95% CI: 0.5 –
214 3.9×10^{-7}) for ASCh and PrSCh, respectively (IC_{50} could not be determined with BSCh). For
215 *V. verrucosa* adductor muscle the IC_{50} values were 1.7×10^{-7} M (95% CI: 1.0 – 2.3×10^{-7}),
216 3.2×10^{-6} M (95% CI: 0.9 – 5.5×10^{-6}) and 1.7×10^{-7} M (95% CI: 0.8 – 2.5×10^{-7}) for ASCh,
217 BSCh and PrSCh, respectively. Significant effect of *iso*-OMPA was observed only in *A. noae*
218 adductor muscle, with up to 60% (PrSCh and ASCh) and 80% (BSCh) reduction with respect
219 to control (Fig. 4). The values of IC_{50} for *iso*-OMPA were 1.5×10^{-4} M (95% CI: 0.6 – $2.4\times10^{-}$
220 ^{4}), 1.6×10^{-4} M (95% CI: 0.8 – 2.4×10^{-4}) and 1.7×10^{-4} M (95% CI: 0.3 – 1.8×10^{-4}), for ASCh,
221 BSCh and PRSCh, respectively. In the gills of both bivalves, ChE activity with BSCh as
222 substrate decreased for 15% in comparison to control at highest concentration of *iso*-OMPA
223 (10^{-3} M), but the effect was not significant.

224

225 *3.3 In vitro exposure to trichlorfon*

226 Since the highest ChE activity in both bivalve tissues was obtained with PrSCh, this substrate
227 was used for further experiments with pesticide inhibition *in vitro*. For comparison purpose,
228 the ChE activity was also measured in *Mytilus galloprovincialis* gills, with ASCh as substrate.
229 The results presented in Fig 5. show a typical dose response curves, with significant inhibition
230 of ChE activity starting at 10^{-7}, 10^{-6} and 10^{-5} M trichlorfon for *A. noae*, *M. galloprovincialis*
231 and *V. verrucosa* tissues, respectively. The IC_{50} values for ChE inhibition by trichlorfon were
232 lower for *A. noae* gills (1.6×10^{-7} M; 95% CI: 0.7 – 2.4×10^{-7}) and adductor muscle (1.1×10^{-7}
233 M; 95% CI: 0.6 – 1.5×10^{-7}) than for *V. verrucosa* gills (1.7×10^{-5} M; 95% CI: 1.1×10^{-5} –
234 2.2×10^{-5}) and adductor muscle (0.9×10^{-5} M; 95% CI: 0.6 – 1.2×10^{-5}) and *M. galloprovincialis*
235 gills (1.0×10^{-6} M; 95% CI: 0.8 – 1.3×10^{-6}).

236

237 **4. DISCUSSION**

238

239 ChEs from invertebrate species typically display a variety of biochemical properties, tissue-
240 specific distribution and activity. Substrate preference of ChE for ASCh is commonly found
241 among invertebrate species, such as crustaceans (Xuereb et al., 2007), gastropods (Gagnaire et
242 al., 2008) and bivalves (Lau and Wang, 2003; Valbonesi et al., 2003; Brown et al., 2004;
243 Yaqin, 2010). In contrast, *A. noae* and *V. verrucosa* ChEs displayed preference for PrSCh
244 over ASCh and particularly BSCh. Similar results were reported for the whole tissue
245 homogenate of bivalves *Corbicula fulminea* (Mora et al., 1999) and *Cerastoderma edule*
246 (Nilin et al., 2012) as well as for some annelid species (Rault et al., 2007; Hannam et al.,
247 2008). Besides, ChE might show a tissue specific preference for either ASCh or PrSCh such
248 as in two *Anodonta* species (Corsi et al., 2007), *Adamussium colbecki* (Romani et al., 2006)
249 and *Cerastoderma glaucum* (Jebali et al., 2011).

