ELSEVIER

Aquatic Toxicology 61 (2002) 169–180

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers

Tamara S. Galloway *, Nicholas Millward, Mark A. Browne, Michael H. Depledge

Plymouth Environmental Research Centre, University of Plymouth, 6, Queen Anne Terrace, Drake Circus, Plymouth PL4 8AA, UK

Received 19 October 2001; received in revised form 29 March 2002; accepted 1 April 2002

Abstract

Carboxylesterases in bivalve molluscs exhibit greater sensitivity to organophosphorous and carbamate pesticides than acetylcholinesterase and are present at higher levels. The aim of the present study was to combine measurement of both acetylcholinesterase and carboxylesterase activities in the marine bivalve *Mytilus edulis* in order to detect the effects of pesticide exposure. Spectrophotometric assays in microtitreplate format were optimised for use with *M. edulis* haemolymph and tissue homogenate samples. This permitted the nature and distribution of the enzymes to be determined. One predominant pharmacological form of activity consistent in its patterns of activation and inhibition with acetylcholinesterase was identified in the haemolymph with an apparent K_m for acetylthiocholineiodide of 1.33 mM. Carboxylesterase activity in the tissues was characterised by its preferential hydrolysis of the substrate analogue phenylthioacetate. Concentration-dependent inhibition of both activities was demonstrated following in vitro incubation with diisopropylfluorophosphate (DFP), paraoxon and eserine in the range 0.1–3.0 mM. When *M. edulis* ($n = 10$) were exposed for 24 h to concentrations of eserine or paraoxon of 0.05–1.0 mM, the percentage inhibition of acetylcholinesterase was in each case greater than for carboxylesterase and reached statistical significance at lower concentrations. In all exposures, a proportion of carboxylesterase activity was present which remained resistant to inhibition by either organophosphorous or carbamate compounds. The ecotoxicological significance of these findings for the environmental monitoring of pesticide exposure is discussed.

Keywords: Acetylcholinesterase; Carboxylesterase; *Mytilus edulis*; Pesticide; Biomarker; Ecotoxicology

1. Introduction

Organophosphorous and carbamate compounds are used extensively as pesticides in urban, agricultural and aquacultural situations. Despite their rapid hydrolysis in air, water and soil, bioac-

* Corresponding author. Tel.: +44-1752-232-904; fax: +44-1752-232-970

E-mail address: tgalloway@plmouth.ac.uk (T.S. Galloway).

0166-445X/02/$ - see front matter
PII: S0166-445X(02)00051-6

cumulation of organophosphorous compounds in sediments has been documented at appreciable levels in estuaries and coastal locations, representing a potential threat to biota (Readman et al., 1992). Organophosphorous and carbamate pesticides exert acute toxicity by inhibiting acetylcholinesterase (E.C 3.1.1.7), a serine hydrolase found in neuromuscular junctions. This leads to susceptible species in accumulation of the neurotransmitter acetylcholine and subsequent hyperpolarisation of the post synaptic membrane. Measurement of peripheral acetylcholinesterase activity levels is a useful, dose dependent means of monitoring exposure to organophosphorous and carbamate compounds in humans and other vertebrate species (Carlock et al., 1999), yet extrapolation of this approach to encompass aquatic invertebrate species that are commonly used in the environmental monitoring of coastal locations, has been problematic (Fulton and Key, 2001). Acetylcholinesterases exhibit genetic and molecular polymorphism and their distributions and physiological roles differ among species (Massoullie et al., 1993; Scaps et al., 1996; Forget and Bocquene, 1999). As a consequence, the degree of inhibition associated with toxicity is highly variable (Lundebye et al., 1997). Baseline data is often lacking, or has revealed large variations both within and between populations (Rattner and Fairbrother, 1991). Activity may be modulated by seasonal and nutritional variables (Edwards and Fisher, 1991; Escartin and Porte, 1997) or by chronic toxicant exposures which can lead, in turn, to elevated levels of activity (Thompson et al., 1991).

In coastal marine environments, the choice of bivalve molluscs such as the blue mussel, *Mytilus edulis* as sentinel species for the ecotoxicological assessment of pollution has many advantages. These organisms are sessile filter feeders, able to accumulate a wide range of contaminants and reflect changes in the contaminant status of the environment. *M. edulis* is found extensively in littoral and shallow temperate waters as large beds in both open waters and brackish estuaries where agricultural runoff is at its highest. Although the occurrence of cholinesterase activity in molluscan haemolymph was first reported many years ago (Von Scramlik, 1941), there have been relatively few published studies of the use of acetylcholinesterase activity as a biomarker of organophosphorous pesticide exposure in bivalves (Najimi et al., 1997; Escartin and Porte, 1997; Radenac et al., 1998; Basack et al., 1998; Narbonne et al., 2001). This reflects low endogenous activity (Najimi et al., 1997; Narbonne et al., 2001) and the relative insensitivity of inhibition by organophosphorous compounds compared with enzymes from other species (Von Wachtendonk and Neef, 1979; Mora et al., 1999).

In addition to their toxic action on acetylcholinesterase, organophosphorous and carbamate compounds may also inhibit other classes of serine hydrolase including carboxylesterases [EC 3.1.1.1] which are typically present in all tissues. Carboxylesterases hydrolyse a wide range of endogenous and exogenous esters (Parkinson, 1996). Their tissue distribution and exact physiological role is often unknown and varies among species but they are believed to be important in the hydrolytic detoxication of some organophosphorous compounds and play an additional protective role as alternative sites of organophosphate binding and phosphorylation (Jokanovic et al., 1996; Walker, 2001). Carboxylesterase activity in bivalve molluscs exhibits higher sensitivity to organophosphorous compounds than acetylcholinesterases (Escartin and Porte, 1997) and its measurable activity is greater (Basack et al., 1998). It is therefore, possible that the combined monitoring of carboxylesterase and acetylcholinesterase activities may provide a more useful indication of organophosphorous pesticide exposure in bivalve species than the measurement of acetylcholinesterase activity alone (Escartin and Porte, 1997; Basack et al., 1998).

