ENZYMATIC RESPONSE OF *CALLINECTES SAPIDUS* AND *GEUKENSIA DEMISSA* AS BIOMARKERS FOR PESTICIDE EXPOSURE

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A Thesis Submitted to the
University of North Carolina at Wilmington in Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

Department of Chemistry

University of North Carolina at Wilmington

2003

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Aquatic Toxicology

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ABSTRACT

The widespread commercial and private use of organophosphate (OP) and carbamate pesticides, and their subsequent presence in aquatic ecosystems, is cause for concern in terms of both environmental and human health. Disruption of acetylcholinesterase (AChE) activity is a primary mode of acute toxicity of OP pesticides to non-target organisms, and this inhibition has been used in limited settings as a biomarker of OP/carbamate exposure. A second hydrolyzing enzyme, carboxylesterase (CBE), is also inhibited upon exposure to these compounds; used in conjunction with AChE activity, it may provide a more inclusive biomarker than AChE activity alone. In this study, two aquatic invertebrates were studied as potential indicator species of pesticide pollution. Enzymes were characterized for Michaelis-Menten kinetics, and *in vitro* and *in vivo* tests were conducted to assess the impact of OP and carbamate compounds on each enzyme, in both hemolymph and tissue in both species. In general, the blue crab, *Callinectes sapidus*, showed higher mortality at lower concentrations than did the ribbed mussel, *Geukensia demissa*. However, *G. demissa* showed more significant enzyme inhibition at sub-lethal concentrations, making it a more effective indicator of a potential threat. Although enzyme inhibition rarely occurred at environmentally relevant concentrations of a single contaminant, the more probable presence of multiple or chronic stressors could effect a measurable response *in situ*.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. Jack Manock, for his guidance and support throughout this research. My committee members, Dr. Paulo Almeida and Dr. Pam Seaton were very generous with their time and advice and helped me immensely. Susan Simmons, in the UNCW Math Department, was also very helpful with statistical analysis of data. Finally, the support and availability of various other faculty, staff, and students from the Chemistry, Biology, and Marine Science Departments were invaluable to me in addressing impromptu questions and making my graduate experience an enjoyable one.

My research and graduate studies at UNCW were funded in part by a research grant from the Center for Marine Science, and a research/teaching fellowship funded by the National Science Foundation.

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INTRODUCTION

At present, environmental regulatory requirements for allowable levels of substances rely heavily on LC_{50} and LC_{10} values (concentrations which result in 50% or 10% mortality of test organisms) derived from acute toxicity tests (Calow and Forbes, 2003; Hendley, 1999). Environmental concentrations are then monitored by traditional chemical analyses, where the concentrations found are correlated to LC values to determine the environmental threat. A criticism of this procedure is that LC_{50} values vary widely across species (Gallo et al., 1995; Mora et al., 2000; Oliveira-Filbo and Paumgartten, 2000; Reyes et al., 2002), and acute toxicity tests generally focus on a single substance without considering possible synergistic effects of multiple or chronic stressors. Traditional monitoring methods are often expensive and analyte-specific, and fail to quantify potential ecological effects such as reduced reproductive ability and compromised immune functions. These effects, though not immediately or obviously lethal, can weaken individual organisms and populations within the ecosystem, increasing susceptibility to low levels of additional chemical insults. In effect, traditional analyses are limited to identifying and quantifying specific analytes present in an ecosystem, without revealing their full effects on the environment.

When a pollutant enters the environment, it first causes an effect within an organism at the biochemical or cellular level, such as loss of membrane integrity (Grundy et al., 1996), appearance of stress proteins (Bagchi et al., 1996), or enzymatic effects (Davies et al., 1994; Escartin and Porte, 1996). These deficiencies may then present themselves as physiological or behavioral changes such as decreased heart rate (Lundebye et al., 1997) or impaired feeding activity (Petrauskien, 2003). Pollutant effects become more

noticeable through time, at higher ecological levels, working from the individual organism to the population as a whole, and finally becoming apparent as an unhealthy ecosystem. As time passes and pollution effects are observed at higher ecological levels, it becomes more difficult to trace an effect back to its source.

A biomarker may be any measurable biochemical, cellular, physiological, or behavioral change in an organism or population that indicates exposure to chemical pollutants (Depledge, 1994). By focusing on the intermediate, sublethal effects of a pollutant, this developing field aims to reveal environmental threats before obvious toxic effects such as death of organisms are observed. An ideal biomarker will also be non-destructive to the indicator organism. This not only avoids further depleting possibly endangered populations, but also allows repeated measurements from single organisms, minimizing problems with inter-individual variations. Non-destructive biomarkers tend to be less time-consuming in their methodology, and often require fewer reagents and less clean-up. In addition to providing an alternative, less expensive method of screening potentially contaminated aquatic environments in United States coastal regions, the use of biomarkers could also help to remedy the deterring expense of traditional environmental monitoring in developing countries (Sanchez-Hernandez, 1998; Wells et al., 2001). The aim of this research is to add to the database of non-destructive biomarkers that may be useful in indicating exposure of coastal ecosystems to organophosphate (OP) or carbamate pesticides.

The health of coastal areas is of special concern for many reasons. Estuaries provide nursery habitat and spawning grounds for numerous species, as well as supporting commercially important organisms such as oysters, mussels, clams, and crabs, and non-

commercial bird and other wildlife populations. Estuaries are also a natural buffer zone between land and coastal waters, absorbing and filtering out various pollutants from surface and groundwater. They are a dynamic environment, naturally experiencing constant fluctuations in temperature, pH, salinity, and dissolved oxygen and nutrients according to season, time of day, and rainfall and storm events. They are a naturally fragile ecosystem, and these traits may make estuaries more sensitive to contaminants than many other ecosystems.

Organophosphorus (OP) and carbamate insecticides make up a group of chemicals currently posing a threat to coastal ecosystems. Because of their relatively fast rate of decomposition, with half-lives on the order of weeks rather than years, these compounds have replaced the organochlorine (OC) pesticides prevalent several decades ago. However, OPs and carbamates may demonstrate severe acute toxicity and/or bioaccumulation in non-target species (U.S. EPA, 1998; 2000; 2003), and are heavily used, resulting in a persistent environmental presence despite their rapid degradation. The U.S. Environmental Protection Agency reported total U.S. pesticide use (including agricultural, industrial, commercial, government, and private sources) as 126 million pounds of active ingredient in 1999, 72% of which are organophosphate compounds (Donaldson et al., 2002). Southeastern North Carolina golf courses may apply up to 45+ pounds of chemicals per acre per year, and according to a list provided by Landfall Country Club, 40% may be OP pesticides (Landfall C. C. Manager, personal communication). These mobile compounds can travel quickly through sandy soil into local groundwater and surface water systems. The estuarine environment is especially susceptible to run-off pollution from agricultural, recreational, industrial and private

lands in the immediate region and miles upstream. Carbaryl concentrations exceeding U.S. Environmental Protection Agency criteria for healthy aquatic life have been documented in the Pungo River and Tar River tributaries in eastern North Carolina (McCarthy et al., 2000). In the Santee River Basin in coastal South Carolina, diazinon, parathion, carbaryl, chlorpyrifos, dieldrin, and malathion were among pesticides found to exceed aquatic life criteria (Maluk and Kelley, 1998).

OP pesticides act by inhibiting the neurological enzyme acetylcholinesterase (AChE; EC 3.1.1.7). The neurotransmitter, acetylcholine travels between nerve cells and binds to a receptor site on the receiving cell. It stimulates and causes the nerve cell to fire until it is hydrolyzed by AChE (Fig. 1; Spiro and Stigliani, 2003). However, if the enzyme is occupied (inhibited) by slow-hydrolyzing compounds such as OPs or carbamates, acetylcholine will not be hydrolyzed, and the nerve cell will continue to fire, resulting in paralysis and death. This enzyme is widespread throughout the animal kingdom, and its inhibition causes neurological response in humans and other animals in this same manner (Carlock et al., 1999; van Gemert et al., 2001). Its activity is thus an obvious choice for a biomarker indicating exposure to OP compounds. Carboxylesterase (CBE, EC 3.1.1.1) is a more general hydrolyser of esters, thought primarily to detoxify xenobiotics in the body, and is also inhibited by many OPs and carbamates (Galloway et al., 2002; Satoh and Hosokawa, 1995; Thompson et al., 1995; Yoon et al., 2003). CBE is found in almost all organisms, though only sporadically in insect species (Gopalan et al., 1997; Sogorb and Vilanova, 2002). This enzyme has also been proposed as a possible biomarker for OP/carbamate exposure, though it has not been studied to the extent that AChE has (Escartin and Porte, 1997; Galloway et al., 2002; Thompson et al., 1995).