250 In general, most prominent ChE activity has been almost regularly detected in the gills tissue,
251 with the highest values reported for green mussel *Perna viridis* [137 nmol min^{-1} mg $prot^{-1}$
252 (Yaqin, 2010)] and *P. perna* [above 200 nmol min^{-1} mg $prot^{-1}$ (Lau and Wang, 2003)].
253 However, ChE activity in the tissues of bivalves most commonly range between 2-30 nmol

254 min^{-1} mg prot^{-1} (Mora et al., 1999; Valbonesi et al., 2003; Matozzo et al., 2005; Lehtonen et
255 al., 2006; Corsi et al., 2007; Bonacci et al., 2008, Nilin et al., 2012). In this study, ChE
256 activity in *A. noae* gills reached values slightly below 10 nmol min^{-1} mg prot^{-1}. ChE activity
257 was was particularly low in *V. verrucosa* gills, displaying values below those reported by
258 Cotou et al (2013) for samples along the Greek coast. The observed variations in the level of
259 ChE activity among bivalves possibly reflect differences in the structure of gills' cilia that are
260 responsible for filtration and feeding. In fact, Corsi et al (2007) recently inferred that higher
261 ChE activity in the gills of *Mytilus sp*. with respect to *Anodonta sp*. could be related to more
262 developed ciliary epithelium of mussels. On the other hand, *A. noae* and *V. verrucosa* exhibit
263 different lifestyle and behaviour, the former being attached to the hard-bottom surface and the
264 latter burrowed in soft sediment. Thus, markedly higher ChE activity level in the gills of *A.*
265 *noae* with respect to *V. verrucosa* could be explained by different routes and extent of
266 exposure of these two bivalves to bioavailable pollutants (Lehtonnen et al., 2006).

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

279 To evaluate the contribution of non-specific esterases, the crude tissue homogenate was
280 incubated with eserine, a general inhibitor of ChE activity. Significant ChE inhibition by
281 eserine found in gills and adductor muscle of *A. noae* and *V. verrucosa*, was also reported
282 previously for gills of *M. galloprovincialis* (Valbonesi et al., 2003; Brown et al., 2004) and
283 european oysters *Ostrea edulis* (Valbonesi et al., 2003) and suggest only negligible
284 contribution of non-specific esterase activity.

285 The sensitivity *in vitro* of ChE to eserine could be used to anticipate general sensitivity to OPs
286 and the utility of an organism as indicator of exposure to OP in environment (Tortelli et al.,
287 2006; Gagnaire et al., 2008). When taking into account the IC_{50} values reported for other
288 bivalve species (Table 1), the sensitivity to eserine could be ranked in decreasing order: *A.*
289 *noae* > *M. galloprovincialis* > *O. edulis* > *Perna perna* > *V. verrucosa* > *Crassostrea*
290 *rhizophorae*. Although it is necessary to consider variations in sample processing and
291 measurement conditions between different laboratories, these results suggest the highest
292 sensitivity of *A. noae* and rather low sensitivity of *V. verrucosa* ChEs to inhibition by eserine
293 in comparison to other bivalve species. In general, a wide range of IC_{50} values for *in vitro*
294 inhibition of ChE is regularly observed, preventing the determination of the most and least
295 sensitive animal taxa. However, the IC_{50} of eserine obtained for *A. noae* tissues is among the
296 lowest values observed so far for freshwater, marine and estuarine vertebrate and invertebrate
297 species (Xuereb et al., 2007; Gagnaire et al., 2008; Valbonesi et al., 2011). Thus, *A. noae*
298 could be regarded as highly susceptible to OPs.

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

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

312 ChE in adductor muscle of *A. noae* displayed preference for PrSCh over ASCh, but the
313 hydrolysing activity was sensitive to both diagnostic inhibitors nearly at the same rate.
314 Interestingly, mild ChE activity was also noticeable on BSCh as substrate, and furthermore, it
315 was sensitive to *iso*-OMPA. However, this ChE form shows little similarity with mammalian
316 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.