To use enzyme activities such as acetylcholinesterase and carboxylesterase as accurate and sensitive biomarkers of water contamination, it is important not only that the enzymes are adequately characterised in the species in question, but also that the assays themselves are sufficiently robust and accessible to permit their use to make measurements of activity of adequate accuracy and precision to detect subtle changes in activity. With this aim, parallel modifications to

the method of Ellman et al. (1961) using substrate analogues specific for either cholinesterase or carboxylesterase activities have been adapted for use in *M. edulis* tissues and body fluids. The distribution and characteristics of acetylcholinesterase and carboxylesterase activities were then determined and the sensitivity of the enzymes to inhibition both in vitro and in vivo to organophosphorous and carbamate compounds was evaluated. The utility of combining these assays as rapid and reliable means of monitoring exposure to pesticides is discussed.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, butyrylthiocholine iodide, propoinylthiocholine iodide, phenylthioacetate, 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB), *tetra*(monoisopropyl)pyrophosphor-tetramide (*iso*-OMPA), physostigmine (eserine), diisopropylfluorophosphate (DFP), diethyl *p*-nitrophenyl phosphate (paraoxon) and phenylmethylsulphonylfluoride (PMSF) were from Sigma Chemical Co., Poole, Dorset. All other reagents and solvents were analytical grade.

2.2. Collection of organisms

Specimens of *M. edulis* of shell length 5–7 cm were collected monthly between May and September from Whitsand Bay, Cornwall and housed in filtered sea water in the aquarium in 10 l glass tanks at $14 = / - 2C$ with a 12-h light:12-h dark cycle. Samples of haemolymph were withdrawn by syringe from the posterior adductor muscle and transferred to small plastic centrifuge tubes on ice. Haemolymph was centrifuged at $5000 \times g$ for 3 min and the supernatant used for subsequent analysis. Tissue homogenate was prepared by excising the whole body from the shell, placing it on filter paper to drain excess fluid, and recording the weight. The tissue was homogenised with a 1:5 ratio (w/v) of 0.1 M Tris–HCl buffer pH 7.2 containing 0.25 M sucrose, centrifuged at $1000 \times g$ and the pellet containing cellular debris discarded. The supernatant fraction was re-centrifuged at $10\,000 \times g$ and the supernatant fraction retained and used for subsequent analyses.

2.3. Enzyme activity

Enzyme activities were assayed at 25 °C in microtitreplate format using modifications to the method of Ellman et al. (1961) in which thiocholine or thioacetate derivatives are hydrolysed by acetylcholineterase or carboxylesterase respectively to yield thiocholine or thioacetate. Subsequent combination with DTNB forms the yellow anion 5-thio-2-nitrobenzoic acid which absorbs strongly at 412 nm. Initial experiments revealed the optimum rate of hydrolysis to occur between pH 7 and 8 and assays were subsequently conducted at pH 7.4. The sample or buffer blank (50 μl) was incubated for 5 min at 25 °C with 150 μl DTNB, 270 μM in 50 mM sodium phosphate, pH 7.4. Measurement of acetylcholinesterase activity was initiated by the addition of acetylthiocholine, 3 mM, and the absorbance recorded at 412 nm. Carboxylesterase activity was assayed in a similar manner with a reduced sample size of 25 μl and with the substitution of phenylthioacetate, 800μM as substrate analogue. Spontaneous substrate hydrolysis was determined in the absence of haemolymph. The results were calculated as nmol substrate hydrolysed min^{-1} mg^{-1} relative to the total protein content of the sample. Total protein was determined using a commercial kit (BioRad) with bovine serum albumin as standard.

2.4. Exposure to pesticides in vitro

Samples were incubated in the presence of the pesticides or specific inhibitors (indicated in the text) for 15 min at 25 °C prior to the addition of substrate. Stock solutions were prepared at 100 mM in acetone and diluted to the desired concentrations with dH_2O. A vehicle control was included for each experimental run and the rate of spontaneous hydrolysis calculated as before. The 50% inhibition concentration (IC50) for both acetylcholinesterase and carboxylesterase activity was determined by the addition of at least five

different concentrations of inhibitors selected to yield between 10 and 90% inhibition. The enzyme activity was determined and the percent inhibition calculated and compared with the control.

2.5. Exposure to pesticides in vivo

The extent of inhibition of enzyme activity was assessed following in vivo exposure of test organisms for 24 h groups of mussels ($n = 10$) were exposed to test solutions of paraoxon, DFP, eserine or solvent control (acetone) in sealed, aerated glass aquaria. The final concentration of solvent did not exceed 0.05% (v/v). Samples were prepared and assayed as described.

2.6. Statistical analysis

All determinations were carried out in triplicate and the results presented as means ± standard deviation (S.D.). Statistical analyses were performed using EXCEL software to determine the one way analysis of variance and assess statistical significance (ANOVA, Student's t-test, Kruskal–Wallis). Differences between means were considered significant for $P < 0.05$. The Michaelis constant was calculated using the graphic method of Eadie–Hoftsee.

3. Results

3.1. Distribution and substrate preference of enzyme activity

Initial experiments sought to determine the distribution of acetylcholinesterase and carboxylesterase activities in preparations of haemolymph or whole body tissue homogenate. Using an initial adaptation of the method of Lundebye et al. (1997), the baseline level of acetylcholinesterase in haemolymph was confirmed to be higher than in the tissues ($P \leq 0.001$) whilst the reverse was true for carboxylesterase ($P \leq 0.001$), (Fig. 1). Subsequently, acetylcholinesterase activity was measured only in haemolymph whilst carboxylesterase activity was measured only in tissue homogenate.

