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Acetylcholinesterase inhibition as a biomarker of adverse effect A study of *Mytilus edulis* exposed to the priority pollutant chlorfenvinphos

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Abstract

The inhibition of acetylcholinesterase (AChE) activity has been used widely as a biomarker of exposure to organophosphorous pesticides (OPs). However, scientific uncertainty about the risk assessment implications of data describing inhibition of cholinesterases in diverse species and tissues has hampered the use of AChE activity as a biomarker of adverse effect. Here, haemolymph AChE activity was combined with biomarkers of cellular integrity, immunotoxicity and physiological status in order to measure exposure to and the effects of the priority pollutant chlorfenvinphos. Laboratory exposures of the blue mussel *Mytilus edulis* to commercial grade chlorfenvinphos (Sapecron®) were conducted over 24, 48 and 96 h. AChE activity in haemolymph of *M. edulis* was highly variable and bore no relationship to either sublethal effects or lethality over the range 0.003–0.03 mg/l chlorfenvinphos. In comparison, concentration dependent inhibition was evident for each of the remaining biomarkers (phagocytic activity, spontaneous cytotoxicity, neutral red retention time, total haemolymph protein). Mussels at the highest exposure concentration showed visual signs of neurotoxicity (impaired neuromuscular control). Haemocyte phagocytic activity and spontaneous cytotoxicity responses were highly sensitive to chlorfenvinphos with significant modulation evident after 24 h exposure to environmentally realistic concentrations of 0.007 mg/l ($P = 0.0003$). Thus the immune function and well being of the mussels was significantly impacted in the absence of measurable inhibition of haemolymph AChE.

Keywords: Immunotoxicity; Acetylcholinesterase; Biomarker; *Mytilus edulis*; Ecotoxicology; Organophosphorous pesticide; Chlorfenvinphos

1. Introduction

The rapid increase in production and use of organophosphorous (OP) and carbamate pesticides has raised concerns about their potential to cause harm to human and non-target wildlife populations. Pesticides enter waterways from agricultural and urban run-off, movement through soil into water courses and after direct application (Schulz and Leiss, 1999)

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and may be transported to estuaries and coastal waters (Readman et al., 1992). The sub-lethal effects of OP pesticides on non-target aquatic organisms that may be essential to the health and maintenance of these vulnerable, highly productive ecosystems are the subject of intense study by scientists and environmental managers (Mineau, 1991; Roast et al., 1999; Werner et al., 2000; Doran et al., 2001; Fulton and Key, 2001).

The OP pesticides are acutely neurotoxic. They are designed to be effective inhibitors of the enzyme acetylcholinesterase located at neuromuscular junctions in the central and peripheral nervous system (Walker et al., 2001). Cholinesterases can also be found in plasma or haemolymph, in mammalian red blood cells and in other organs although the physiological functions of AChE in tissues other than nerves are not known (Carlock et al., 1999). The inhibition of these peripheral enzymes does, however, provide a convenient, non-destructive means of monitoring exposure to pesticides and has been widely implemented by regulatory agencies. While there is agreement that such inhibition is a biomarker of exposure, there is controversy over whether inhibition, especially in body fluids, constitutes an adverse effect (Costa, 1998; Carlock et al., 1999; EPA, 2003) unless accompanied by physical manifestation of toxicity.

Aquatic invertebrates are essential to investigations of the sub-lethal effects of pollutants as they are an integral part of all aquatic biomes and biotypes, and the effects of toxicants could alter the structure and functioning of aquatic ecosystems. The blue mussel *Mytilus edulis* occurs abundantly in estuaries throughout the United Kingdom. It is a sessile filter-feeder and its low rates of metabolic transformation (Moore et al., 1989) and ability to accumulate pollutants have encouraged its extensive use in research protocols and monitoring programmes to reflect changes in the contaminant status of the environment (Wade et al., 1998; Moore et al., 1999).

Acetylcholinesterase activity has been identified and biochemically characterised in many aquatic invertebrates (reviewed by Fulton and Key, 2001). Bivalve and prosobranch molluscs in particular have high levels of AChE activity in the haemolymph (Srivatsan, 1999). We have identified cholinesterase activity in the haemolymph in *M. edulis* consistent in its patterns of activation and inhibition with AChE (Galloway et al., 2002a). Concentration-dependent inhibition of the enzyme was demonstrable after in vitro and in vivo exposure to both OP and carbamate pesticides, suggesting that the activity of haemolymph AChE might provide a rapid, accessible means of monitoring pesticide exposure. However, in order to be of use in defining hazard in the risk assessment procedure, there is a need to appreciate how haemolymph AChE activity relates to toxicity. Jensen et al. (1997) investigated the relationship between enzyme activity and sub-lethal toxicity in the carabid beetle *Pterostichuscupreus* using computer-aided video tracking as a general effect biomarker, and reported a correlation between locomotor behaviour and inhibition of AChE activity. Based on this, they were able to deduce a mechanistic relationship between molecular and behavioural responses. It has proven more difficult, in sessile aquatic species, to definitively link inhibition of AChE with the adverse neurophysiological and behavioural signs of toxicity that would indicate a biologically significant event.