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

Species	tissue	IC_{50} (M) *	Reference
Arca noae	gills	1.6×10^{-9} (1.3-1.9×10^{-9})	this work
	adductor muscle	$1.3\times10^{-9*}$(0.7-1.8×10^{-9})	this work
Mytilus galloprovincialis	gills	2.1×10^{-8}	Valbonesi et al., 2003
Ostrea edulis	gills	$1.0\times10^{-7*}$	Valbonesi et al., 2003
Perna perna	gills	4.6×10^{-6} (4.0-5.1×10^{-6})*	Monserrat et al., 2002
Venus verrucosa	gills	2.1×10^{-6} (1.1-3.1×10^{-6})	this work
	adductor muscle	1.1×10^{-6} (0.8-1.5×10^{-6})	this work
Crassostrea rhizophorae	gills	1.0×10^{-6} (0.6-1.2×10^{-6})*	Monserrat et al., 2002

IC_{50} – Concentration of eserine that inhibits 50% of ChE activity after 30 min of *in vitro* incubation

(*) - duration of incubation reaction was not reported

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478 Figure captions:

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

483 Fig 2. ChE inhibition in *Arca noae* gills (○) and adductor muscle (□) and *Venus verrucosa* gills (Δ)
484 and adductor muscle (◊) by eserine with ASCh as substrate. Results represent the mean ± S.D. of five
485 pooled samples (N=5). Control values of ChE activity were 4.8 ± 1.2 nmol min^{-1} mg $prot^{-1}$ for *A. noae*
486 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.*
487 *verrucosa* gills and 6.8 ± 0.4 nmol min^{-1} mg $prot^{-1}$ for *V. verrucosa* adductor muscle. Filled symbols
488 indicate inhibitor concentrations at which the remaining activity was significantly different from the
489 respective control ($p<0.05$).

490 Fig. 3. ChE inhibition in gills and adductor muscle of *Arca noae* and *Venus verrucosa* by BW284c51,
491 with ASCh (○) BSCh (Δ) and PCTC (□) as substrate. Control values for ChE activity were 5.6 ± 1.4,
492 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
493 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
494 *V. verrucosa* gills, 6.9 ± 1.6, 0.8 ± 0.3 and 8.2 ± 2.4 nmol min^{-1} mg $prot^{-1}$ for *V. verrucosa* adductor
495 muscle, with ASCh, BSCh and PrSCh as substrate, respectively. Results represent the mean ± S.D. of
496 five pooled samples (N=5). Filled symbols indicate inhibitor concentration at which the remaining
497 activity was significantly different from the respective control ($p<0.05$).

498 Fig. 4. ChE inhibition in gills and adductor muscle of *Arca noae* and *Venus verrucosa* by *iso*-OMPA,
499 with ASCh (○), BSCh (Δ) and PrSCh (□) as substrate. Control values for ChE activity were 5.7 ± 1.8,
500 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
501 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
502 *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
503 muscle, with ASCh, BSCh and PrSCh as substrate, respectively. Results represent the mean ± S.D. of
504 five pooled samples (N=5). Filled symbols indicate inhibitor concentration at which the remaining
505 activity was significantly different from the respective control ($p<0.05$).

506 Fig. 5. ChE inhibition in *Arca noae* gills (○) and adductor muscle (□), *Venus verrucosa* gills (Δ) and
507 aductor (◊) and *Mytilus galloprovincialis* gills (▽) by trichlorfon. Control values of ChE activity were
508 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),
509 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*
510 adductor muscle) and 13.3 ± 3.3 nmol min^{-1} mg $prot^{-1}$ (*M. galloprovincialis* gills). Results represent
511 the mean ± S.D. of five pooled samples (N=5). Filled symbols indicate inhibitor concentrations at
512 which the remaining activity was significantly different from the respective control ($p<0.05$).