The limits of detection of the enzyme activities were calculated by measuring the reaction rate for ten replicates of an activity equivalent to zero (buffer blank). The limits of detection were determined to be three S.D. from the mean of this rate which gave a sensitivity of detection of 1.02 nmol min^{-1} mg^{-1} for acetylthiocholine hydrolysis and 0.39 nmol min^{-1} mg^{-1} for phenylthioacetate hydrolysis. The reproducibility of the method was assessed using ten separate measurements of a control sample. For the acetylthiocholine hydroly-

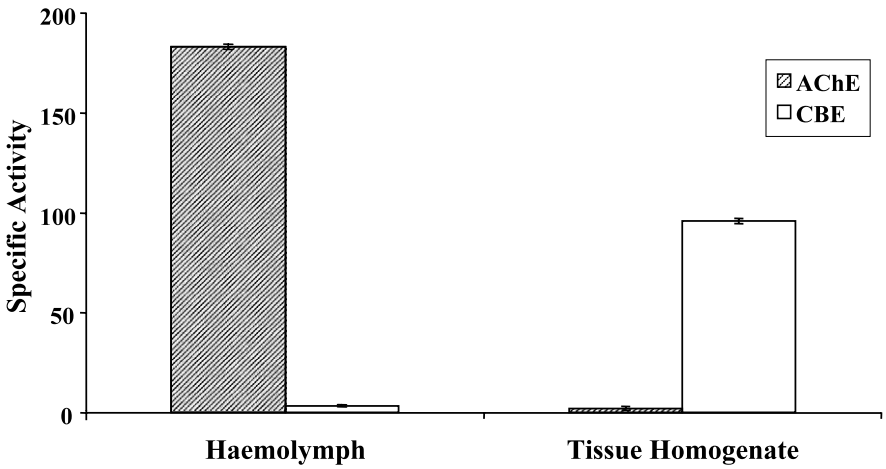

Fig. 1. Acetylcholinesterase and carboxylesterase specific activities in the haemolymph and tissues of *M. edulis*. All values are the mean ± standard error of the mean (SEM) of ten individual mussels each assayed in triplicate and presented as nmol min^{-1} mg^{-1}.

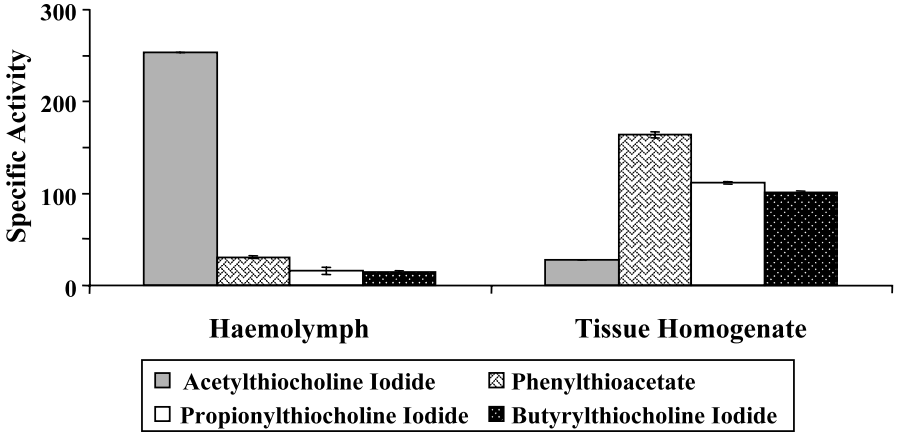

Fig. 2. Substrate preference of haemolymph and tissue homogenate preparations. Activity was determined in the presence of concentrations of individual substrates in the range 0.01–3.0 mM and the maximum rate recorded. Values are the mean ± SEM of triplicate determinations and are expressed as nmol min^{-1} mg^{-1}.

sis, the mean control value was 183 ± 15.2 nmol min^{-1} mg^{-1} representing a coefficient of variation of 8.3%. For phenylthioacetate hydrolysis, the mean value was 95.8 ± 14.5 nmol min^{-1} mg^{-1} representing a coefficient of variation of 15.1%.

The substrate preference of the haemolymph preparation was determined by comparing rates of reaction with concentrations in the range 0.1–3 mM of the substrate analogues acetylthiocholine iodide, butyrylthiocholine iodide, propionylthiocholine iodide and phenylthioacetate, each of which may be preferentially hydrolysed by different classes of esterase (Fig. 2). Acetylthiocholine iodide was the preferred substrate for the haemolymph samples ($P \leq 0.001$). The hydrolysis of the alternative substrate analogues was much lower, indicating that acetylcholinesterase activity was the predominant activity in the haemolymph. Tissue homogenate showed a preferential ability to hydrolyse phenylthioacetate ($P \leq 0.001$) with propionyl, butyryl and acetyl thiocholine analogues hydrolysed in decreasing order of preference.

Incubation of tissue homogenate with 3 mM *iso*-OMPA, a potent inhibitor of butyrylcholinesterase but not of acetylcholinesterase (Barahona and Sanchez-Forun, 1999) inhibited the enzyme activity by 77 ± 1.61%. Incubation with 3 mM PMSF, a general inhibitor of enzyme activities containing active site serine residues, reduced the level of activity to negligible levels as did denaturing the enzymes by boiling the samples for 2 min. The hydrolysis of the substrate analogues was therefore, entirely due to the action of serine dependent enzymes and not spontaneous hydrolysis.