In addition to neurotoxicity, the adverse effects of OP exposure may alter other body functions. The ability of OP pesticides to interfere with the immune system and exert immunotoxic effects through both anti-cholinergic and non-cholinergic pathways has been recognised in recent years (Barnett and Rodgers, 1994; Vial et al., 1996; Galloway and Handy, 2003). The defense systems of aquatic invertebrates are highly sensitive to homeostatic disturbance (Smith, 1991; Galloway and Depledge, 2001) raising the possibility that assays of immune function could provide a biologically relevant means of monitoring the adverse effects of OP exposure in invertebrates such as *M. edulis*.

The aim of this study was to use a combination of biomarkers to characterise the relationship between exposure to an OP pesticide, toxic damage and adverse health effects in *M. edulis* following laboratory exposures. Chlorfenvinphos was chosen as test compound because it is a priority pollutant under current EU legislation, registered for use on a wide range of vegetable crops under trade names including Dermatorn and Sapecron®. It is relatively resistant to hydrolysis and volatilisation and this results in an aquatic half life of several days to weeks and consequently residues have been detected at environmental

monitoring sites (USPHS, 1996). The testing regime included haemolymph AChE activity as a biomarker of exposure combined with biomarkers of cellular viability (neutral red retention time), immunotoxicity (phagocytosis, spontaneous cytotoxicity activity, cell count) and metabolic state (total haemolymph protein). In addition, the physiological well-being of the mussels was non-quantitatively assessed by observing shell-gape and byssal thread attachment. The ecotoxicological relevance of the results is discussed.

2. Materials and methods

Chlorfenvinphos was obtained as an emulsifiable concentrate (Sapecron®, Ciba-Geigy Agrochemicals, Cambridge, UK) containing 90%, v/v chlorfenvinphos. An aqueous stock solution was freshly prepared in distilled water prior to use. Acetylthiocholine iodide and 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB) were from Sigma Chemical Co, Poole, Dorset. Sheep erythrocytes were from Tissue Culture Services, UK. All other reagents and solvents were analytical grade. All glassware was acid washed prior to use.

2.1. Sample collection and preparation

Mussels were collected from Whitsand Bay, Cornwall, UK (Grid Reference: SX387518) between April and August 2002. Organisms were chosen to be of similar size (5–7 cm) and reproductive stage and were transferred to 20 l tanks of filtered seawater until required. They were fed regularly and kept under a 12 h light/dark cycle at a constant temperature of 14 ± 2 °C. A fixed scalpel was used to prize the shell open slightly and haemolymph was extracted from the posterior adductor muscle using a 1 ml syringe with a 21 guage needle. Samples were diluted with an equal volume of physiological saline (0.02 M HEPES pH 7.4, 0.4 M $NaCl_2$, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$) and placed on ice until analysis.

2.2. In vitro exposure

Samples of haemolymph (50 μl) were incubated for 30 min in triplicate at 25 °C with 10 μl aliquots of chlorfenvinophos in the range 0.4–30 mg/l. The samples were sealed tightly to prevent evaporation and gently shaken to maximise exposure of the haemolymph to the pesticide. Samples were then removed and assayed for AChE activity as described.

2.3. In vivo exposures

The mussels were prepared by removing any barnacles and rinsing off debris and placed in 2 l glass tanks containing 1.5 l filtered sea water with secured airlines. In order to limit the release of pesticide vapour, tanks were then secured with parafilm and double contained in sealed 20 l tanks. For each concentration five organisms were exposed in duplicate tanks for 24, 48 and 96 h during which time none of the organisms was fed. The water in each tank was replaced every 24 h in order to ensure consistency of exposure. Haemolymph samples were taken and analysed as described.

2.4. Acetylcholinesterase assay

The AChE activity was determined in haemolymph samples using a modification of the method of Ellman (1961) as described by Galloway et al. (2002a). Briefly, samples or buffer blanks (50 μl) were incubated for 5 min in microtitre plates at 25 °C with 150 μl DTNB, 270 μM in 50 mM sodium phosphate, pH 7.4. Measurement of enzyme activity was initiated by the addition of acetylthiocholine iodide, 3 mM, and the absorbance recorded at 412 nm. 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 protein in the sample. Total protein was determined using a commercial kit (BioRad) with bovine serum albumin as standard.