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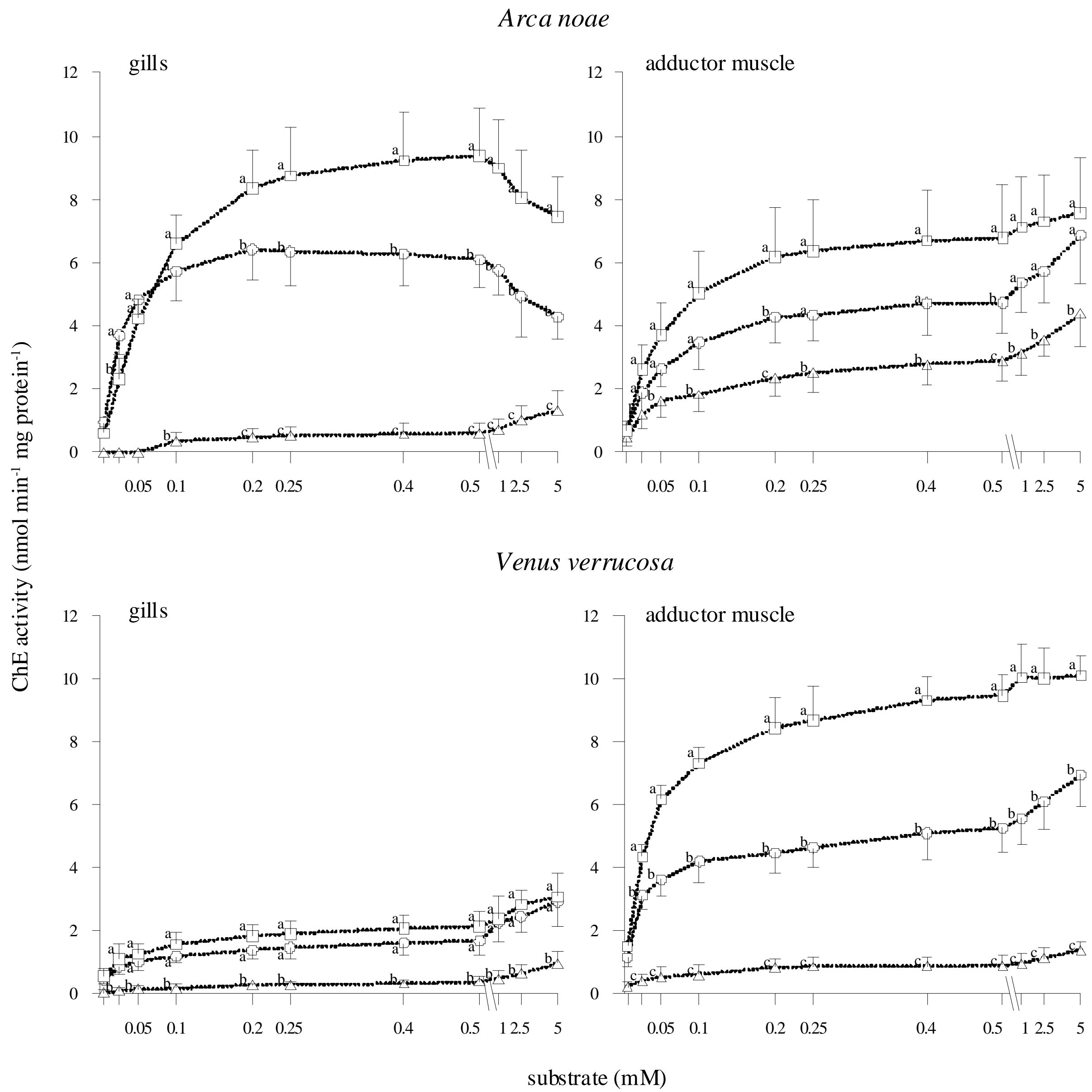