3.2. Characterisation of acetylcholinesterase activity

The concentration of acetylthiocholine iodide was varied to give concentrations in the range 0.05–12.0 mM and the basic protocol followed as described (Fig. 3). The K_m value calculated using the Eadie–Hofstee equation was 1.3 mM. The maximum rate of hydrolysis, V_{max}, was achieved at 3 mM, and this concentration was used for subsequent analysis in the knowledge that it was not rate limiting over the 15 min time period chosen. A distinguishing characteristic of acetylcholinesterase enzymes is the occurrence of substrate inhibition at high concentrations and this phenomenon can be seen clearly in Fig. 3a. A series of dilutions of both haemolymph and tissue homogenate were made in buffer to confirm linear kinetics. Reaction rates were directly proportional to enzyme concentration across the range of dilu-

tions tested confirming that Michaelis Menten kinetics were supported in this range (Fig. 3b). The sample volume subsequently chosen typically yielded reaction rates for individual mussels in the upper third of this range, allowing for the accurate detection of inhibition. The presence of the detergent Triton X 100 did not influence the measurable activity indicating that in haemolymph the enzyme was globular rather than membrane bound (data not shown).

3.3. Characterisation of carboxylesterase activity

The concentration of phenylthioacetate was varied to give concentrations in the range 40–800 μM and the basic protocol followed as described (Fig. 3c). An apparent K_m value of 200 μM was estimated for the measured activity. The maximum rate of hydrolysis, V_{max}, was achieved at 800 μM, and this concentration was used for subsequent analysis. At higher concentrations, a precipitate formed on addition of the sample, making confirmation of substrate inhibition at higher doses impractical. A series of dilutions of tissue homogenate were made in assay buffer to confirm linear kinetics (Fig. 3d). Reaction rates were directly proportional to enzyme concentration across the range of dilutions tested confirming that Michaelis Menten kinetics were supported in this range. A sample volume of 25 μl was chosen for subsequent analysis which typically gave activity values for individual mussels in the upper ranges of the assay yet was low enough to prevent precipitation with phenylthioacetate.

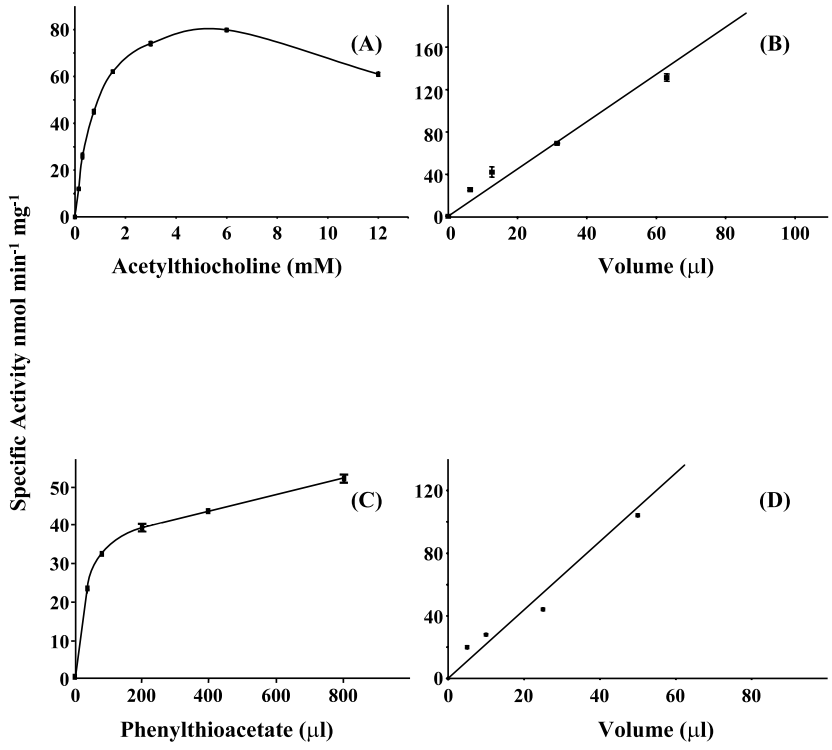

Fig. 3. Characterisation of acetylcholinesterase activity in *M. edulis* haemolymph: (a) the relationship between substrate (acetylthiocholine iodide) concentration and reaction rate; (b) the relationship between sample volume and reaction rate. Characterisation of carboxylesterase activity in *M. edulis* tissue homogenate; (c) the relationship between substrate (phenylthioacetate) concentration and reaction rate; (d) the relationship between sample volume and reaction rate.