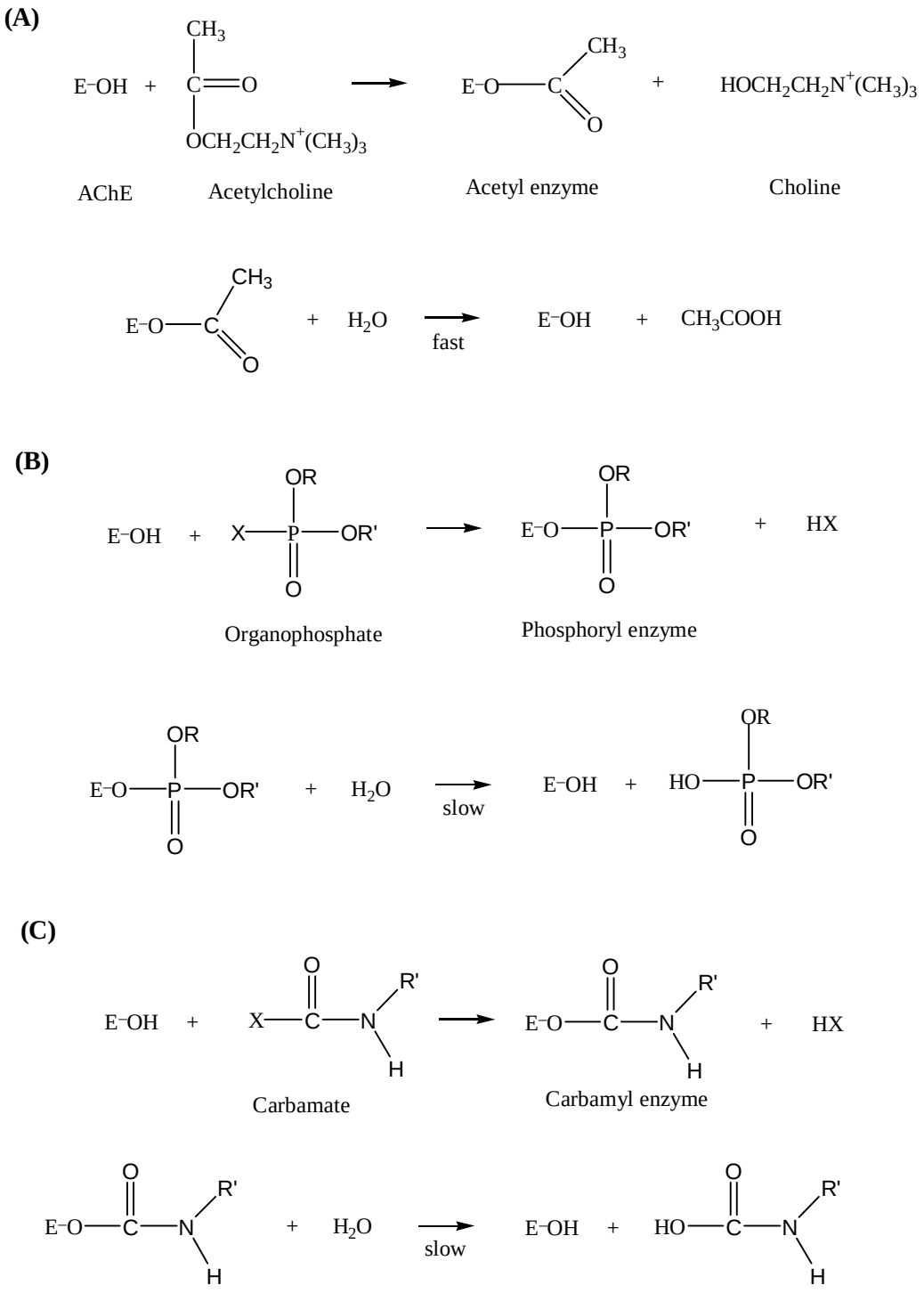
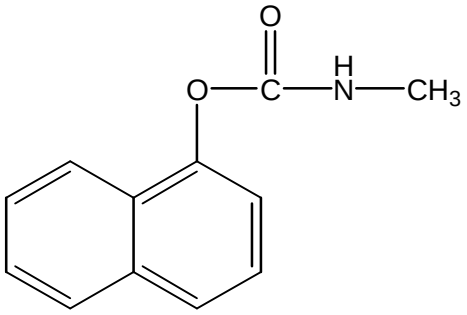


Figure 1. Reaction and subsequent hydrolysis of acetylcholinesterase (AChE) with **(A)** its natural substrate, acetylcholine, **(B)** organophosphate compound, and **(C)** carbamate compound, where E is the enzyme AChE, X is a leaving group, and R and R' are alkyl groups. Organophosphates and carbamates are considered to be AChE inhibitors because they bind to the enzyme like its normal substrate, but hydrolyze at a much slower rate, preventing its interaction with acetylcholine.

There have been few definitive studies done on crustacean enzyme activity as an OP/carbamate biomarker (Lundebye et al., 1997; Escartin and Porte, 1996). In southeastern North Carolina, and much of the eastern coast of the U.S., blue crabs, *Callinectes sapidus*, are a commercially important, and populous species. Phylogenetically, they are more similar to the insects targeted by pesticides than are the fish or mollusks that have been more extensively studied. It is therefore hypothesized that blue crab enzyme activity will be more susceptible to inhibition by OPs/carbamates, and a more sensitive biomarker of exposure. A recent study showed that juvenile blue crabs exposed to high levels of the carbamate pesticide carbaryl (300 μg/L) showed no change in respiratory rate or death until they molted, at which point 100% mortality was observed (Roer, personal communication, 2002). In this case, reported LC_{50} values are of little relevance, and a biomarker that demonstrates the presence of a sublethal effect would be more useful in ensuring the continued existence of a healthy population.

The objectives of this research were to compare enzyme character and OP/carbamate-induced inhibition of AChE and CBE in the blue crab, *Callinectes sapidus* and the ribbed mussel, *Geukensia demissa* with the results of other published studies investigating different marine species; and to determine the usefulness of these species' enzymatic responses as biomarkers for OP/carbamate exposure. Two organophosphates (paraoxon and diisopropylfluorophosphate (DFP)) and two carbamates (carbaryl and eserine) were tested (Fig. 2). Paraoxon is a metabolite of the OP insecticide parathion, which is produced when the parent compound is biologically metabolized, and is the toxic element of the pesticide (Thompson et al., 1995). Its use in the United States is limited to certain agricultural crops; it is not available for residential or commercial use (U.S. EPA, 2000).



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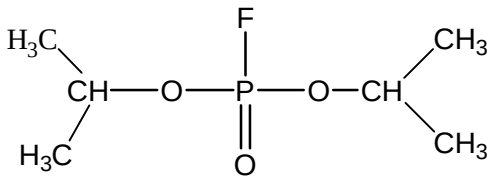

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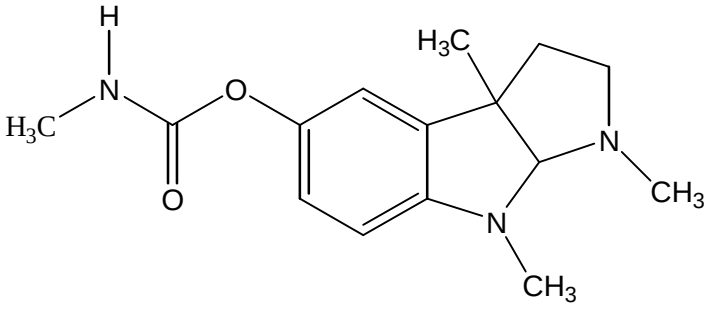

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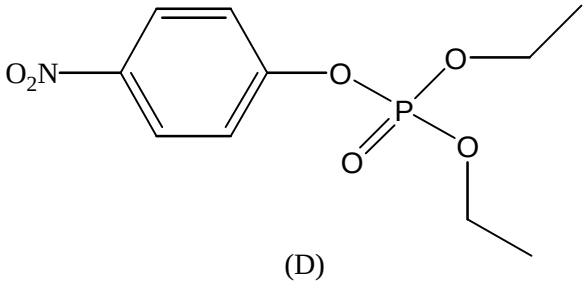


(D)

Figure 2. Chemical structure of test compounds: **(A)** Carbaryl is a carbamate insecticide heavily used in the U.S. **(B)** DFP (Diisopropylfluorophosphate) is an organophosphate often used to study the biochemical effects of OP compounds in humans and other animal species. **(C)** Eserine (physostigmine) is a carbamate used in medicine, which has shown inhibition towards AChE. **(D)** Paraoxon is a more toxic metabolite of the OP insecticide parathion.

DFP is a chemical used to study enzymatic effects of OP exposure in various species, particularly mammals (Kamata et al., 2001; Simonian et al., 1999; Stone et al., 2000). Carbaryl is a carbamate pesticide used extensively in U.S. agricultural, commercial, and private sectors (Maluk and Kelley, 1998; McCarthy et al., 2000; U.S. EPA, 2003). Eserine (physostigmine) is a carbamate used in the medical field to treat glaucoma and Alzheimer's disease, and to protect against nerve agent poisoning; extensive studies have been conducted on its enzymatic effects in mammals (Sienkiewicz-Jarosz et al., 2003; Wetherell et al., 2002).

MATERIALS AND METHODS

Chemicals and Equipment

Acetylthiocholine iodide, 5,5'-dithiobis-2-dinitrobenzoic acid (DTNB), tetra(monoisopropyl)pyrophosphor-tetramide (iso-OMPA), and physostigmine (eserine) were purchased from Sigma (St. Louis, MO). Butyrylthiocholine iodide was purchased from TCI (Tokyo, Japan). Phenylthioacetate was purchased from ICN Biomedicals (Aurora, OH). Diisopropylfluorophosphate (DFP) and diethyl *p*-nitrophenyl phosphate (paraoxon) were purchased from Aldrich (Steinheim, Germany). Carbaryl was purchased from Chem Service (West Chester, PA). All other reagents and solvents were analytical grade. Microplate reader was purchased from MTX Lab Systems, Inc. (Vienna, VA), and a centrifuge 5415 D was purchased from Eppendorf (Westbury, NY).

Sample collection

Ribbed mussels, *Geukensia demissa* were collected from the Intracoastal Waterway at Wrightsville Beach and kept in a 10-gallon aquarium in filtered, aerated seawater. They were fed 2 mL of shrimp-based invertebrate food three times per week and their water was changed weekly or if it became discolored. If individuals died in the holding tank, they were removed immediately.

Hemolymph samples were taken from the posterior adductor muscle of *G. demissa* using a 1-mL syringe containing an equal volume (0.2 - 0.3 mL) of marine bivalve physiological saline (0.017 M HEPES, 0.44 M NaCl, 0.11 M $MgSO_4$, 0.01 M KCl, 0.013 M $CaCl_2$ in de-ionized water, pH 7.3) to avoid coagulation, then centrifuged at 5000 x *g* for three minutes. Tissue samples were prepared by excising the digestive gland, draining excess fluid with a paper towel, and adding Tissue Homogenization Buffer (0.1 M Tris-HCl buffer pH 7.2 containing 0.25 M sucrose) (THB) in a 1:4 (w/v) ratio. Tissue samples were centrifuged at 10,000 x *g* for 10 minutes.

Blue crabs, *Callinectes sapidus* were taken from the Intracoastal Waterway behind Masonboro Island and kept separated in aquaria in filtered seawater. They were fed frozen shrimp and their water was changed every four days.

Hemolymph samples were taken from the basal arthrodial membrane of blue crabs using a 1-mL syringe containing an equal volume (0.2 - 0.3 mL) of marine crustacean physiological saline (0.1 M D-Glucose, 0.07 M NaCl, 0.03 M sodium citrate dihydrate, 2.6 mM citric acid monohydrate in de-ionized water, pH 7.5) to prevent coagulation. Tissue samples were taken by causing the crab to autotomize a walking leg, removing the

tissue, and adding THB in a 1:4 (w/v) ratio. Hemolymph and tissue samples were centrifuged at 12,000 x g for 10 minutes.

For all centrifuged samples, the supernatant was transferred to a clean micro-centrifuge tube and stored at 4°C until analysis (< 4 hours).

Characterization of Enzyme activity

A modification of Ellman's colorimetric technique (Ellman et al., 1961) was used to analyze AChE activity (Fig. 3). Aliquots of hemolymph or tissue supernatant (50 µL) were incubated in microtitre plate wells for five minutes with 150 µL of 270 µM DTNB to allow any endogenous reaction to occur. Substrate hydrolysis was then initiated by adding 50 µL of the cholinesterase substrate acetylthiocholine iodide (ACTC, 6 mM). CBE activity was measured similarly, using the carboxylesterase substrate phenylthioacetate (PTA, 3 mM). The sample volume for all crab CBE assays in this study was reduced to 25 µL based on preliminary data that suggested non-linearity. However, subsequent data confirmed linearity to 60 µL, so 50 µL aliquots could be used for every assay (Fig. 4). The thiocholine or thioacetate products react with DTNB to form the yellow thiolate anion of 5-thio-2-nitrobenzoic acid. Initial absorbance and ten-minute absorbance at 414 nm were measured. Enzyme activity was observed to be linear in this time period at the substrate concentrations used (Fig. 5), so the change in absorbance was divided by ten to get activity per minute. Michaelis-Menten kinetics were confirmed with both substrates in mussels and crabs.