2.5. Phagocytosis

The phagocytosis activity of haemocytes was determined by measuring the uptake of zymosan particles (from *Saccharomyces cerevisiae*) dyed with Neutral Red dye. The method was exactly as described by Pipe et al. (1995) in which particle uptake by adhered cells is estimated by absorbance at 540 nm against a standard curve prepared using zymosan particles in the range 1.56–100 × 10^7 ml^{-1}. The protein concentration of each haemolymph sample from

the phagocytosis assay was obtained using the BCA Protein Assay (Pierce 23223/4).

2.6. Spontaneous cytotoxicity response

Immune function was also assessed by measuring the ability of haemocytes to lyse erythrocyte target cells using a method adapted from Raftos and Hutchison (1995) by Galloway et al. (2002b). Haemocytes were harvested and adjusted to a density of 2×10^8 cells/ml in Tris-buffered saline with calcium (TBS-Ca, 10 mM Tris pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$). Sheep erythrocytes were washed and suspended in TBS-Ca and incubated at a 1:1 ratio with haemocytes for 1 h at 20 °C in round-bottomed microtitreplates. After pelleting by centrifugation, the percent lysis was determined by measuring the release of haemoglobin into the supernatant by its absorbance at 405 nm. Results were expressed as percent lysis relative to the maximum lysis achieved using a solution of 0.2% Triton-X 100 in distilled water.

2.7. Cellular viability

The viability of haemocytes was determined by measuring their ability to retain Neutral Red dye (Babich and Borenfreund, 1994). Samples of haemolymph (50 μl) were incubated in triplicate in flat-bottomed microtitreplates in order to allow a monolayer of cells to adhere to the wells. After 45 min, non-adhered cells were discarded and the plates washed with physiological saline. A solution of 0.004% neutral Red dye in physiological saline (200 μl) was added to each well and the cells incubated for a further 3 h at room temperature. The wells were washed and an acidified solution of 1% acetic acid, 20% ethanol added to resolubilise the dye. The plate was gently shaken for 10 min before reading the absorbance at 540 nm.

2.8. Behavioural observations

During water changes, redosing and/or sampling of haemolymph the following observations were recorded: (1) The ability of organisms to attach to the glass beaker, (2) the ease with which shells were opened with a fixed scalpel and (3) the length of time it took for shells to re-close after sampling.

2.9. Statistical analysis

All data sets were analysed using Statgraphics v5.0. A variance check was firstly performed to ascertain whether the standard deviations between concentrations and times were significantly different ($P < 0.05$). If the standard deviations were different the data were transformed. A one-way ANOVA, allowing comparison of means, was used on data sets that showed no significant difference between the standard deviations, either before or after transformation. However, if the standard deviations were still significantly different after transformation a non-parametric Kruskal–Wallis test was performed to look at differences between the medians. For analysis of multiple data sets i.e. between time periods a multiple-sample comparison test was used first followed by an ANOVA or Kruskal–Wallis test as before.

3. Results

3.1. Acetylcholinesterase activity

The sensitivity of the haemolymph enzyme activity to inhibition by chlorfenvinphos was first determined following in vitro exposure of haemolymph to increasing concentrations of the pesticide. From the results shown in Fig. 1, an IC50 value for in vitro exposure of 2.3 mg/l was calculated from plotting a regression line through the linear portion of the graph and using the plot equation to calculate the concentration (x) at which 50% inhibition was observed. A series of in

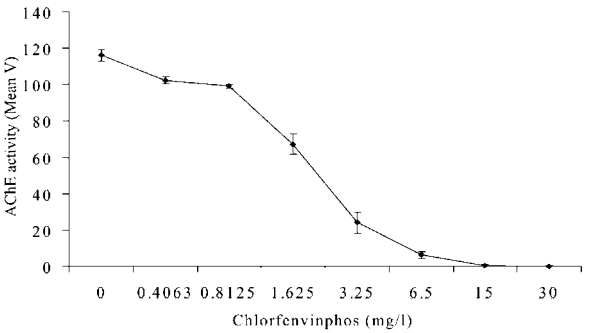

Fig. 1. Acetylcholinesterase (AChE) activity (Mean V) in *M. edulis* haemolymph after an in vitro exposure to Chlorfenvinphos (mg/l).

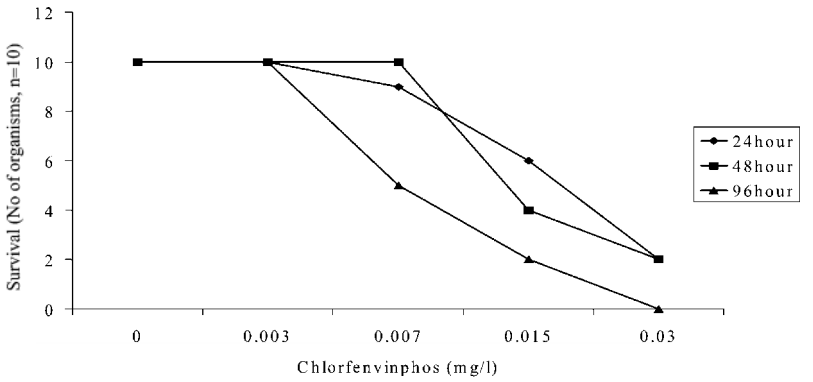

Fig. 2. Survival of *M. edulis* after 24, 48 and 96 h exposure to Chlorfenvinphos (mg/l).