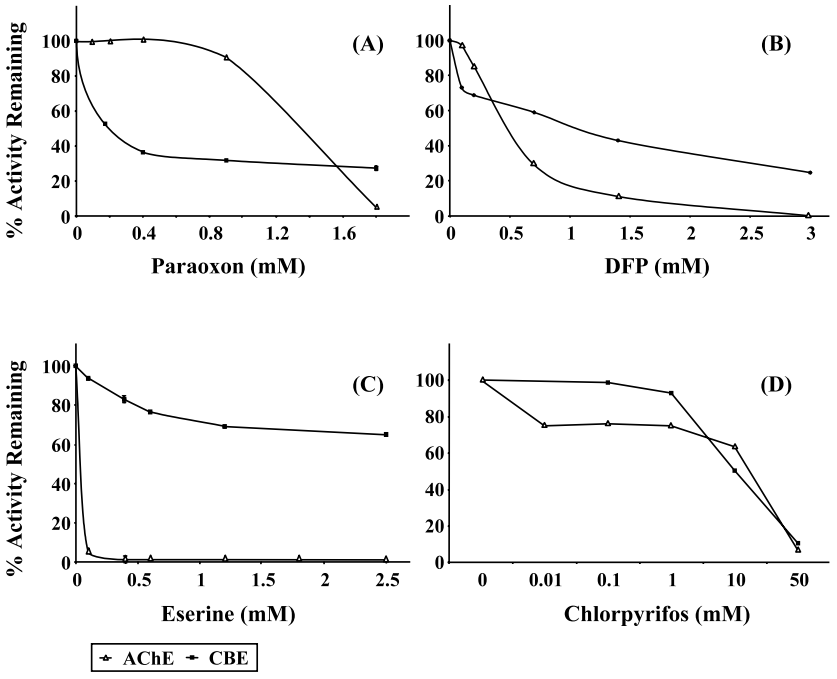

Fig. 4. The effect of organophosphorous and carbamate compounds on esterase activities in *M. edulis*. Acetylcholinesterase activity was determined in haemolymph and carboxylesterase in whole body tissue homogenate. Values are the mean ± SEM of triplicate determinations. Results are expressed as a percent of the vehicle control (unexposed) (a) activity; (b) eserine; (c) paraoxon; (d) DFP; (e) Chlorpyrifos.

3.4. Inhibition of enzyme activity

The sensitivity of the enzyme activity in haemolymph and tissue homogenate to inhibition by organophosphorous and carbamate compounds was initially investigated in vitro. The selected compounds were the carbamate eserine, a potent inhibitor of cholinesterases, and the organophosphorous compounds DFP and paraoxon. These compounds were chosen as they do not require metabolic activation prior to use. Chlorpyrifos, a phosphorothioate, was also included. In Fig. 4 the inhibition curves obtained for both acetylcholinesterase and carboxylesterase activities are shown. The IC50 values calculated from these graphs are presented in Table 1. For each compound tested, a proportion of the apparent carboxylesterase activity remained insensitive to inhibition by each of the inhibitors applied. The inhibition concentration has been calculated after subtraction of this pesticide resistant activity.

3.5. In vivo exposure to cholinesterase inhibitors

The effects of eserine, paraoxon and DFP were then determined in vivo. Initially, *M. edulis* ($n = 10$) were exposed to a single sublethal dose of 1 mM of each compound over 24 h and the acetylcholinesterase and carboxylesterase activities measured as described (Fig. 5). The degree of

Table 1
Inhibition of enzyme activities by DFP, eserine, paraoxon and chlorpyrifos following in vitro exposure

Compound	Acetylcholinesterase (mM)	Carboxylesterase (mM)
Eserine	<0.01	Not determined*
Paraoxon	1.83	0.4
DFP	0.82	1.1
Chlorpyrifos	15.0	10.1

Results are expressed as IC50 values and represent the mean of three separate triplicate determinations. *, ≥2.5 mM.

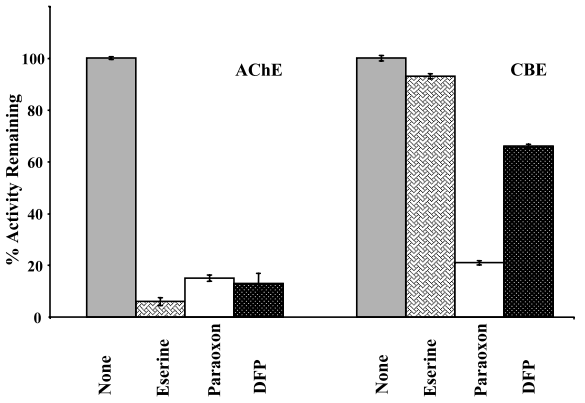

Fig. 5. The effect of exposure of *M. edulis* ($n = 10$) to a single dose of 3 mM paraoxon, DFP or eserine for 24 h expressed as a percentage of the vehicle control (unexposed) activity.

inhibition caused by these exposure concentrations was greater for acetylcholinesterase than for carboxylesterase ($P \leq 0.001$). When expressed as percent inhibition compared with the control, eserine, paraoxon and DFP at 1 mM inhibited acetylcholinesterase activity in the haemolymph by 96 ± 0.12, 85 ± 4.92 and $90 \pm 0.75\%$, respectively. Carboxylesterase activity was inhibited by 8.9 ± 0.98, 81 ± 12.0 and 46 $16 \pm 11.4\%$ by these same exposure concentrations. There was a statistically significant difference between each treatment group and the control for both activities ($P \leq 0.001$).

The concentration dependent inhibition of acetylcholinesterase and carboxylesterase activities was then determined for the carbamate eserine and the organophosphorous paraoxon in the range 0.05–1.0 mM and the enzyme activities determined as before (Figs. 6 and 7). For eserine (Fig. 6) there was a significant difference in enzyme activity between control and exposed animals for each of the treatment groups ($P \leq 0.001$). For acetylcholinesterase activity, exposure to 0.05 mM eserine caused inhibition of activity of $85 \pm 1.5\%$ and subsequently, no significant difference was detectable among the treatment groups themselves ($P = 0.07$). Carboxylesterase activity varied significantly between each of the treatment concentrations ($P \leq 0.002$) and reached a level of $39 \pm 0.73\%$ inhibition relative to the control at 0.5 mM eserine. Following exposure to paraoxon

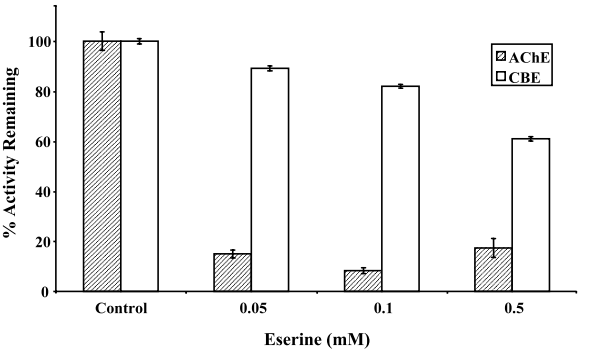

Fig. 6. The effect of 24 h in vivo exposure of *M. edulis* ($n = 10$) to eserine. Results are expressed as a percent of the vehicle control (unexposed) activity.