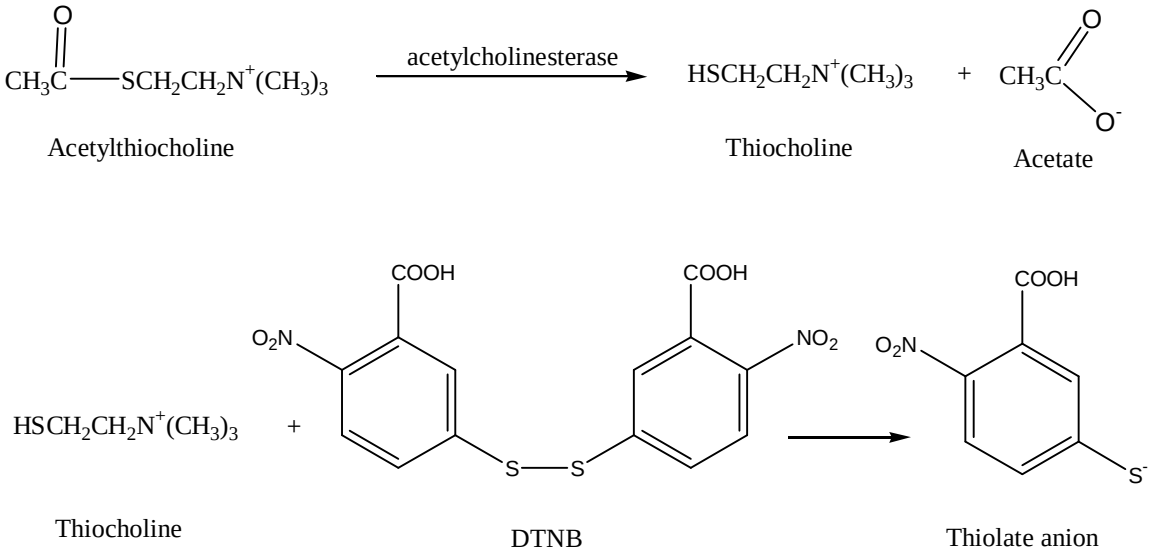


Figure 3. Colorimetric reaction used to determine enzyme activity. Substrate acetylthiocholine (or phenylthioacetate for carboxlyesterase assay) is hydrolyzed by AChE (or CBE) to thiocholine (or thioacetate), which reacts with DTNB to form the yellow thiolate anion of 5-thio-2-nitrobenzoic acid, a strong absorber at 414nm. Absorbance is directly proportional to thiocholine concentration, which depends on enzyme activity.

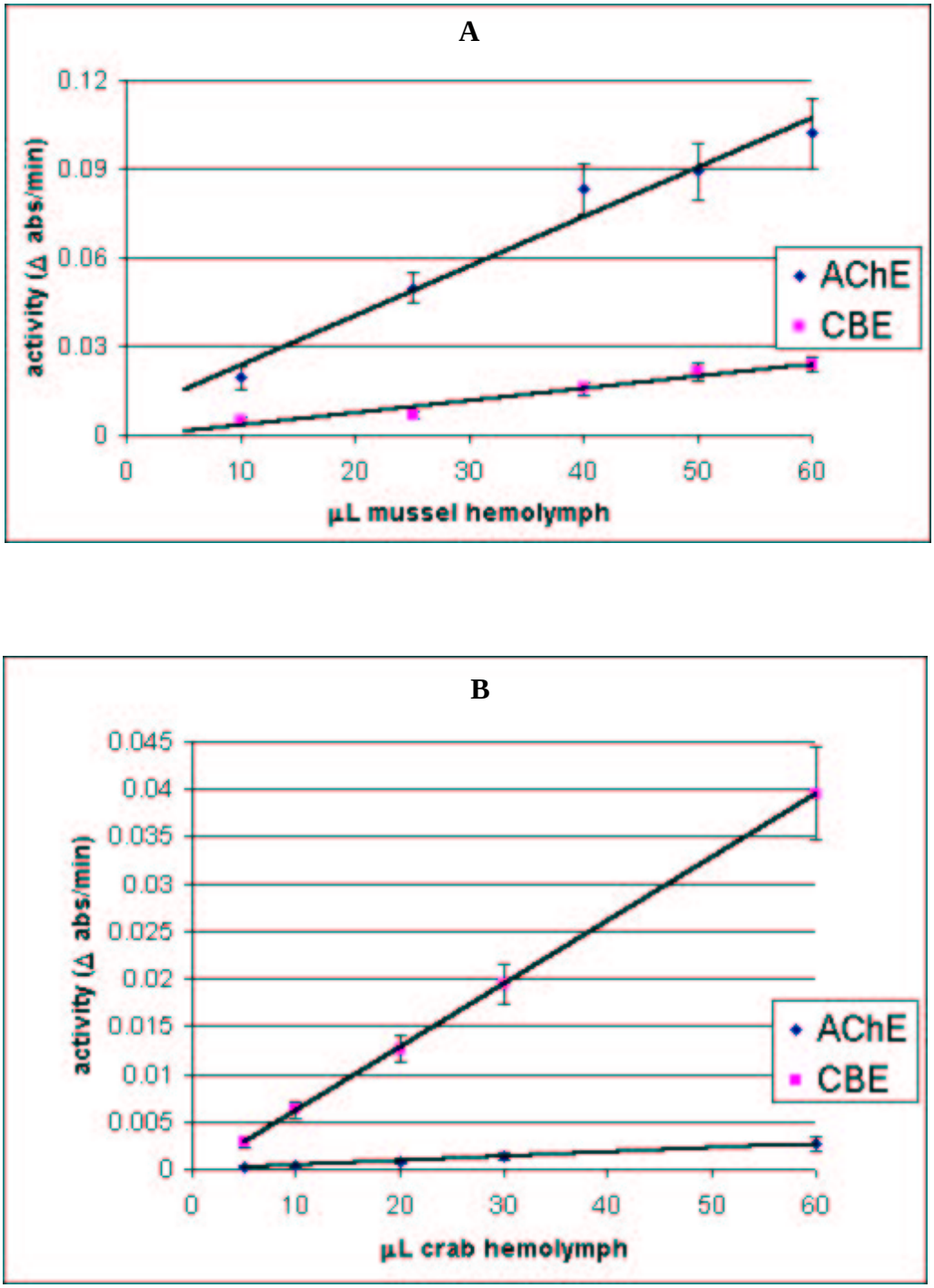


Fig. 4. Rate of enzyme activity relative to sample volume for **(A)** mussels and **(B)** crabs. Basic protocol was followed using both substrates (acetylthiocholine iodide and phenylthioacetate), varying only hemolymph volume. For all trendlines, $R^2 \geq 0.969$.

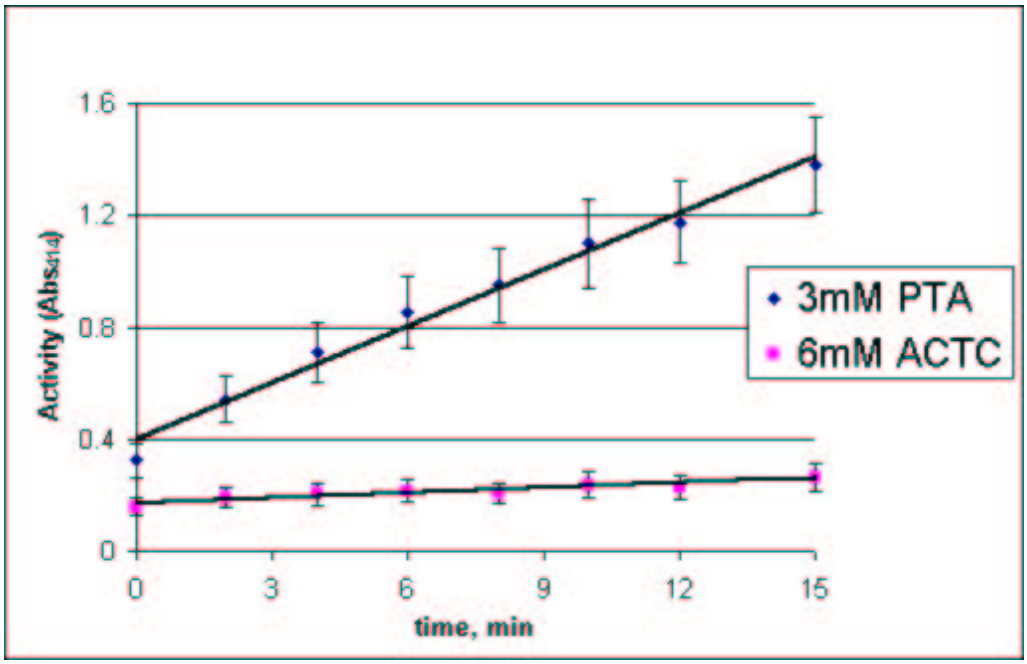


Fig. 5. Enzyme activity of blue crab (Δ Abs_{414}/min) at substrate concentrations used for assays (3 mM phenylthioacetate and 6 mM acetythiocholine iodide) over a 15-minute period. After confirming linearity, assays were subsequently conducted for ten minutes and the change in absorbance divided by ten to arrive at Δ Abs_{414}/min. R^2 = 0.985 and 0.8843, for PTA and ACTC, respectively.

In the Michaelis-Menten model:

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrows}} ES \xrightarrow{k_3} E + P$$

where [*E*] represents the enzyme (AChE) and [*S*] represents the substrate (ACh or ACTC), the rate equation for the formation of the complex [*ES*] is

$$\frac{\partial[ES]}{\partial t} = k_1[E] \bullet [S] - (k_2 + k_3)[ES].$$

With [E_o], initial enzyme concentration, equal to [*ES*] + [*E*], at steady-state when

$$\frac{\partial[ES]}{\partial t} = 0,$$

the concentration of the complex [*ES*] is given as:

$$[ES] = \frac{k_1[S] \bullet [E_0]}{(k_2 + k_3 + k_1[S])}.$$

Thus, the rate of formation of the product, [*P*] is equal to:

$$\frac{\partial[P]}{\partial t} = v = k_3[ES] = \frac{k_3[E_0] \bullet [S]}{K_m + [S]},$$

where

$$K_m = \frac{k_2 + k_3}{k_1}.$$

When [*S*] >>> K_m, the rate equation becomes:

$$\frac{\partial[P]}{\partial t} = k_3[E_0] = V_{\max}.$$

With V_{max} defined as the limiting rate, the equation becomes

$$v = \frac{V_{\max}[S]}{K_m + [S]}.$$

Transforming the equation by taking the reciprocal of both sides gives:

$$\frac{1}{v} = \left(\frac{K_m}{V_{\max}}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V_{\max}}.$$

Thus, plotting $\frac{1}{v}$ versus $\frac{1}{[S]}$ gives a linear relationship with $\frac{1}{V_{\max}}$ equal to the intercept and $\frac{K_m}{V_{\max}}$ equal to the slope. This is often referred to as the Lineweaver-Burk transformation.