vivo sublethal exposures to environmentally realistic concentrations of chlorfenvinphos ranging from 0.003 to 0.03 mg/l was then performed. The survival of organisms at each exposure time is shown in Fig. 2. from which a 96 h LC50 value of 0.009 mg/l was calculated from plotting a regression line through the linear portion of the graph and using the plot equation to calculate the concentration (x) at which 50% (y) of organisms died. Acetylcholinesterase activity was highly variable both between and within each study group (Fig. 3). Although there were some statistical differences between groups, they followed no obvious trend and a concentration dependent alteration was not detected. Neither was there any significant difference in activity with increasing times of exposure using an ANOVA ($P = 0.4961$).

3.2. Phagocytosis activity

The phagocytosis activity of haemocytes was significantly affected by exposure to chlorfenvinphos (Fig. 4). At all three time periods stimulation of activity was observed at the lowest concentrations of 0.003 and 0.007 mg/l compared with the control. Stimulation at the lowest concentration became increasingly significant with time (ANOVA, $P = 0.0003$ at 96 h). Concentration dependent inhibition of activity was observed above 0.007 mg/l, with significant decreases between each concentration from 0.007 to 0.03 mg/l (Kruskal–Wallis, $P = 0.003$). There was a highly significant change in activity when exposed over 96 h compared with 24 and 48 h (Kruskal–Wallis, $P = 0.00005$). This would indicate that phagocytosis was being increasingly disrupted with increasing exposure time.

3.3. Spontaneous cytotoxicity activity

The ability of haemocytes to recognise and lyse an allogeneic cell type (sheep red blood cells) followed a similar pattern of change to that of phagocytosis activity (Fig. 5). At all time periods there was a slight stimulation of cytotoxicity at the lowest concentration. Above 0.003 mg/l, concentration dependent inhibition of cytotoxicity occurred. After 24 h a significant decrease at 0.007 and 0.015 mg/l was observed compared with the lowest concentration of 0.003 mg/l ($P = 0.015$). After 48 h highly significant inhibition occurred at the two highest concentrations of 0.015 and 0.03 mg/l compared with the two lowest concentrations (0.003 and 0.007 mg/l) and the control ($P = 0.0004$). Kruskal–Wallis tests revealed no significant change over time ($P = 0.39$) and this may have been due to the high variability found within the sample set.

3.4. Neutral red retention

The viability of haemocytes was significantly affected by exposure to chlorfenvinphos after 96 h (Fig. 6). There was a small increase in retention time at the lowest concentration at each time point although this was significant only at 24 h. Concentration dependent decreases in viability were then observed between 0.007–0.015 mg/l at 24 h ($P = 0.02$) and between 0.007–0.03 mg/l at 96 h.

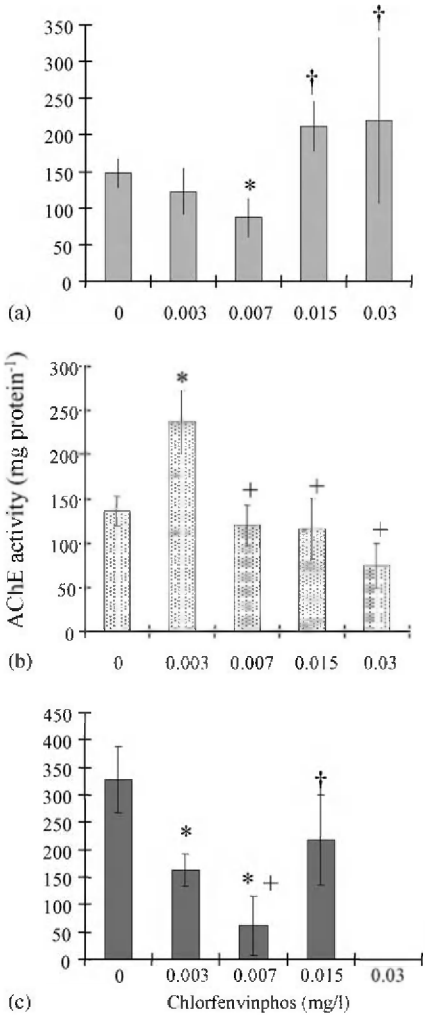

Fig. 3. AChE activity (mg protein^{-1}) in *M. edulis* after (a) 24 h, (b) 48 h and (c) 96 h exposure to Chlorfenvinphos. Asterisk (∗) indicates significant difference from control (0 mg/l) ($P < 0.01$), (+) indicates significant difference from 0.003 mg/l ($P < 0.01$), (†) indicates significant difference from 0.007 mg/l ($P < 0.01$).