(Fig. 7) there was a significant difference in acetylcholinesterase activity between control and exposed animals for each of the treatment groups ($P \leq 0.005$). Exposure to 1.0 mM resulted in a reduction of activity to $17.9 \pm 1.06\%$ relative to the control. The inhibition of carboxylesterase activity reached statistical significance at concentrations of 0.1 and 1.0 mM ($P \leq 0.001$). At the highest exposure concentration (1.0 mM) $27.6 \pm 2.95\%$ of the control activity remained.

4. Discussion

This study has sought to characterise the measurable esterase activity in selected tissues and body fluids of *M. edulis* in order that their utility

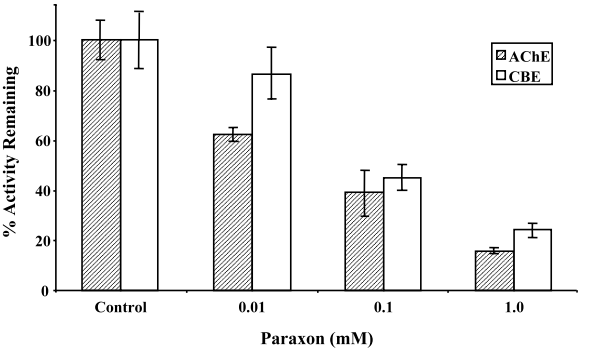

Fig. 7. The effect of 24 h in vivo exposure of *M. edulis* ($n = 5$) to paraoxon. Results are expressed as a percent of the vehicle control (unexposed) activity.

as accurate and sensitive biomarkers of organophosphorous and carbamate exposure might be determined. An additional consideration for their use in environmental monitoring has been the need for such assays to be accessible to personnel who may have limited experience of biochemical techniques or access to specialised monitoring equipment (Depledge, 2000).

The choice of a common endpoint for the detection of cholinesterase or carboxylesterase activity was made to simplify both reagent preparation and detection, the only difference between the methods being the nature of the substrate analogue. Both methods proved reproducible and sensitive for the detection of small changes in activity in microliter sample volumes. The added advantage of using haemolymph is the opportunity to employ repeated, non-destructive sampling. For reasons of simplicity in sample preparation, only whole body tissue homogenate rather than the dissection of individual tissues was considered.

4.1. Characterisation of enzyme activities

In higher vertebrates, esterases are generally classified into three groups, cholinesterases, arylesterases and carboxylesterases base on their catalytic properties, inhibition specificities and tissue distributions (Mounter and Whittaker, 1953). The differentiation of these enzyme activities in many invertebrate species remains largely undescribed and the vertebrate classification system may be inappropriate to use (Walker and Thompson, 1991). Bearing this in mind, the results presented here verify the existence of one predominant enzyme activity, classifiable as an acetylcholinesterase on the basis of its substrate preference, pH optimum and high dose substrate inhibition in the haemolymph of *M. edulis* (Von Wachtendonk and Neef, 1979). The level of activity was comparatively low, a characteristic reported for acetylcholinesterases in mollusc haemolymph (Winners et al., 1977; Najimi et al., 1997; Narbonne et al., 2001). The apparent K_m of 1.33 mM for acetylthiocholine iodide calculated for this unpurified preparation is in the same order of magnitude as values reported for acetylcholinesterase purified from the gill of *Mytilus* species (Mora et al., 1999). The hydrolysis of other substrate analogues, albeit at considerably lower levels, is in agreement with previous reports of four distinct cholinesterase activities in the haemolymph of *M. edulis*, only one of which could be classified as an acetylcholineterase based on its substrate preference and susceptibility to inhibitors (Von Wachtendonk and Neef, 1979). Thus, interpretation of acetylcholinesterase activity measurements in *M. edulis* haemolymph may be made on the basis that a single predominant pharmacological form is present.

Many different substrate analogues have been used in the measurement of carboxylesterase activities which typically exhibit a level of promiscuity in their substrate reactions. Phenylthioacetate was chosen as a substrate for monitoring carboxylesterase activity in this study because of its preferential hydrolysis (Fig. 2), ease of detection using parallel methodology to the detection of acetylcholinesterase activity and because hydrolysis of this substrate by acetylcholinesterase is negligible (Basack et al., 1998) allowing differentiation of the two activities. Carboxylesterase activity as measured by this method was higher in whole body tissue homogenate than in haemolymph in agreement with previous reports (Basack et al., 1998). High levels of esterase activity have been reported in the digestive gland (Ozretic and Krajnovic-Ozretic, 1992) and gill tissues (Escartin and Porte, 1997) of *Mytilus* species although for the purposes of this study, the exact location of individual carboxylesterases was not determined. The measured activity could be inhibited completely with PMSF, a general inhibitor of serine hydrolases and was also inhibited partially by iso-OMPA, confirming that this activity is distinct from acetylcholinesterase. Although the predominant activity in the tissue preparation was carboxylesterase, some cholinesterase activity was also present showing an order of thiocholine substrate preference of butyryl > propionyl $\gg$ acetyl. An alternative conclusion, that a single broad-spectrum activity was present, able to hydrolyse each derivative cannot be ruled out but is less likely. Carboxylesterases are in general specific for simple carboxylic esters and lack the anionic bind-

ing site considered necessary for interaction with cholinesterase derivatives (Maxwell, 1992).