For the enzymes studied, V_{max} was determined graphically from Michaelis-Menten and Lineweaver-Burk plots (see Figures 8 and 9). From the V_{max} value, the substrate concentration was selected to ensure the maximum rate for the reaction. K_m was also determined from the Lineweaver-Burk plot. Since K_m is equal to $\frac{k_2 + k_3}{k_1}$, it is a measure of the enzyme-substrate binding strength when $k_2 >> k_3$, and is useful in comparing enzymes.

Total protein was determined by incubating 4 µL of supernatant, diluted to 0.2 – 1.4 mg/mL with 200 µL of BioRad reagent (1:5 dilution in de-ionized water) for 15 minutes. The absorbance at 595 nm was measured, with bovine serum albumin as standard.

Mussel hemolymph regularly showed total protein concentrations lower than the calibration curve, though enzyme activity was apparent. For this reason, mussel data could not be normalized for protein. However, crab samples always showed measurable protein concentrations, so crab data was normalized for total protein using the following equation:

$$\frac{\Delta A_{414} \bullet Vol_T \bullet 1000}{1.36 \times 10^4 \bullet Vol_S \bullet [protein]_T}$$

where

ΔA_{414} = change in absorbance per minute

Vol_T = total assay volume (mL)

Vol_S = sample volume (mL)

1.36×10^4 = extinction coefficient of TNB (M^{-1} cm^{-1})

$[protein]_T$ = total protein concentration (mg mL^{-1})

Specific activity units are nmol min^{-1} mg^{-1}.

Results calculated as specific activity typically showed the same trends as non-normalized results, based solely on change in absorbance at 414 nm per minute, and reported as activity (Δ Abs_{414}/min). In order to compare more accurately trends between organisms, the non-normalized values are reported here for both crab and mussel data. *In vitro* and *in vivo* results are reported as percent activity remaining, with activity from acetone control groups set at 100%.

In vitro pesticide exposure

Hemolymph and tissue supernatant samples from individual organisms were incubated for 15 minutes with 10 µL of varying concentrations of carbaryl, DFP, eserine, or paraoxon in acetone, with both acetone and de-ionized water controls. Enzyme activity assays were then carried out as described above, and IC_{50} values were determined as the concentration of pesticide resulting in 50% inhibition of enzyme activity.

In vivo pesticide exposure

Live mussels were placed in 600-mL beakers (three mussels per beaker) containing aerated filtered seawater spiked with varying concentrations of carbaryl, DFP, eserine, or paraoxon in acetone, with acetone controls, for 24 hours.

Live blue crabs were separated in aquaria containing 6 L of filtered seawater spiked with varying concentrations of carbaryl, DFP, eserine, or paraoxon in acetone, with acetone controls, for 24 hours.

Enzyme activity was measured in hemolymph and tissue as described above, and *in vivo* IC_{50} values were determined from graphed data.

RESULTS

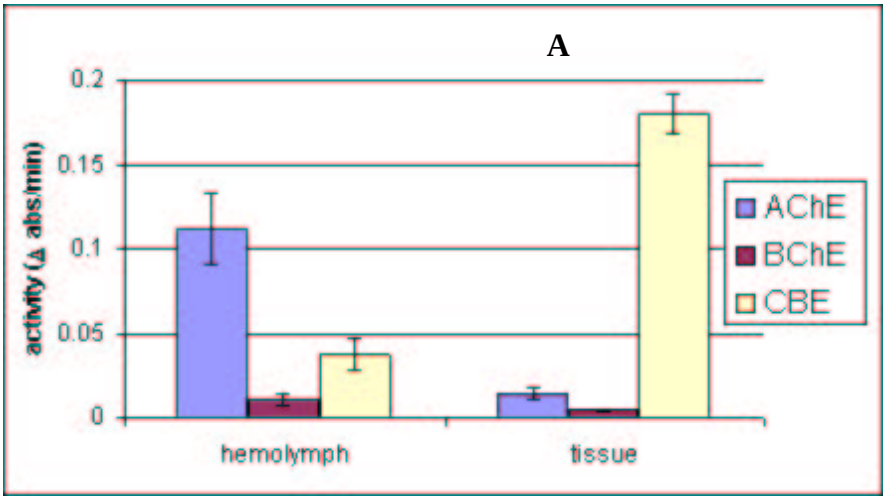
Characterization of enzyme activity

Limits of detection for this procedure were determined to be three standard deviations from the mean change in absorbance per minute of ten replicates of a buffer blank. For ACTC hydrolysis, the limits of detection were found to be 0.003 and 0.0009 absorbance units/min for mussels and crabs, respectively. For PTA hydrolysis, limits of detection were 0.009 and 0.001 absorbance units/min for mussels and crabs, respectively.

Enzyme distribution was determined by assaying hemolymph and tissue supernatant samples with the acetylcholinesterase (AChE) substrate acetylthiocholine iodide (ACTC), the butyrylcholinesterase (BChE) substrate butyrylthiocholine iodide (BCTC), or the carboxylesterase (CBE) substrate phenylthioacetate (PTA), and comparing change in

absorbance over ten minutes at 414 nm (Fig. 6). In blue crabs, hydrolysis of PTA predominated in both tissue and hemolymph, with tissue AChE showing much greater activity than AChE activity in hemolymph. According to the change in absorbance, CBE showed similar levels of activity in both hemolymph and tissue. When values were normalized for total protein, however, significantly greater CBE activity was seen in tissue than in hemolymph. In mussels, AChE activity was dominant in hemolymph and CBE predominated in tissue, with both enzymes showing a much greater rate of hydrolysis than in blue crabs. Low levels of BCTC hydrolysis were observed in hemolymph and tissue samples for both organisms.

Substrate concentrations ranging from 0.3 – 12 mM were tested to determine K_m and V_{max} values. V_{max} occurred at 3 mM ACTC in mussels (Fig. 7), and was approached at this concentration in crabs, but crab AChE continued to increase slightly with [ACTC] up to 12 mM. Mussel and crab hemolymph showed K_m values of 1.0 mM and 0.92 mM ACTC, respectively. Based on this data, 6 mM ACTC was used for the duration of this study for all AChE assays in order to maximize rate of reaction. V_{max} clearly occurred by 2 mM PTA in mussel tissue (Fig. 8), but the K_m value of 2.3 mM PTA was derived from a Lineweaver-Burke plot with a linear correlation coefficient, $R^2 = 0.5$, indicating variance in the data. V_{max} was not attained in crab hemolymph with PTA as substrate up to 12 mM, but dissolution of the substrate became a problem at concentrations greater than 3 mM. Subsequent analyses were conducted at 3 mM PTA. To ensure that assays were performed in the linear region of enzyme activity, a range of enzyme activities was tested, by varying sample volume. Hemolymph AChE and CBE activity were linear to 60 µL for mussels and crabs (see Fig. 4). All subsequent assays were performed using 50 µL samples, except 25 µL samples were used for blue crab CBE assays.



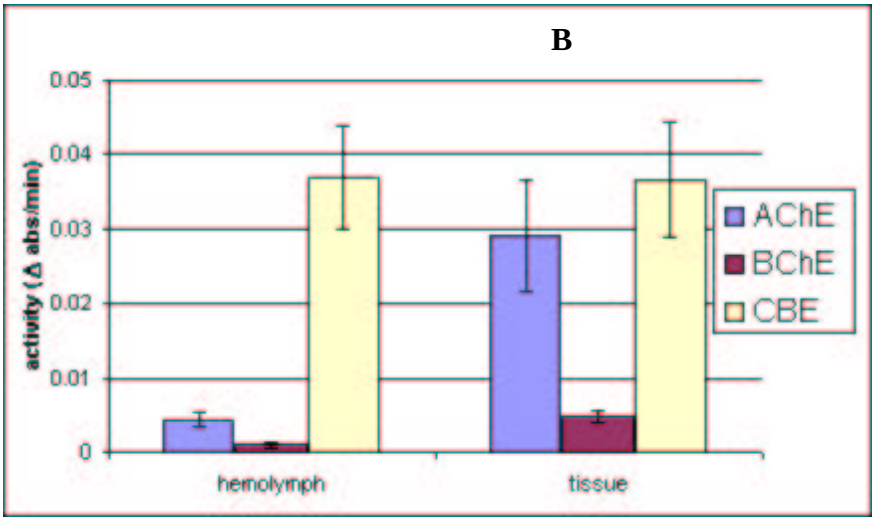


Fig. 6. Enzyme distribution in mussels (A) and crabs (B). Activity was measured by reacting 50 mL hemolymph and tissue samples with the cholinesterase substrate acetylthiocholine iodide, the butyrylcholinesterase substrate butyrylthiocholine iodide, and the carboxylesterase substrate phenylthioacetate. Mean activities were as follows: mussel hemolymph: 0.11+0.02 (AChE), 0.01+0.004 (BChE), and 0.038+0.009 (CBE) absorbance units per minute; mussel tissue: 0.014+0.003 (AChE), 0.004 + 0.0007 (BChE), and 0.18+0.01 (CBE) absorbance units per minute; crab hemolymph: 0.004+0.001 (AChE), 0.001+0.0004 (BChE), and 0.037+0.007 (CBE) absorbance units per minute; crab tissue: 0.029+0.007 (AChE), 0.005+0.0009 (BChE), and 0.037+0.008 (CBE) absorbance units per minute.