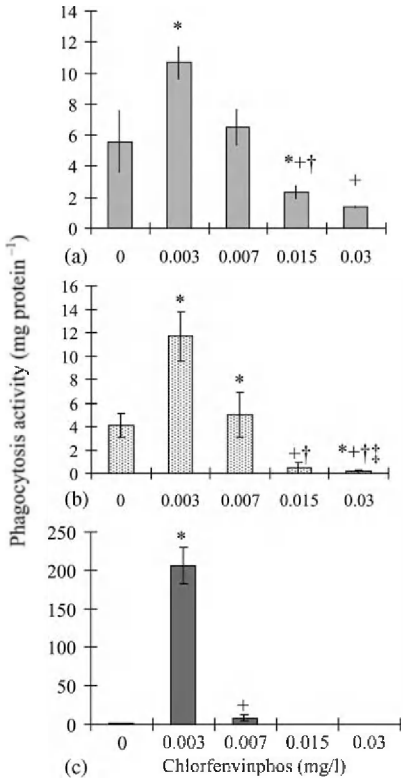

Fig. 4. Phagocytosis activity (mg protein^{-1}) in *M. edulis* after (a) 24 h, (b) 48 h and (c) 96 h exposure to Chlorfenvinphos. Asterisk (∗) indicates significant difference from control (0 mg/l) ($P < 0.05$), (+) indicates significant difference from 0.003 mg/l ($P < 0.05$), (†) indicates significant difference from 0.007 mg/l ($P < 0.05$), (‡) indicates significant difference from 0.015 mg/l ($P < 0.05$).

Comparison of neutral red retention over the differing time periods, using an ANOVA, revealed no significant difference ($P = 0.5313$). Thus the viability of haemocytes was not increasingly affected by length of exposure to chlorfenvinphos.

3.5. Haemocyte number

With increasing concentrations of chlorfenvinphos there was a small decrease in haemocyte number (Fig. 7). This was significant after 24 h at the highest concentration of 0.03 mg/l. After 48 h a Kruskal–Wallis test indicated a significant increase at 0.007 mg/l from the control followed by a decrease at 0.015 mg/l ($P = 0.0367$). However, after 96 h no significant differences were detectable. Over longer periods of time (48 and 96 h) there was a decrease in haemocytes compared to earlier time points (ANOVA, $P = 0.039$).

3.6. Haemolymph total protein

Total protein content of haemolymph declined after 48 and 96 h exposure to chlorfenvinphos of 0.007 mg/l and above ($P = 0.0045$) (Fig. 8). Analysis of the

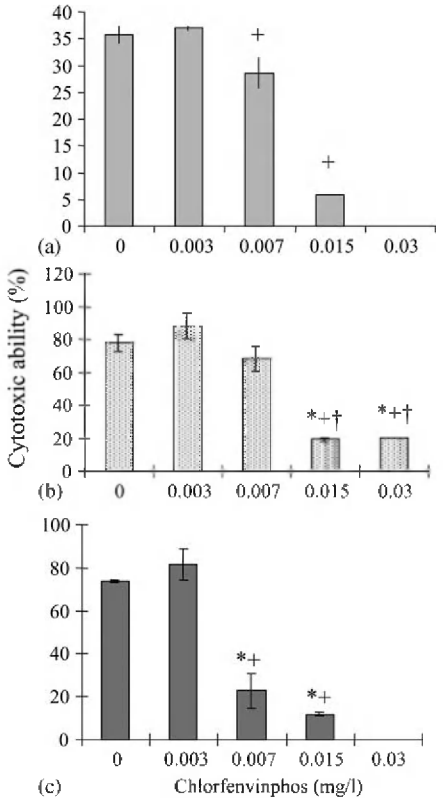

Fig. 5. Cytotoxic ability (%) of *M. edulis* after (a) 24 h, (b) 48 h and (c) 96 h exposure to Chlorfenvinphos. Asterisk (*) indicates significant difference from control (0 mg/l) ($P < 0.05$), (+) indicates significant difference from 0.003 mg/l ($P < 0.05$), (†) indicates significant difference from 0.007 mg/l ($P < 0.05$).