4.2. Interpretation of combined esterase activity measurements

Characterisation of the relative sensitivities of the two enzyme activities to carbamate and organophosphorous compounds following in vitro incubation confirmed the sensitivity of both enzymes to the exposure-dependent effects of these compounds. The sensitivity of the enzymes was similar, with carboxylesterase slightly more sensitive to paraoxon and chlorpyrifos. The inhibition seen at high concentrations of the phosphorothioate chlorpyrifos may be due to the presence of chlorpyrifos oxon or other contaminants in the preparation. The manufacturer's quoted purity was 99.5%. Carboxylesterase activity was insensitive to the carbamate eserine except at high concentrations. Similar patterns of inhibition were also evident following in vivo exposures. Both enzyme activities showed sensitivity in vitro to concentrations of pesticide in the same order of magnitude although the percent inhibition of acetylcholinesterase activity was consistently greater. Acetylcholinesterase activity could be used to detect in vivo exposure to 0.05 mM eserine and 0.01 mM paraoxon with statistical significance. Despite the similar in vitro sensitivities of the enzymes, the percent inhibition of carboxylesterase activity was smaller at was significantly inhibited only by higher amounts of pesticide. The observation of a high percentage of pesticide resistant carboxylesterase activity is striking and has been reported by others in invertebrate (Maxwell, 1992; Boone and Chambers, 1996; Basack et al., 1998) and vertebrate studies (Lassiter et al., 1999). The inhibition of enzyme activity seen in tissue preparations following in vivo exposure provides a net reflection of the sensitivity of the organism in terms of uptake, biotransformation and detoxification patterns. Conversely, the relative insensitivity of the enzyme to environmentally relevant pesticide concentrations may make it difficult to accurately measure small fluctuations in activity against a varying background of inter-individual activities. In other species, the combined monitoring of measures of behaviour and neurological function has helped to define the toxicological significance of exposure (Beauvais et al., 2000). Adequate means of assessing behavioural changes in bivalves would no doubt be of benefit in interpretation of this kind of data, as would further insight into the physiological roles of the enzymes. Although the acetylcholinesterase activity described here has been defined as such based on substrate preference and inhibitor sensitivity, there is no indication other than this that it is involved in neuromuscular activity. The toxic effects of organophosphorous pesticide exposure can also be evaluated by measuring other forms of toxicity which can occur in addition to their effects on esterase targets, for example the alkylating potential of compounds such as dichlorvos can cause damage to DNA, detectable using assays of genotoxicity (Sbrana and Musio, 1995).

5. Conclusions

The measurement of acetylcholinesterase activity in *M. edulis* haemolymph using the described methodology provides a rapid, relatively inexpensive and reliable means of non-destructively assessing the exposure of mussels (and potentially other invertebrates) to organophosphorous and carbamate pesticides. Inhibition of activity is exposure concentration dependent and the interpretation of inhibition can be made on the basis that one predominant pharmacological form of the enzyme is present. Parallel determination of carboxylase activity can be achieved with the need for just one additional reagent and with minimal extra expenditure of time. However, the interpretation of carboxylesterase activity must take account of the large percentage of pesticide resistant activity apparent using the method described. Whilst these assays provide a useful means of assessing the exposure of bivalves to pesticides, interpretation of the ecotoxicological significance of such exposure should be made in conjunction with other multi-level indicators of toxicity, such as physiological and behavioural changes of evidence of genotoxic damage.

Acknowledgements

This work was supported financially by The Nuffield Foundation, DEFRA and the University of Plymouth. Many thanks to Sarah MacMahon for her help in preparing the manuscript.

References

Barahona, M.V., Sanchez-Forun, S., 1999. Toxicity of carbamates to the brine shrimp *Artemia salina* and the effects of atropine, BW 284c51, iso-OMPA and 2-PAM on carbaryl toxicity. Environ. Pollut. 104, 469–476.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J., Kesten, E.M., 1998. Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorous pesticides. Bull. Environ. Contam. Toxicol. 61, 569–576.

Beauvais, S.H., Jones, S.B., Brewer, S.K., Little, E.E., 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout *Onchorhynchus mykiss* and their correlation with behavioural measures. Environ. Toxicol. Chem. 19, 1875–1880.

Boone, J.S., Chambers, J.E., 1996. Time course of inhibition of cholinesterase and aliesterase activities and non protein sulfhydryl levels following exposure to organophosphorous insecticides in mosquitofish (*Gambusia affinus*). Fundam. Appl. Toxicol. 29, 202–207.

Carlock, L.L., Chen, W.L., Gordon, E.B., Killeen, J.C., Manley, A., Meyer, L.S., Mullin, L.S., Pendino, K.J., Percy, A., Sargent, D.E., Seaman, L.R., 1999. Regulating and assessing the risks of cholinesterase-inhibiting pesticides: divergent approaches and interpretations. J. Toxicol. Environ. Health 2, 105–160.

Depledge, M.H., 2000. Rapid assessment of marine pollution. In: Bui C.Q. et al. (eds.), Proceedings of the IOC Vietnam–UK Joint Workshop on Marine Pollution Assessment, Hanoi, June 8 2000, Bui Vet My, Vietnam, pp. 5–16.

Edwards, C.A., Fisher, S.W., 1991. The use of cholinesterase measurements in assessing the impacts of pesticides on terrestrial and aquatic invertebrates. In: Mineau, P. (Ed.), Cholinesterase Inhibiting Insecticides, Their Impact on the Environment. Elsevier, Amsterdam, pp. 255–275.

Ellman, G.L., Courtney, K.O., Anders V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Escartin, E., Porte, C., 1997. The use of cholinesterase activities from *Mytilus galloprovincialis* in pollution monitoring. Environ. Toxicol. Chem. 10, 2090–2095.