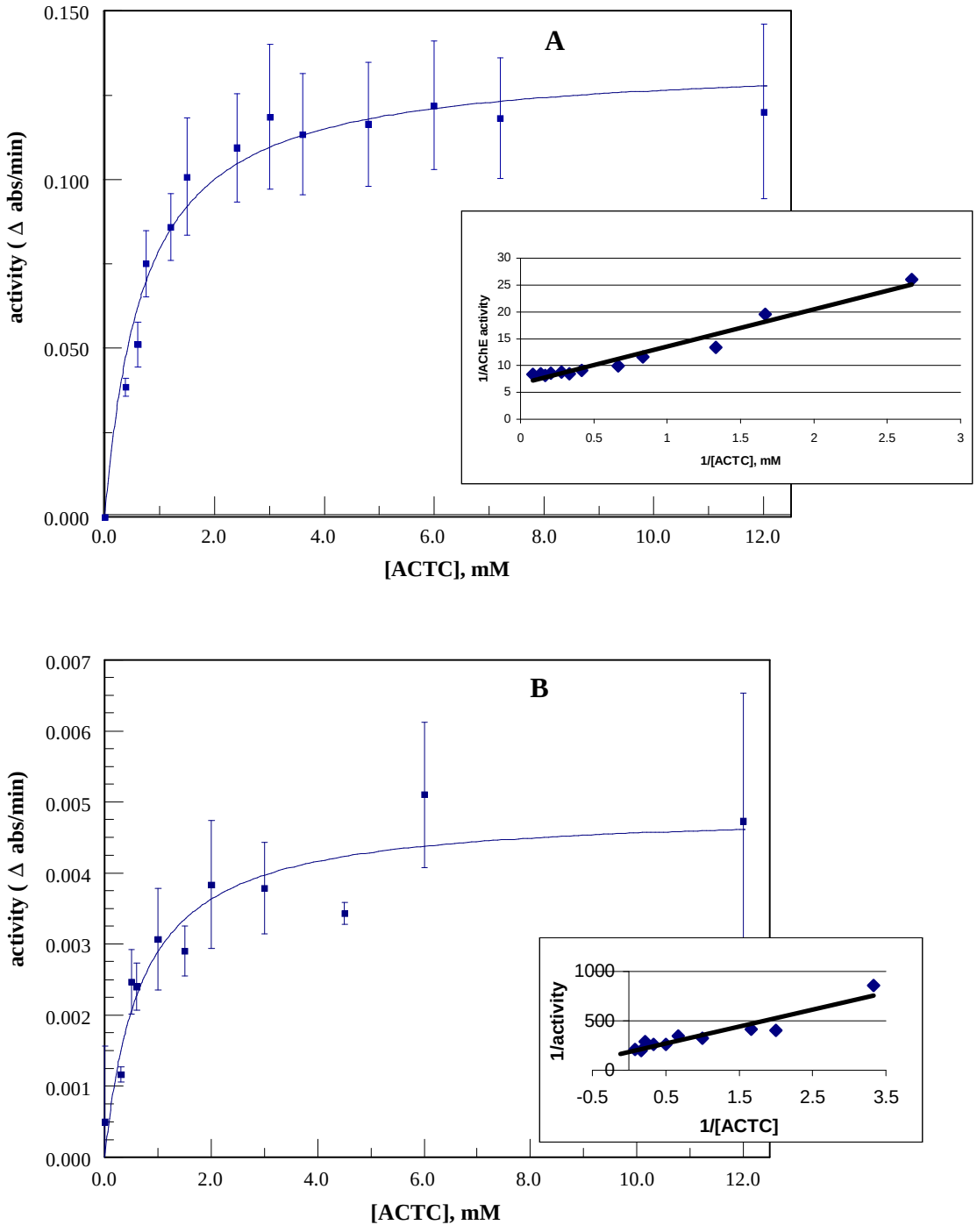


Fig. 7. Characterization of AChE activity relative to substrate concentration in **(A)** mussels and **(B)** crabs, with respective Lineweaver-Burk plots. K_m values for mussels were 0.70 mM and 1.1 mM ACTC from the Michaelis and Lineweaver-Burk plots, respectively. K_m values for crabs were 0.69 mM and 0.92 mM ACTC from the Michaelis and Lineweaver-Burk plots, respectively.

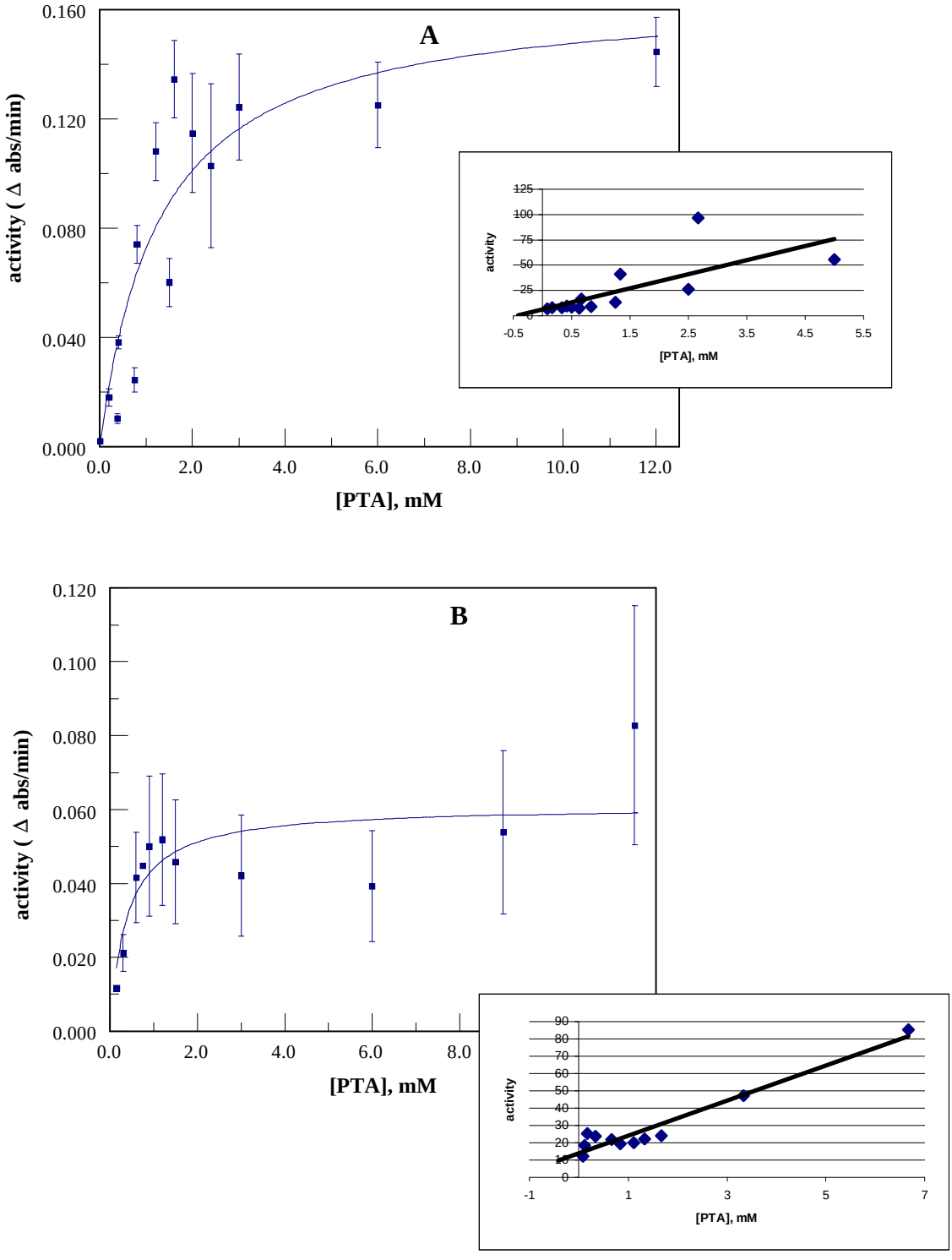


Fig. 8. Characterization of CBE activity relative to substrate concentration in **(A)** mussels and **(B)** crabs, with respective Lineweaver-Burk plots. K_m value for mussels, determined by the Michaelis plot, was 1.3 mM. K_m from Lineweaver-Burk plot is not reported due to variance in the data. For crabs, K_m values were 0.38 mM and 0.72 mM PTA from the Michaelis and Lineweaver-Burk plots, respectively.

In vitro pesticide exposure

In order to determine the direct effect of OP and carbamate pesticides on enzyme activity, centrifuged hemolymph and tissue samples were exposed *in vitro* to carbaryl, DFP, eserine, or paraoxon. The enzyme activity shown by an individual sample exposed to acetone was set at 100%, and the activities of the same sample exposed to varying pesticide concentrations were reported as percent activity remaining, relative to the acetone control. An average IC_{50} value (the concentration of pesticide resulting in 50% inhibition of enzyme activity) was determined for each enzyme in both tissue and hemolymph for each species (Table 1).

In mussels, hemolymph AChE was more sensitive to all inhibitors than tissue AChE (Fig. 9). Compounds in order of lowest to highest IC_{50} values (most inhibiting to least inhibiting) are eserine < DFP < carbaryl < paraoxon, with paraoxon having an IC_{50} value a thousand times greater than eserine. CBE also showed greater sensitivity in hemolymph than in tissue, except with paraoxon. Paraoxon was the only compound that resulted in at least 50% tissue CBE inhibition in the concentrations tested. In hemolymph CBE, the IC_{50} values increased from eserine < DFP < paraoxon < carbaryl, again with an order of magnitude difference in IC_{50} concentrations between eserine and carbaryl.

In crabs, AChE inhibition for each compound varied little between hemolymph and tissue (Fig. 10), except for paraoxon, which showed a tissue IC_{50} value of 7 μM, but had not reached 50% inhibition at the maximum concentration tested (17 μM) in hemolymph. AChE was most sensitive to carbaryl and eserine. Hemolymph CBE was more sensitive to paraoxon and DFP exposure than tissue CBE, while neither medium showed 50% inhibition at the maximum concentrations of carbaryl or eserine tested.