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Fig 1

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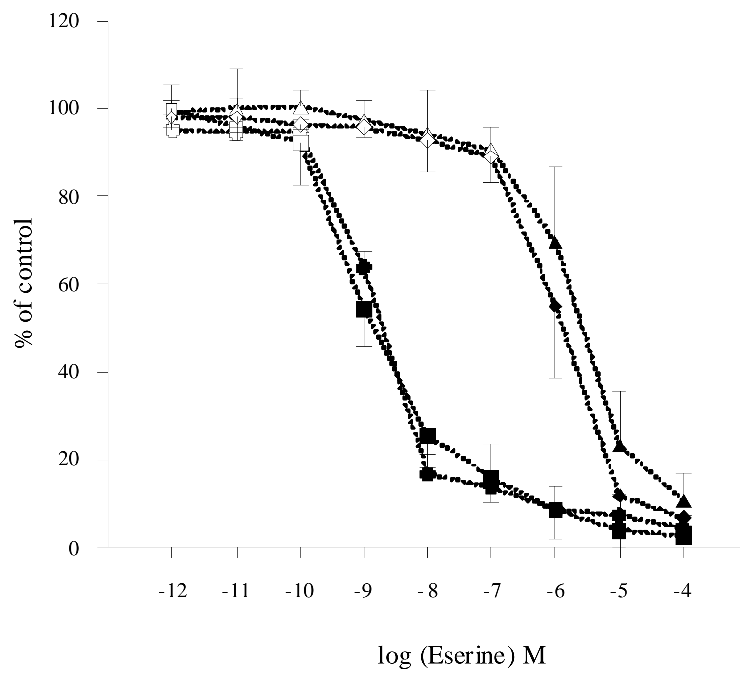

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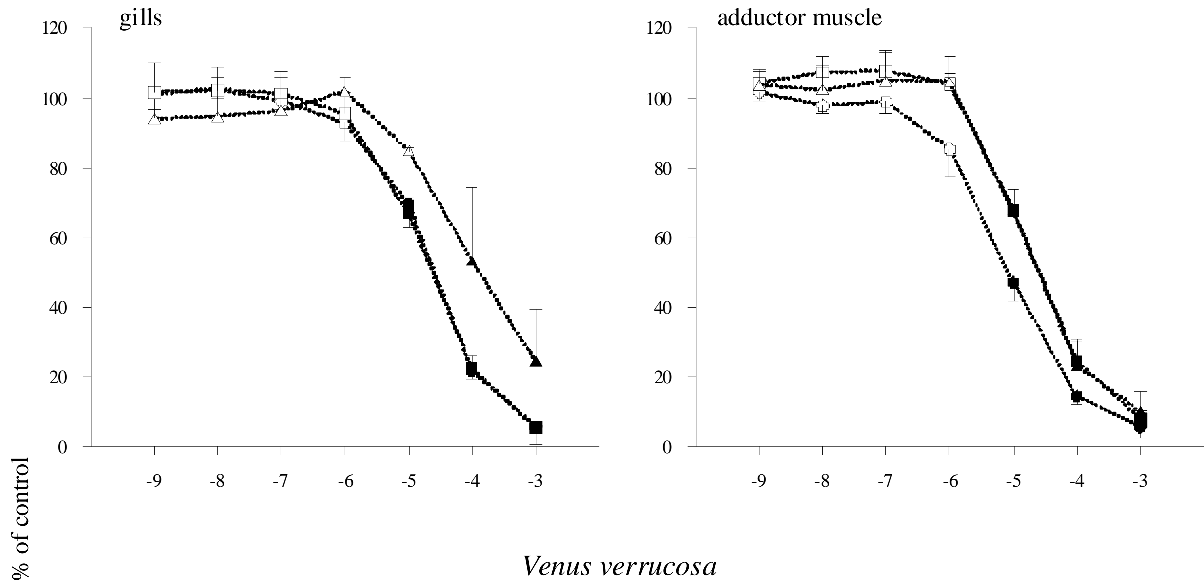