Forget, J., Bocquene, G., 1999. Partial purification and enzymatic characterisation of acetylcholinesterase from the intertidal marine copepod *Tigriopus brevicorni*. Comp. Biochem. Physiol. 123B, 345–350.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorous insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37–46.

Jokanovic, M.M., Kosanovic, M., Maksimovic, M., 1996. Interaction of organophosphorous compounds with carboxylesterases in the rat. Arch. Toxicol. 70, 444–450.

Lassiter, T.L., Barone, S., Moser, V.C., Padilla, S., 1999. Gestational exposure to chlorpyrifos: dose response profiles for cholinesterase and carboxylesterase activity. Toxicol. Sci. 52, 92–100.

Lundebye, A.K., Curtis, T.M., Braven, J., Depledge, M.H., 1997. Effects of the organophosphorous pesticide dimethoate on cardiac and acetylcholinesterase activity in the shore crab, *Carcinus maenus*. Aquat. Toxicol. 40, 23–26.

Massoullie, J., Pezzementi, L., Bon, S., Krejci, E., Valette, F.M., 1993. Molecular and cellular biology of cholinesterase. Prog. Neurobiol. 41, 31–91.

Maxwell, D.M., 1992. Detoxification of organophosphorous compounds by carboxylesterases. In: Chambers, J.E., Levi, P.E. (Eds.), Organophosphates, Chemistry, Fate and Effects. Academic Press, San Diego, CA, p. 183.

Mora, P., Fournier, D., Narbonne, J.F., 1999. Cholinesterases from the marine mussels *Mytilus galloprovincialis* and *M. edulis* and from the freshwater bivalve *Corbicula fluminea*. Comp. Biochem. Physiol. 122C, 353–361.

Mounter, L.A., Whittaker, V.P., 1953. The hydrolysis of esters of phenol by choinesterases and other esterases. Biochem. J. 54, 551–559.

Najimi, S., Bouhaimi, A., Daubeze, M., Zekhnini, A., Pellerin, J., Narbonne, J.F., Moukrim, A., 1997. Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker in Agadir marine bay (S Morocco). Bull. Environ. Contam. Toxicol. 58, 901–908.

Narbonne, J.F., Mora, P., Michel, X., Budzinski, H., Garrigues, M.P., LaFaurie, M., Salaun, J.P., Berge, J.B., Den Besten, P., Pagano, G., Porte, C., Livingstone, D., Hansen, P.D., Herbert, A., 2001. Biological markers of environmental contamination in marine ecosystems. J. Toxicol. Toxin Rev. 18, 205–220.

Ozretic, B., Krajnovic-Ozretic, M., 1992. Esterase heterogeneity in mussel *Mytilus galloprovincialis*: effects of organophosphate and carbamate pesticides in vitro. Comp. Biochem. Physiol. C103, 221–225.

Parkinson, A., 1996. Biotransformation of xenobiotics. In: Klassen, C.D. (Ed.), Casarett and Doull's Toxicology. The Basic Science of Poisons, fifth ed. McGraw-Hill, New York.

Radenac, G., Bocquene, G., Fichet, D., Mirimand, P., 1998. Contamination of a dredged material disposal site (La Rochelle Bay, France). The use of the acetylcholinesterase activity of *Mytilus edulis* (L) as a biomarker of pesticides: the need for a critical approach. Biomarkers 3, 305–315.

Rattner, B.A., Fairbrother, A., 1991. Biological variability and the influence of stress on cholinesterase activity. In: Mineau, P. (Ed.), Cholinesterase Inhibiting Insecticides,

Their Impact on the Environment. Elsevier, Amsterdam, pp. 89–110.

Readman, J.W., Liong Wee Kwong, L., Mee, L.D., Bartocci, J., Nilve, G., Rodriguez-Solano, J.A., Gonzalez-Farias, F., 1992. Persistent organophosphorous pesticides in tropical marine environments. Mar. Poll. Bull. 24, 398–402.

Sbrana, I., Musio, A., 1995. Enhanced expression of common fragile site with occupational exposure to pesticides. Cancer Genet. Cytogenet. 82, 123–127.

Scaps, P., Demuynck, S., Descamps, M., Dhainaut, A., 1996. Biochemical and enzymatic characterisation of an acetylcholinesterase from *Neiris diversicolor* (Annelida, Polychaeta): comparison with the cholinesterases of *Eisenia fetida* (Annelida, Oligochaeta). Biol. Bull. 190, 396–402.

Thompson, H.M., Walker, C.H., Hardy, A.R., 1991. Changes in activity of avian serum esterases following exposure to organophosphorous insecticides. Arch. Environ. Contam. Toxicol. 20, 514–521.

Von Scramlik, E., 1941. Ergebnisse der Biologie pp. 131–134.

Von Wachtendonk, D., Neef, J., 1979. Isolation, purification and molecular properties of an acetylcholinesterase (EC 3.1.1.7) from the haemolymph of the sea mussel *Mytilus edulis*. Comp. Biochem. Physiol. 63C, 279–286.

Walker, C.H., 2001. Organic Pollutants. Taylor & Francis, New York, pp. 35–36.

Walker, C.H., Thompson, H.M., 1991. Phylogenetic distribution of cholinesterases and related esterases. In: Mineau, P. (Ed.), Cholinesterase Inhibiting Insecticides. Elsevier, Amsterdam, pp. 1–18.

Winners, W., Neep, J., von Wachtendonk, D., 1977. Distribution of cholinesters and cholinesterases in haemolymphs and smooth muscles of molluscs. Comp. Biochem. Physiol. C61, 121–131.