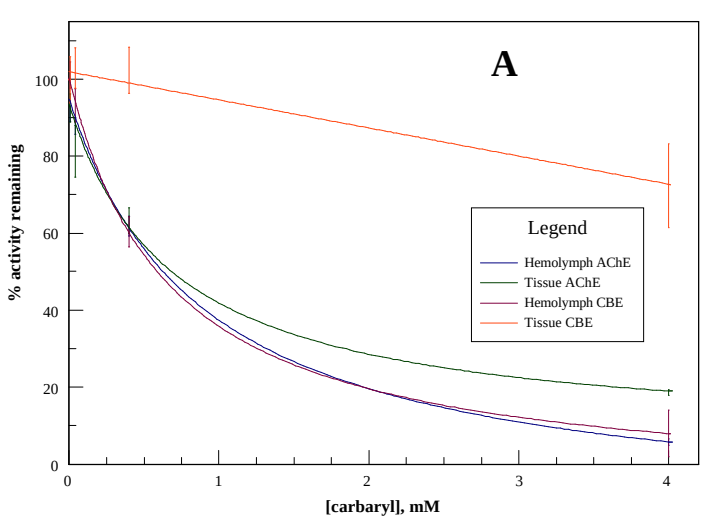
MUSSELS				
	HEMOLYMPH		TISSUE	
Inhibitor	AChE	CBE	AChE	CBE
Carbaryl	600	600	700	IC_{70} = 4000*
DFP	40	20	200	IC_{55} = 800*
Eserine	1	3	7	No inhibition to 50µM
Paraoxon	2000	600	1000	200

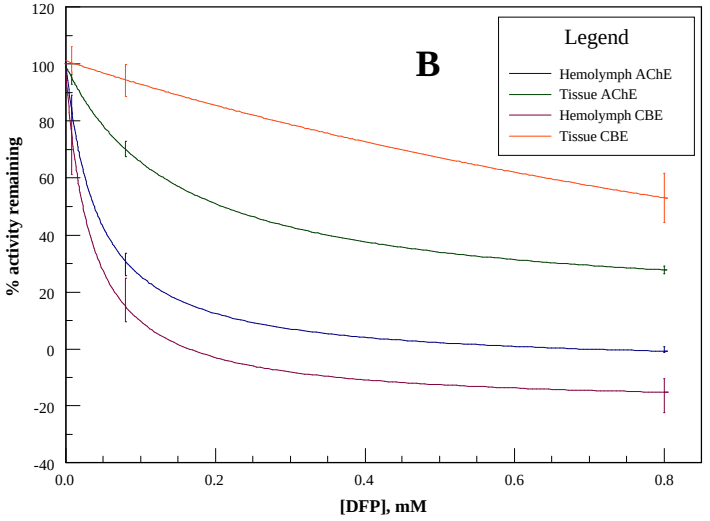
CRABS				
	HEMOLYMPH		TISSUE	
Inhibitor	AChE	CBE	AChE	CBE
Carbaryl	3	IC_{60} = 30*	1	IC_{85} = 40*
DFP	500	20	900	IC_{75} = 3000*
Eserine	5	IC_{95} = 20*	4	IC_{85} = 20*
Paraoxon	IC_{70} = 20*	0.3	5	20

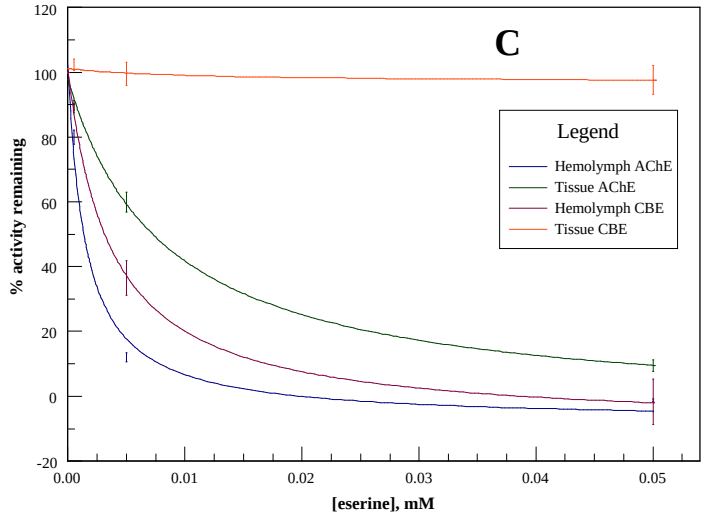
Table 1. IC_{50} values (in µM) derived from15-minute *in vitro* exposure of organisms to test compounds (sample size, n = 3-12). Values given are taken from *in vitro* data shown in Figures 9 and 10.

*Enzyme activities that did not reach 50% inhibition in the range tested are shown as IC_x, where x is the % activity remaining at the highest concentration tested.

Fig. 9. Response of mussel enzyme activity to 15-minute *in vitro* exposure to varying concentrations of **(A)** carbaryl, **(B)** DFP, **(C)** eserine, and **(D)** paraoxon. Each data point is the average of 3-12 different organisms. Activity of samples exposed to acetone (control) was set at 100%.


A
% activity remaining
[carbaryl], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE


B
% activity remaining
[DFP], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE


C
% activity remaining
[eserine], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE

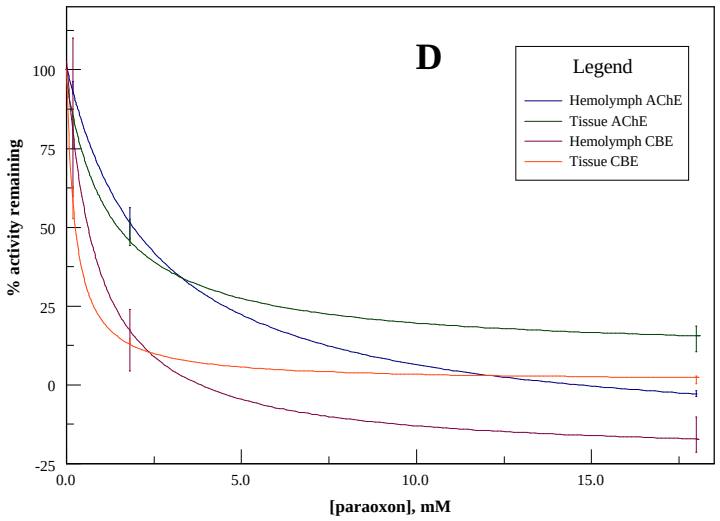
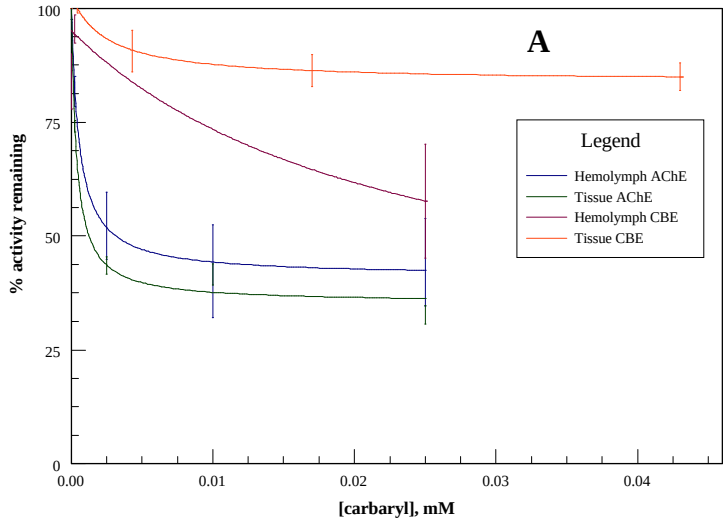
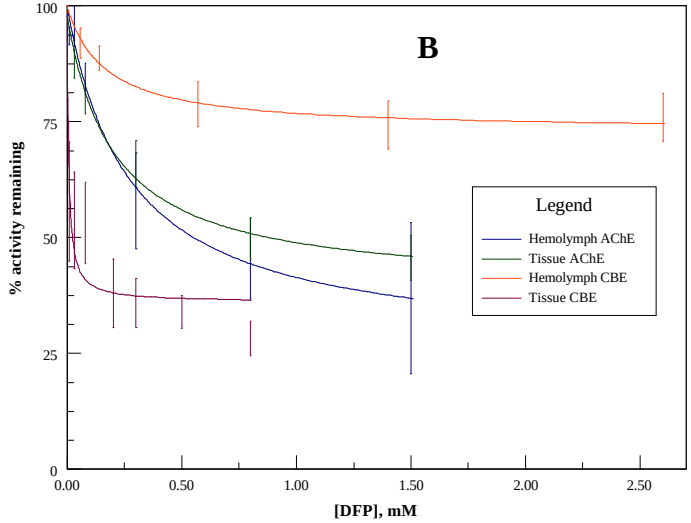
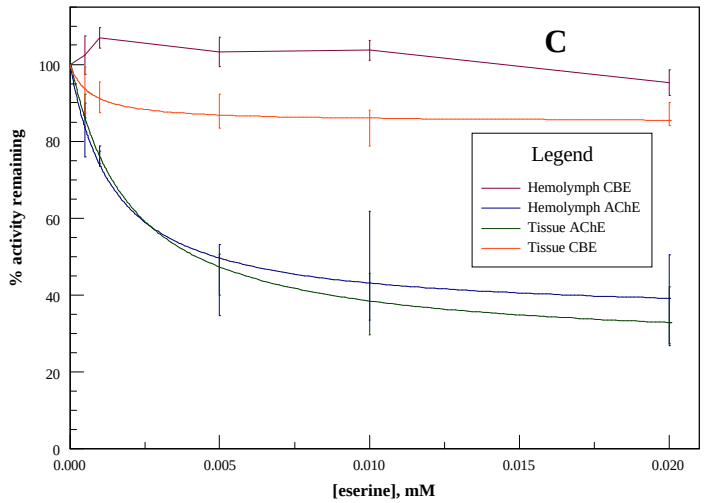
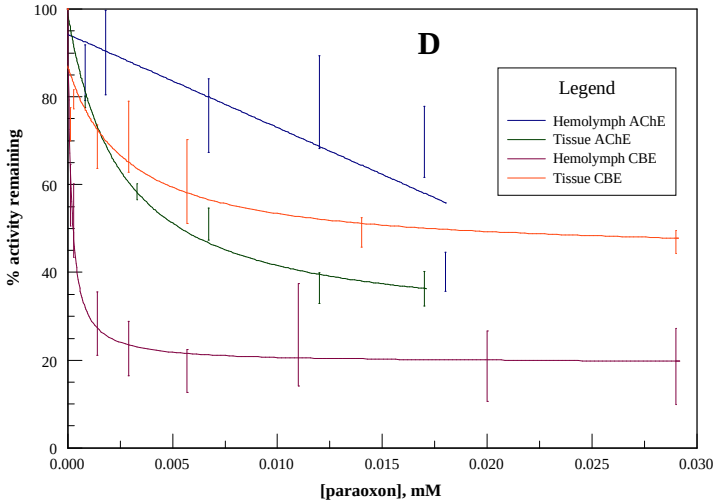

D
% activity remaining
[paraoxon], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE

Fig. 10. Response of crab enzyme activity to 15-minute *in vitro* exposure to varying concentrations of **(A)** carbaryl, **(B)** DFP, **(C)** eserine, and **(D)** paraoxon. Each data point is the average of 3-12 different organisms. Activity of samples exposed to acetone (control) was set at 100%.


A
% activity remaining
[carbaryl], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE


B
% activity remaining
[DFP], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE


C
% activity remaining
[eserine], mM
Legend
Hemolymph CBE
Hemolymph AChE
Tissue AChE
Tissue CBE


D
% activity remaining
[paraoxon], mM
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE

In vivo pesticide exposure

In vivo tests were performed to determine the enzymatic inhibition brought about by exposure of living organisms to OP and carbamate compounds. Organisms were exposed for twenty-four hours, and IC_{50} values were determined as above, with enzyme activity of control organisms set at 100% (Table 2).