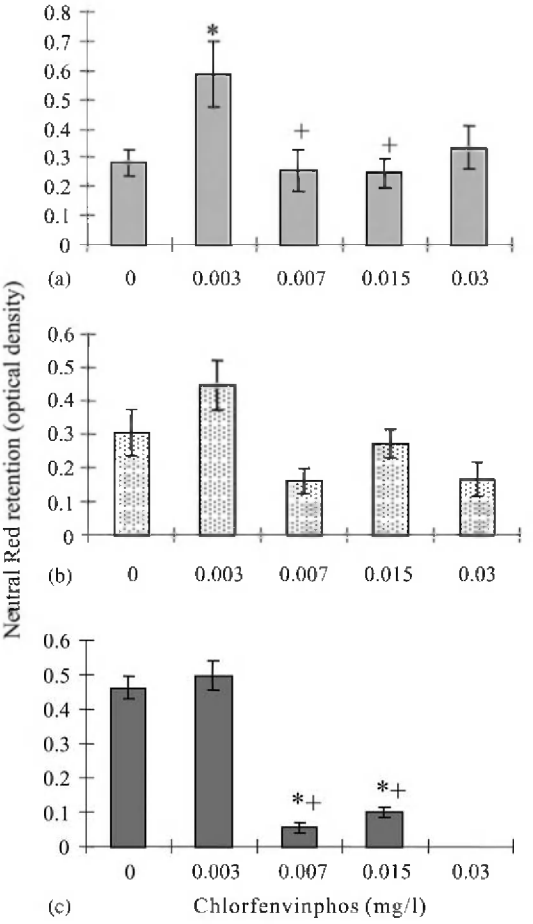

Fig. 6. Neutral red retention (optical density) in *M. edulis* haemolymph after (a) 24 h, (b) 48 h and (c) 96 h exposure to Chlorfenvinphos. Asterisk (*) indicates significant difference from control (0 mg/l) ($P < 0.05$), (+) indicates significant difference from 0.003 mg/l ($P < 0.05$).

different time periods using ANOVA revealed no time-dependent change.

3.7. Behavioural observations

Mussels exposed to concentrations higher than 0.003 mg/l did not attach to the tank or to each other whilst the byssal threads of those in the control tanks were joined tightly. All shells in the control and 0.003 mg/l tanks were shut firmly and required a fixed scalpel to open and to keep open. At higher concentrations the shells were easily opened, even by hand at the highest concentration of 0.03 mg/l, and remained open for sustained periods consistent with a decline in neuromuscular control.

4. Discussion

This study illustrates the relative insensitivity of haemolymph AChE activity following exposure of mussels to sublethal, environmentally realistic concentrations of chlorfenvinphos, despite evidence of detrimental effects to immunological and physiological parameters.

The lack of inhibition following in vivo exposures is surprising. In a previous study, haemolymph AChE could be used to detect in vivo exposure of *M. edulis* to 2.6 mg/l paraoxon with statistical significance

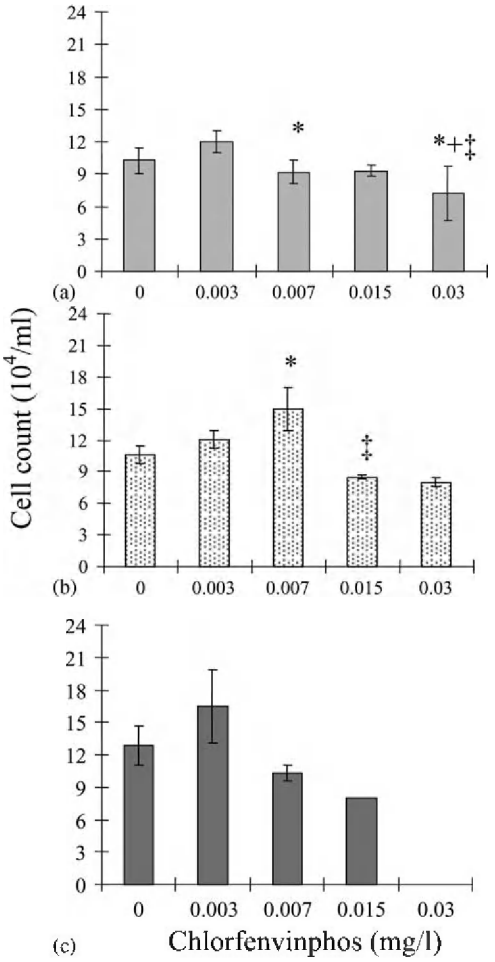

Fig. 7. Haemovyte cell counts (10^4/ml) in *M. edulis* after (a) 24 h, (b) 48 h and (c) 96 h exposure to Chlorfenvinphos. Asterisk (*) indicates significant difference from control (0 mg/l) ($P < 0.05$), (+) indicates significant difference from 0.003 mg/l ($P < 0.05$), (‡) indicates significant difference from 0.015 mg/l ($P < 0.05$).