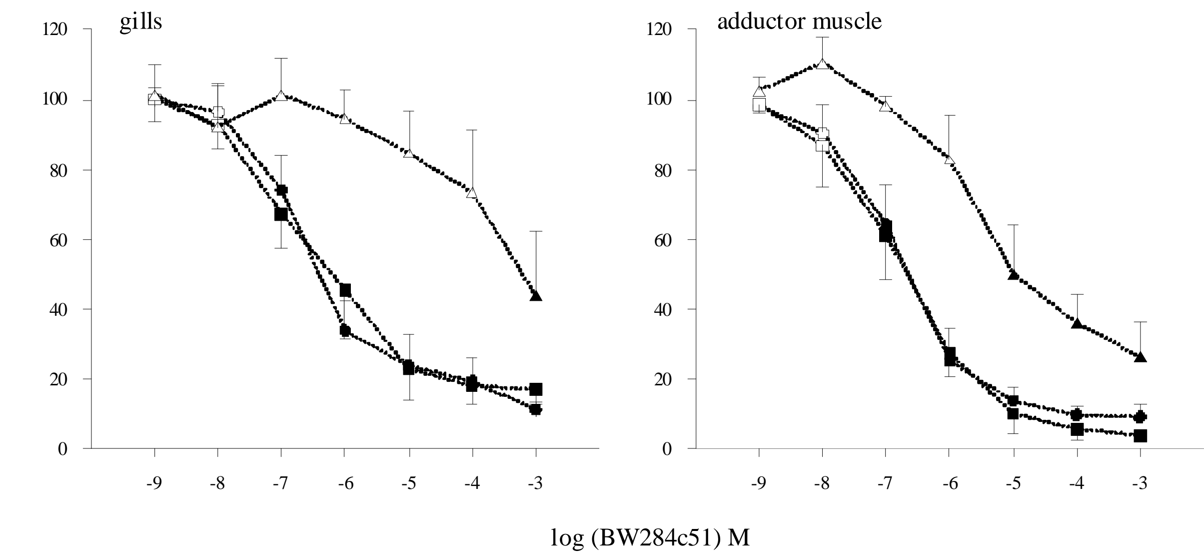

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Fig 3

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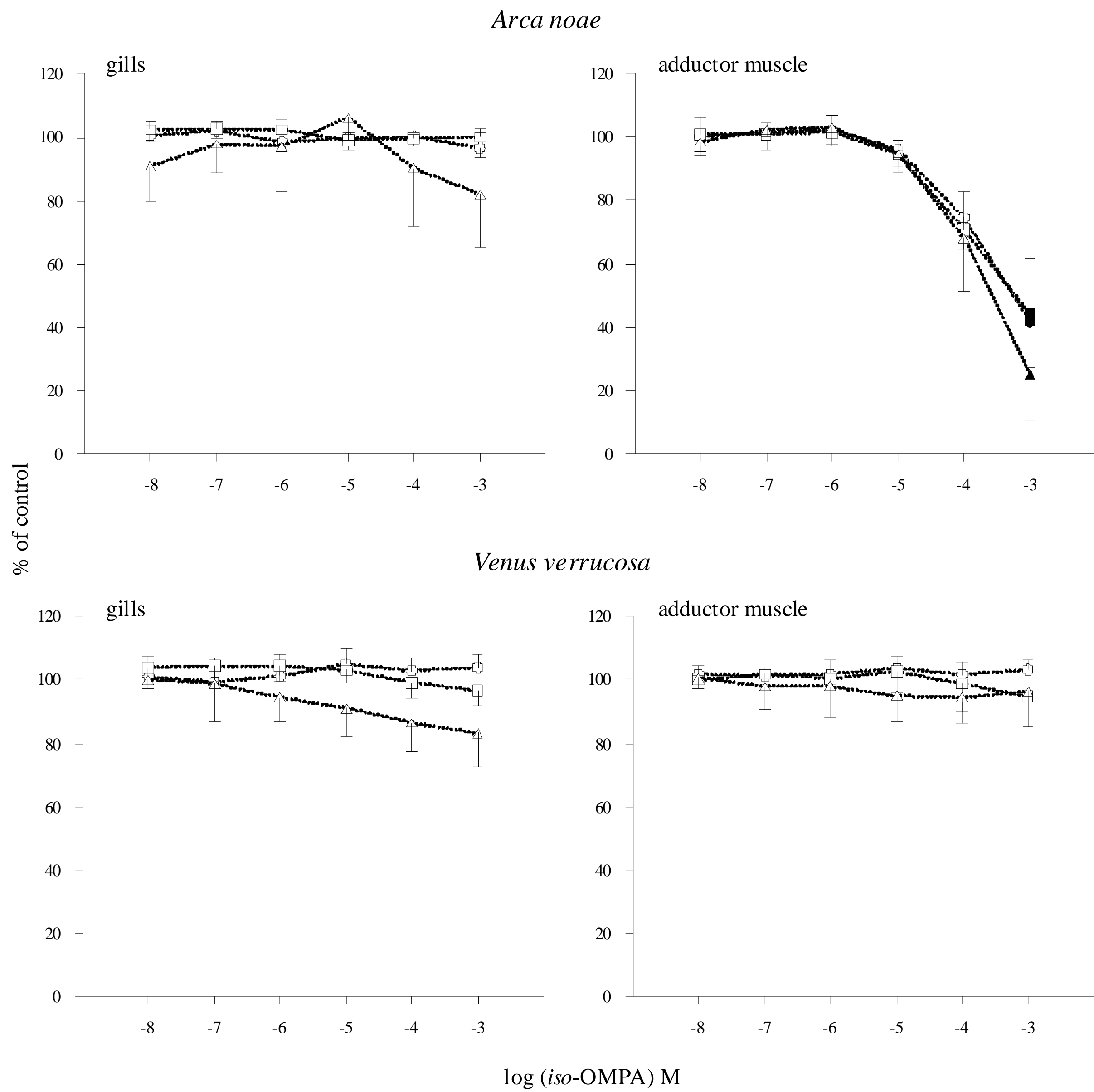

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563 Fig 4