In mussels, hemolymph AChE was inhibited by all compounds, in the following order of decreasing effect (IC_{50}): eserine > DFP > paraoxon > carbaryl (Fig. 11). Hemolymph CBE showed similar IC_{50} values, except 50% inhibition was not reached at the highest concentration of carbaryl tested (40 μM). Neither enzyme in mussel tissue was inhibited to 50% at non-lethal concentrations.

In crabs, hemolymph AChE was not inhibited to 50% at non-lethal concentrations of any compound tested (Fig. 12). Tissue AChE showed 50% inhibition only by eserine and DFP. CBE in either medium never reached an IC_{50} value at non-lethal concentrations except with DFP.

DISCUSSION

The results of this study indicate that in general, hemolymph is the more sensitive medium for measuring enzyme inhibition in both mussels and crabs. Although initial tests of enzyme distribution showed very low levels of carboxylesterase activity in mussel hemolymph, IC_{50} values (*in vitro* and *in vivo*) were established at lower contaminant concentrations in hemolymph CBE than in tissue CBE in almost every case. It was initially proposed that this disparity may be due to ineffective homogenization of tissue samples. However, significant activity was apparent in tissue CBE in enzyme

MUSSELS

	HEMOLYMPH		TISSUE		DEATH OCCURRED
Inhibitor	AChE	CBE	AChE	CBE	
Carbaryl	20	IC_{60} = 40*	IC_{60} = 40*	No inhibition to 40 μM	400
DFP	5	4	IC_{80} = 90*	IC_{80} = 90*	90
Eserine	0.03	0.02	0.1	No inhibition to 0.1 μM	0.1
Paraoxon	6	5	IC_{70} = 10*	10	100

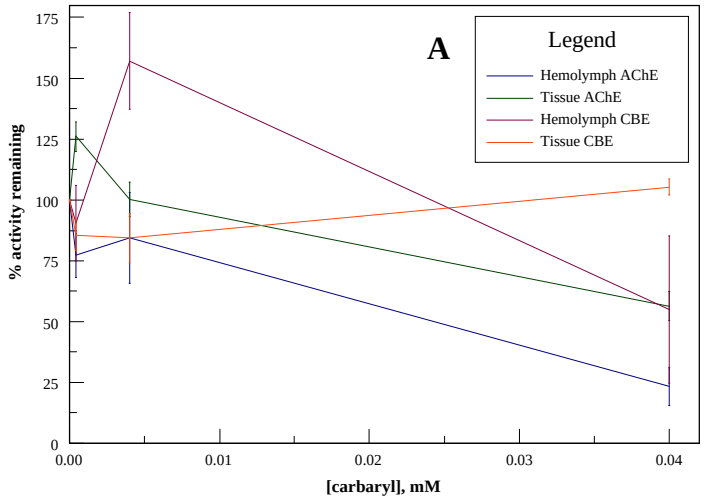
CRABS

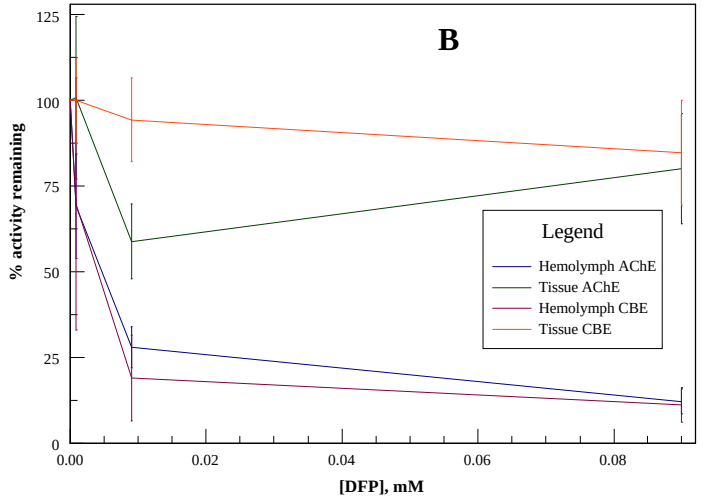
	HEMOLYMPH		TISSUE		DEATH OCCURRED
Inhibitor	AChE	CBE	AChE	CBE	
Carbaryl	IC_{80} = 1.5*	IC_{70} = 1.5*	IC_{60} = 1.5*	IC_{90} = 1.5*	1.5
DFP	No inhibition to 30 μM	5	30	30	30
Eserine	IC_{70} = 100*	IC_{70} = 100*	10	IC_{70} = 100*	> 100
Paraoxon	No inhibition to 0.5 μM	No inhibition to 0.5 μM	No inhibition to 0.5 μM	No inhibition to 0.5 μM	1

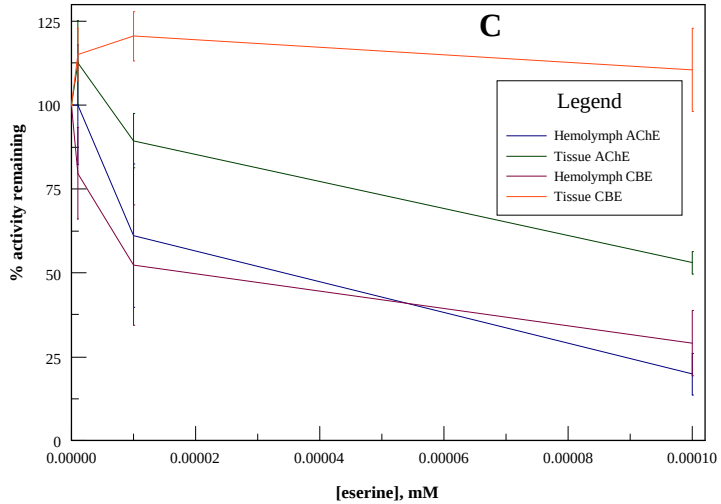
Table 2. IC_{50} values (in μM) derived from 24-hour *in vivo* exposure of organisms to test compounds (sample size n = 3-6). Values given are taken from *in vivo* data shown in Figures 11 and 12. The concentration at which death began to occur in test organisms is reported in the final column. No crabs died during exposure to eserine.

*Enzyme activities that did not reach 50% inhibition in the range tested are shown as IC_x, where x is the % activity remaining at the highest concentration tested.

Fig. 11. Response of mussel enzyme activity to 24-hour *in vivo* exposure to varying concentrations of **(A)** carbaryl, **(B)** DFP, **(C)** eserine, and **(D)** paraoxon. Each data point is the average of 3-12 different organisms. Activity of organisms exposed to acetone (control) was set at 100%.


A
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[carbaryl], mM
0
25
50
75
100
125
150
175
0.00
0.01
0.02
0.03
0.04


B
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[DFP], mM
0
25
50
75
100
125
0.00
0.02
0.04
0.06
0.08


C
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[eserine], mM
0
25
50
75
100
125
0.00000
0.00002
0.00004
0.00006
0.00008
0.00010

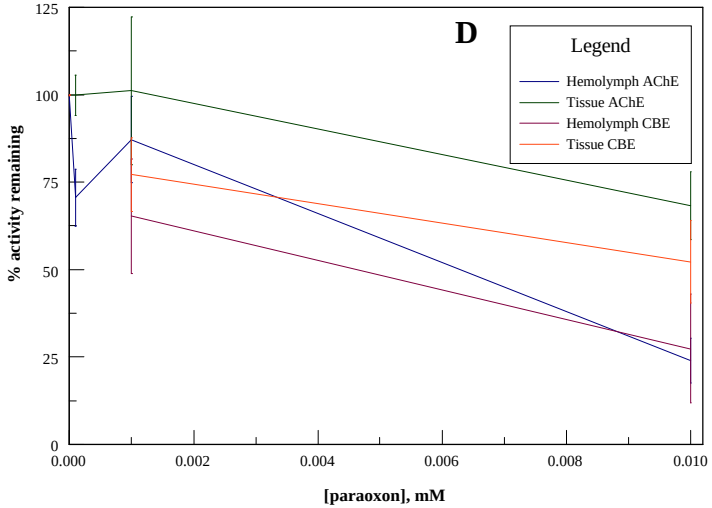
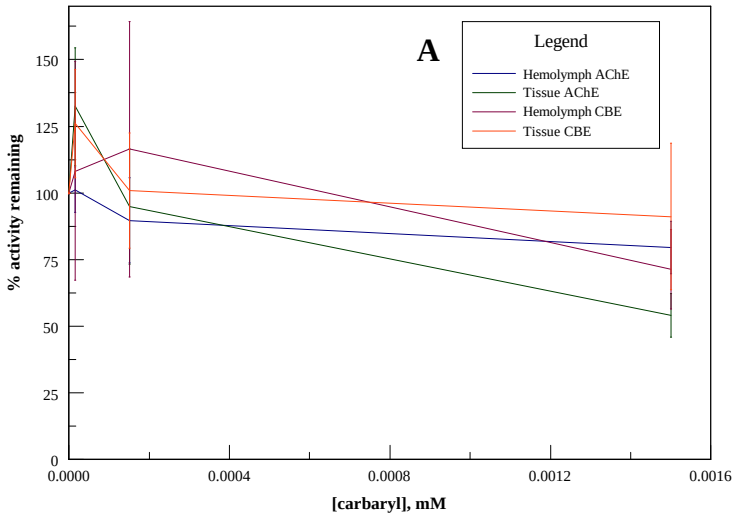
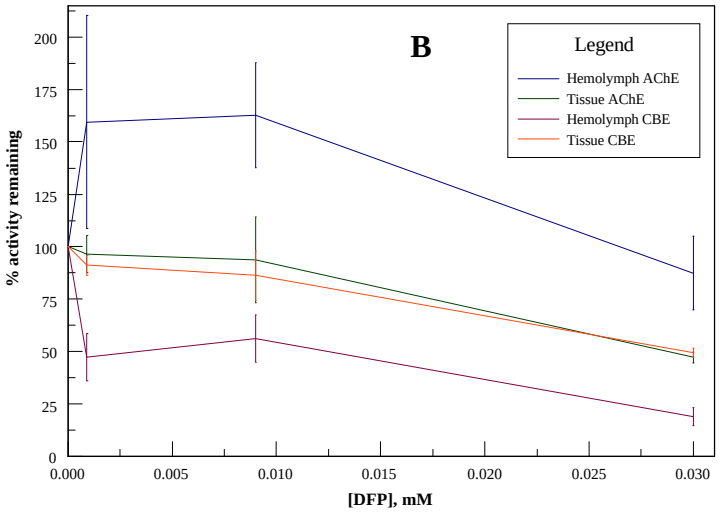
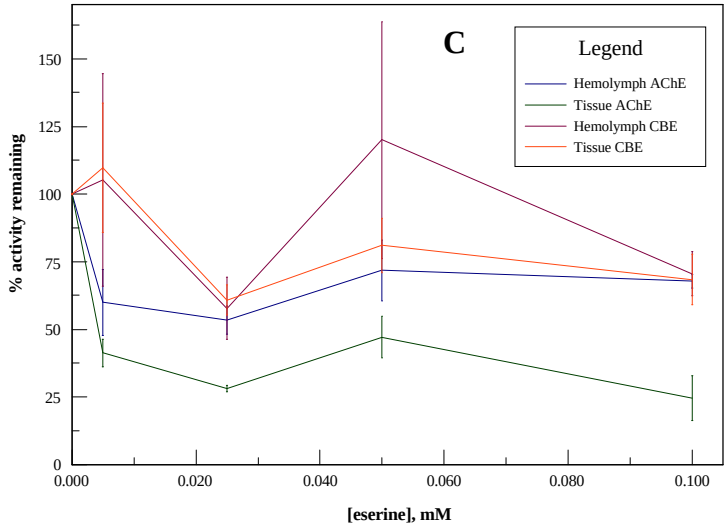
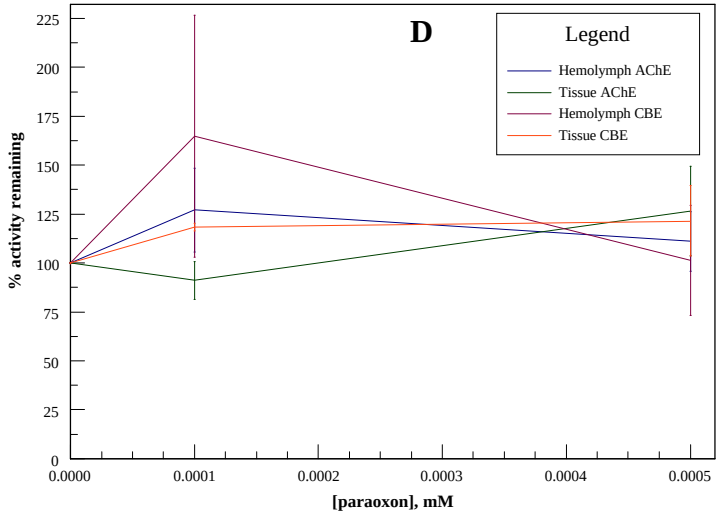