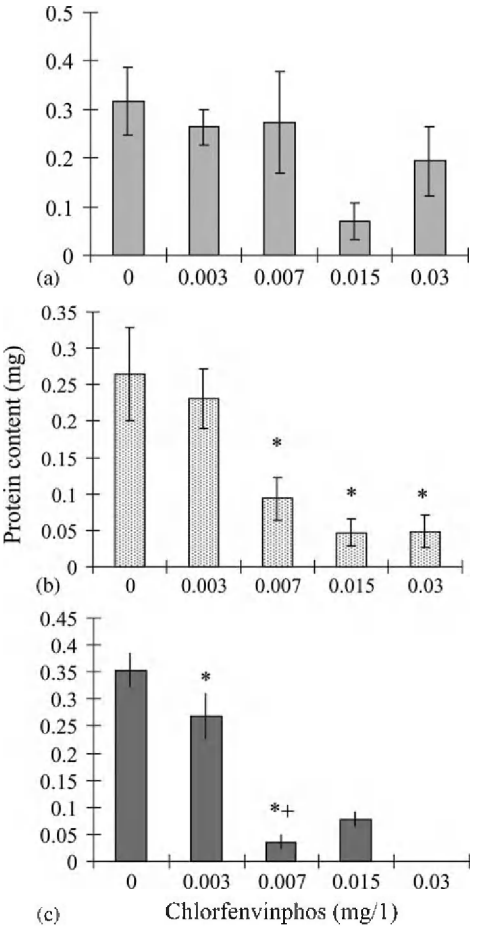

Fig. 8. Total protein content (mg) of haemolymph in *M. edulis* after (a) 24 h, (b) 48 h and (c) 96 h exposure to Chlorfenvinphos. Asterisk (*) indicates significant difference from control (0 mg/l) ($P < 0.05$), (+) indicates significant difference from 0.003 mg/l ($P < 0.05$).

(Galloway et al., 2002a). The haemolymph enzyme was certainly sensitive to the inhibitory effects of chlorfenvinphos in vitro, with an IC50 value of 2.3 mg/l, although there are reported occurrences of AChE variants insensitive to OP inhibition. For example, Bocquene et al. (1997) could differentiate between at least two AChE variants co-existing in the oyster *Crassostrea gigas*, one sensitive to paraoxon and the other insensitive. In a study of the benthic clam *Scapharca inaequivalvis* collected from sites in the Northern Adriatic, different patterns of AChE expression were revealed in clams from polluted sites (Talesa et al., 2002). The presence of an OP insensitive soluble haemolymph AChE was tentatively linked to the presence, at the site of collection, of mixed classes of chemical pollutants.

A lack of correlation between in vitro and in vivo exposures has been highlighted in other invertebrate species. Exposure of nerve tissue from the shrimp *Penaeus duorarum* to methyl paraoxon resulted in complete inhibition of AChE activity. However, there was no measurable inhibition of AChE in nervous tissue after in vivo exposures, in spite of the moribund state of the exposed shrimp (Schoor and Brausch,

1980). Laboratory studies have confirmed the relative insensitivity of molluscs when exposed in vivo. For example, the gill AChE IC50 values calculated for the oyster *C. Gigas* following exposure to fenitrothion and phosalone were 38.8 and 183.9 mg/l, respectively, (Bocquene et al., 1995). These concentrations are much higher than the amounts of chlorfenvinphos used here, albeit they are for different compounds and organisms.

The extent of AChE inhibition may have been related to time after initial exposure. Mussels were not tested until 24 h after the start of the experiment. Rapid recovery from AChE inhibition has been previously observed in *M. edulis* exposed to dichlorvos (McHenery et al., 1997) and an intermediate analysis of AChE activity could be advantageous in future studies. Chlorfenvinphos is relatively stable in aqueous solution and replacement of the tanks every 24 h should have ensured a constant level of exposure, although short half lives for some OPs have been observed in laboratory exposures (Dauberschmidt et al., 1996) presumably due to hydrolysis and adsorption to the glass walls of the aquaria, silicon adhesive and to the pump.

The metabolism of chlorfenvinphos is complex and in mammals involves oxidative 0-deethylation and subsequent reactions to yield a range of glucuronides and other metabolites (WHO, 1972). The metabolism of chlorfenvinphos has not been described in detail in *M. edulis* and it is only possible to speculate on its in vivo fate in the present study. It has been suggested that high levels of carboxylesterase activity, such as that detectable in *M. edulis* tissues protect organisms from OP toxicity by providing alternative sites of inhibitory phosphoryl binding (Jokanovic et al., 1996) and by direct detoxication of OP compounds (Maxwell and Brecht, 2001). Mussels can avoid contaminant exposure by shell clamping, a protective mechanism that has been seen in *M. edulis* when subjected to OP pesticide contamination (Mohan et al., 1987). This is discounted here by the visual observation of gaping shells and concentration dependent inhibition of other cellular processes.