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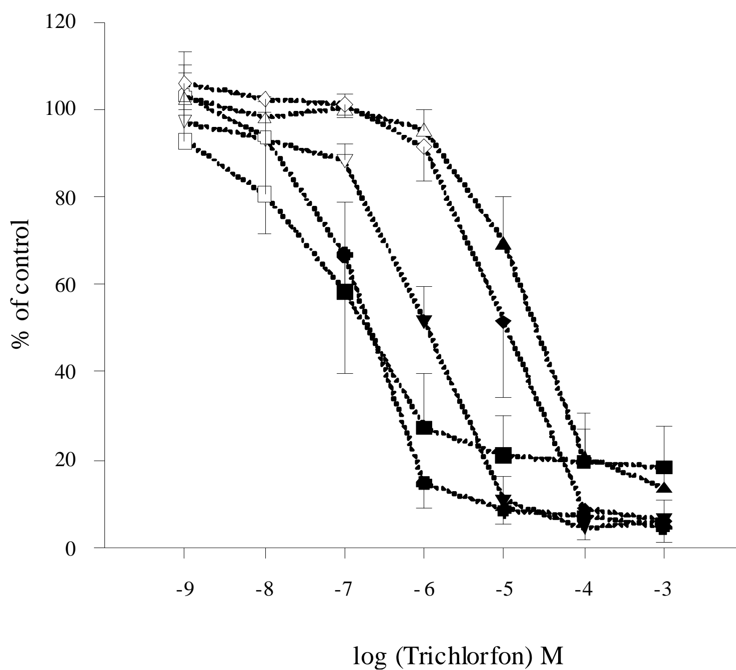

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583 Fig 5

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