D
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[paraoxon], mM
0
25
50
75
100
125
0.000
0.002
0.004
0.006
0.008
0.010

Fig. 12. Response of crab enzyme activity to 24-hour *in vivo* exposure to varying concentrations of **(A)** carbaryl, **(B)** DFP, **(C)** eserine, and **(D)** paraoxon. Each data point is the average of 3-12 different organisms. Activity of organisms exposed to acetone (control) was set at 100%.


A
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[carbaryl], mM
0
25
50
75
100
125
150
0.0000
0.0004
0.0008
0.0012
0.0016


B
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[DFP], mM
0
25
50
75
100
125
150
175
200
0.000
0.005
0.010
0.015
0.020
0.025
0.030


C
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[eserine], mM
0
25
50
75
100
125
150
0.000
0.020
0.040
0.060
0.080
0.100


D
Legend
Hemolymph AChE
Tissue AChE
Hemolymph CBE
Tissue CBE
% activity remaining
[paraoxon], mM
0
25
50
75
100
125
150
175
200
225
0.0000
0.0001
0.0002
0.0003
0.0004
0.0005

distribution tests, indicating efficient transfer of enzyme activity to the aqueous medium. A comparison was also made between tissue homogenization methods, and there was no significant difference in activity between homogenization by grinding and shaking and by shaking alone. Regardless, the relative sensitivity of hemolymph enzymes to OP and carbamate compounds is promising to the search for a non-destructive biomarker. One difficulty with using enzyme inhibition as a biomarker for contaminant exposure is the significant variability of responses. This variability encompasses differences between invertebrate species, differences among individuals within a species, and differing responses to individual compounds within a chemical class. Studies done on the inhibitory effects of OP and carbamate compounds on an array of aquatic invertebrates have produced varied results (Table 3). Dembele et al. (2000) found that 1nM (0.36 ppb) chlorpyrifos inhibited brain AChE activity in the common carp, *Cyprinus carpio* by 85%, while Boone and Chambers (1997) found that 50% inhibition in brain AChE activity in the mosquitofish, *Gambusia affinis,* required a 50-fold greater concentration. The latter study also found that 60 nM (17 ppb) paraoxon resulted in 50% inhibition of muscle AChE activity, while the present study concluded that 500 nM (140 ppb) paraoxon had no inhibitory effect on muscle AChE activity in the blue crab, *Callinectes sapidus*. Cusack (1998) found that 0.08 mM (15 ppm) acephate caused 28% muscle AChE inhibition in the ribbed mussel, *Geukensia demissa*, while 4 mM (750 ppm) acephate showed no inhibitory effect in the fiddler crab, *Uca pugilator*.

Individual variation in enzyme activity was also noted. Varo et al. (2003) have demonstrated an age-dependent correlation in AChE inhibition in European sea bass (*Dicentrarchus labrax*) exposed to the OP pesticide dichlorvos, but no correlation

Organism	Treatment	Effect	Author
Tropical scallop *Euvola ziczac*	$2.85x10^{-4}$ nM (0.1 ppt) chlorpyrifos	45% hemolymph AChE inhibition	Owen, et al., 2002
Common carp *Cyprinus carpio*	1nM (0.36 ppb) chlorpyrifos	85% brain AChE inhibition	Dembele, et al., 2000
Mosquitofish *Gambusia affinis*	6 nM (2 ppb) chlorpyrifos	50% muscle AChE inhibiton	Boone and Chambers, 1997
	50 nM (18 ppb) chlorpyrifos	50% brain AChE inhibition	
Mosquitofish *Gambusia affinis*	60 nM (17 ppb) paraoxon	50% muscle AChE inhibition	Boone and Chambers, 1997
Blue crab *Callinectes sapidus*	0.5 μM (140 ppb) paraoxon	no effect on hemolymph or muscle AChE (100% mortality at 1μM)	Caveny, present study
Ribbed mussel *Geukensia demissa*	6 μM (1.8 ppm) paraoxon	50% hemolymph AChE inhibition	Caveny, present study
Shore crab *Carcinas maenas*	8.7 μM (2 ppm) dimethoate	24% hemolymph AChE inhibition	Lundebye, et al., 1997
Blue mussel *Mytilus edulis*	10 μM (2.8 ppm) paraoxon	38% hemolymph AChE inhibition	Galloway, et al., 2002
Fiddler crab *Uca pugilator*	80 μM (15 ppm) acephate	28% muscle AChE inhibition	Cusack, 1998
Ribbed mussel *Geukensia demissa*	4mM (750 ppm) acephate	no effect on muscle AChE	Cusack, 1998

Table 3. Comparison of AChE sensitivity among aquatic organisms.

between size of *G. demissa*, or sex or size of *C. sapidus* and enzyme activity was observed among control organisms in this study. Another important factor in intra-species variability should be the general health of the organism, and the extent of exposure to other stressors. Increased volumes of water across gill tissues would increase the amounts of contaminants an organism is exposed to, so organisms moving or filtering more rapidly, such as those located in areas where food is less plentiful, may show more potential for inhibitory effects. This may be difficult to account for when collecting specimens from different test sites, but an adequate sample size should produce an average result that can be compared to averaged enzyme activities of organisms from "clean"/control sites.

Finally, significant differences in enzymatic and whole organism response were observed for different test compounds, even of the same chemical class. In this study, death occurred in blue crabs at significantly lower contaminant concentrations than in ribbed mussels in all compounds tested except eserine. *In vivo* 24-hour exposure to 0.1 μM eserine resulted in 25% mortality in mussels, while no crabs died as a result of 24-hour exposure to 100 μM eserine. Carbaryl, the other carbamate studied, gave opposite results: 40% crab mortality was seen at 1.5 μM, and 100% mortality at 5 μM in a 24-h exposure period, while no mortality occurred in mussels at 40 μM (400 μM carbaryl did result in 100% mortality). These two compounds should act in a physiologically similar manner, reversibly inhibiting acetylcholinesterase; however, a number of studies have shown insensitivity to OPs/carbamates among specific enzymes in some species (Bocquene et al., 1997; Da Silva et al., 2003; Galloway et al., 2002; Mora et al., 1999). In general, AChE and CBE activities in this study followed similar trends; however there

were some discrepancies (see Figures 12 and 13). From these results, it appears that monitoring activities of a combination of enzymes would make enzymatic inhibition a more effective biomarker of exposure. Eserine is the only compound tested that elicited greater sensitivity from an enzyme in crab tissue than in hemolymph. This single incidence, however, does not lessen the advantages and simplicity of using a non-destructive biomarker, and does not warrant the regular monitoring of tissue enzymes to determine contaminant exposure.

In some cases in this study, death occurred in the organisms before enzyme activity was significantly inhibited. In enzymes found in hemolymph, this only occurred in crabs. Given this, it would seem that *G. demissa* provide a better non-destructive biomarker, despite the fact that they are often less sensitive to chemical insults than *C. sapidus*. Other advantages of using mussels over crabs is the potential for containing them in a mesh bag in an area exposed to runoff, and monitoring the same individuals over time to detect changes in aquatic health. Mussels are easier organisms to work with physically than crabs, and this researcher found that hemolymph drawn from blue crabs tended to thicken and become useless for enzyme assays much more quickly than hemolymph drawn from mussels.

Although this study found contaminant concentrations resulting in measurable enzyme inhibition to be greater than those normally found in monitored aquatic environments (Loewy et al., 1999; Neumann et al., 2003), the usefulness of this biomarker should not be discounted. Runoff into actual aquatic sites will rarely be comprised of a single chemical, so organisms will be faced with multiple stressors, the

cumulative concentrations of which may exceed allowable concentrations of any given one.

In conclusion, the results of this study indicate that the ribbed mussel, *G. demissa* is a better indicator organism than the blue crab, *C. sapidus*, due to its enzymatic inhibition at contaminant concentrations well below lethal levels. The sensitivity of hemolymph enzymes together with the advantages of a non-destructive technique suggest that hemolymph, rather than tissue, is the preferred source. Since AChE and CBE responded differently to different contaminants in some cases, and because performing assays for both enzymes requires little more effort than an assay for a single enzyme, it seems that an assay for both enzymes would produce more valuable information. However, based on the results of this study, it cannot be said definitively that *G. demissa* is an appropriate sentinel species for indicating pesticide exposure. From the *in vivo* data shown in Table 2, it is predicted that a body concentration of contaminant would have to reach the high-ppb to low-ppm range in order to produce 50% enzyme inhibition. The likelihood of this occurring would depend not only on the cumulative concentrations of contaminants released into the water column, but also on the efficiency with which these organisms metabolize and depurate their body burden. Other research has shown that bivalves can be a suitable sentinel species for pesticide contamination (Owen et al., 2002), so perhaps a different species of bivalve would be more sensitive to pesticides of concern.

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