The high level of variability in AChE activity made it difficult to detect any statistically significant changes between exposure groups. Inter-individual variability in AChE activity has been noted before (Rattner and Fairbrother, 1991) although without knowing the exact role of AChE in *M. edulis* it is possible only to speculate as to the cause. The role of acetylcholine in neurotransmission in molluscs has not yet been clearly defined (Heyer et al., 1973; Mercer and McGregor, 1982; Dauberschmidt et al., 1996) although AChE is present in both cholinergic and non-cholinergic neurons and in the central ganglia of the nervous system (Giller and Schwartz, 1971). Non-catalytic morphogenic activities have been described for AChE variants, raising speculation that the soluble enzyme may function as a growth factor (Soreq and Seidman, 2001). For example in the prosobranch mollusc *Aplysia californica*, haemolymph AChE was able to promote neurite growth in both cholinergic and dopaminergic neurons (Srivatsan, 1999). Whether this has any relevance to the high level of inter-individual variation is not known, but it does illustrate the difficulty in the interpretation of toxicity data when the functions and characteristics of the target are not yet completely understood.

Immune function assays revealed a concentration dependent inhibition of function, with reduced phagocytic ability, altered cytology and decreased cell viability at environmentally realistic concentrations of chlorfenvinphos. Interestingly, at the lowest exposure concentration (0.003 mg/l), each of these functions was stimulated. This kind of biphasic low dose stimulation has been observed previously in haemocyte responses to many different chemicals (Anderson, 1981; Cheng and Sullivan, 1984; Cheng, 1988; Coles et al., 1995; McCormick-Ray, 1987; Carissan et al., 1997; Pipe and Coles, 1995; Livingston et al., 2000) and appears to be a general response to low dose stimulation. Enhanced immune activity at low, non-cholinergic doses is also a characteristic feature of the immune response of mammals to OP pesticides; much of this data relates to parathion, malathion, diazinon and chlorpyrifos (Pruett, 1992; Galloway and Handy, 2003). Most experimental models have identified decreased immune functions following acute exposures (sufficient to induce symptoms of neurotoxicity and/or AChE inhibition), (Pruett, 1992). The mechanism by which chlorfenvinphos affects phagocytosis and spontaneous cytotoxicity responses is most likely by inhibition of serine hydrolases. These enzymes are susceptible to inhibition by OP compounds and play vital roles in immune functions, both as components of the cell membrane and in the signal transduction

pathways that modulate the activation, proliferation and, subsequently the effector functions of immune cells (Coffey and Hadden, 1985; Stepanovic et al., 1998). The reactivity of OP pesticides can also lead to direct oxidative damage to cell membranes, proteins and DNA, although these cytotoxic effects are reported at extremely high concentrations (Cao et al., 1999) or after prolonged exposures (Handy et al., 2002).

Examination of the time course of exposure shows that whilst phagocytosis was affected within 24 h of exposure and spontaneous cytotoxicity within 48 h, the viability of haemocytes was not significantly affected until 96 h after exposure. Thus a specific inhibition of effector function was augmented after prolonged exposure with a decrease in cell viability, in line with the above model. The low concentration at which modulation of phagocytosis and spontaneous cytotoxicity became statistically detectable (0.007 mg/l) illustrates their sensitivity in the detection of adverse effects.

The physiological effects of exposure were suggested by a decrease in haemolymph protein and cell count and in shell-gaping and decreased byssal thread attachment at higher concentrations and exposure times (these latter observations were not quantified). Protein catabolism is a recognized effect of exposure to OP pesticides (Marinovich et al., 1994), affecting the general health status of the organisms and the integrity of physical barriers to infection. The importance of shell clamping as a protective mechanism against OP toxicity has been discussed by Mohan et al. (1987). As the mussels were unable to close their shells due to decreased neuromuscular control of the adductor muscle, they would be more susceptible to the effects of pollution and predation. Byssal thread attachment is a general indicator of health status in mussels. Taken together, these observations illustrate the impact of chlorfenvinphos on the well-being and survival of the mussels.

5. Conclusion

This study has illustrated how a combination of biomarkers can be used to characterise the relationship between exposure to an OP pesticide, toxic damage and adverse health effects in *M. edulis* following laboratory exposures. Measurement of haemolymph AChE activity was not able to detect either exposure to or the adverse effects of environmentally realistic concentrations of the priority pollutant chlorfenvinphos. In comparison, concentration dependent alterations were evident for each of the remaining biomarkers (phagocytic activity, spontaneous cytotoxicity, neutral red retention time, total haemolymph protein). Phagocytosis and spontaneous cytotoxicity responses could be used to detect in vivo exposure to 0.007 mg/l chlorfenvinphos, illustrating the sensitivity of immune functions to the adverse effects of OP pesticides. From these results, the inhibition of haemolymph AChE in *M. edulis* cannot be considered to represent a biologically significant consequence of exposure to chlorfenvinphos.

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