Environ Monit Assess (2010) 165:331–340
DOI 10.1007/s10661-009-0949-y

Acetylcholinesterase activity in *Corbicula fluminea* Mull., as a biomarker of organophosphate pesticide pollution in Pinacanauan River, Philippines

Kimberly S. Beltran · Glorina N. Pocsidio

Received: 6 November 2008 / Accepted: 18 April 2009 / Published online: 12 May 2009

Abstract Organophosphates are known to inhibit the enzyme acetylcholinesterase. In this study, the AChE activity from the total soft tissues of *Corbicula fluminea* Mull. was used as a biomarker of organophosphate pollution in Pinacanauan River. Clams were collected from two different sites and at different seasons of the year. A colorimetric assay on the total soft tissues of the clams showed a directly proportional relationship between enzyme activity and condition of the riverine system. In vitro experiments on the total soft tissue, adductor muscles, digestive glands, and gills were conducted to assess the degree of localization of AChE as well as the sensitivity and tolerance of the enzymes in these tissues to varying concentrations of malathion. The degree of enzyme localization from highest to lowest is as follows: adductor muscle > gills > digestive gland whereas sensitivity to OP from greatest to least is: gills > adductor muscles > digestive gland.

K. S. Beltran (✉) · G. N. Pocsidio
Institute of Biology, College of Science,
University of the Philippines,
Diliman, Quezon City, 1101, Philippines
e-mail: kimbys2003@yahoo.com

K. S. Beltran
Department of Biology, College of Arts
and Sciences, University of the Philippines,
Ermita, Manila, Philippines

Keywords Acetylcholinesterase · *Corbicula fluminea* · Malathion · Pinacanauan River

Introduction

The rapid increase in the world's population today has led to a vast demand for food, which paved the way for people to seek ways on how to increase and improve crop yields (Eto 1974). The introduction of pesticides has been one of the major breakthroughs in the field of agriculture today. Organophosphate (OP) pesticides are among the most widely used since these compounds are known to be effective in combating pests, at the same time are readily degraded and hence, are less persistent in various environmental compartments. The mechanism of toxicity of OPs involves the inhibition of the enzyme acetylcholinesterase (AChE) that is responsible for the rapid hydrolysis of the neurotransmitter acetylcholine (ACh). The inhibition of the AChE enzyme leads to excessive amounts of acetylcholine in synapses and neuromuscular junctions resulting to functional signs and symptoms of cholinergic toxicity. Thus, AChE has been extensively used as a biomarker of OP pollution both in terrestrial and aquatic environments. Although AChE has been widely used as a specific biomarker of OP pollution, very few studies have examined the effects of OP insecticides to aquatic invertebrates especially those

belonging to the benthic class, particularly clams and mussels (Day and Scott 1990).

In this study, a freshwater bivalve mollusk species, the Asiatic clam *Corbicula fluminea* Mull. was studied for its possible utility as biomonitor of OP pollution. As cited by various authors, the obvious advantages for bivalves in environmental monitoring include the following: (1) limited mobility and abundance which would make sampling and collection relatively easy and inexpensive (Hartley and Johnston 1983; Muncaster et al. 1990; Mora et al. 1999); (2) they are not dependent on any type of host to complete their reproductive cycle (Barnes and Riggert 2000); (3) a single adult provides sufficient tissue to permit analysis for organic and inorganic contaminants allowing for a measure of individual variability not permitted by use of smaller organism (Muncaster et al. 1990); (4) they are known to be sensitive to different types of pollutants and have the ability to accumulate organic pollutants and heavy metals (Soucek et al. 2001).

The present work provides some necessary data that could be the basis for the possible use of the clam *C. fluminea* AChE activity in vivo or in vitro for environmental monitoring. Specifically, it determined (1) levels of changes in AChE activity in the soft body tissues of clams collected from two sites along the Pinacanauan River in Northern Philippines that differ in their level of associated agricultural practices, in particular, the use of organophosphate pesticides, at different seasons of the year; (2) if changes in AChE activity of the clam can be correlated with the level of OPs in the water, abiotic factors, and seasonal changes; and (3) differential distribution of clam tissue AChE and sensitivity of tissue enzymes to malathion, an OP commonly used in the locality.

Materials and methods

Sampling location

The site of collection of bivalves was the Pinacanauan River, a major tributary of the Cagayan River. It is situated in the Northern Philippines between 17°27′30″ N and 121°50′ E (Fig. 1). The Pinacanauan River traverses the municipalities of Peñablanca, Tuguegarao, San

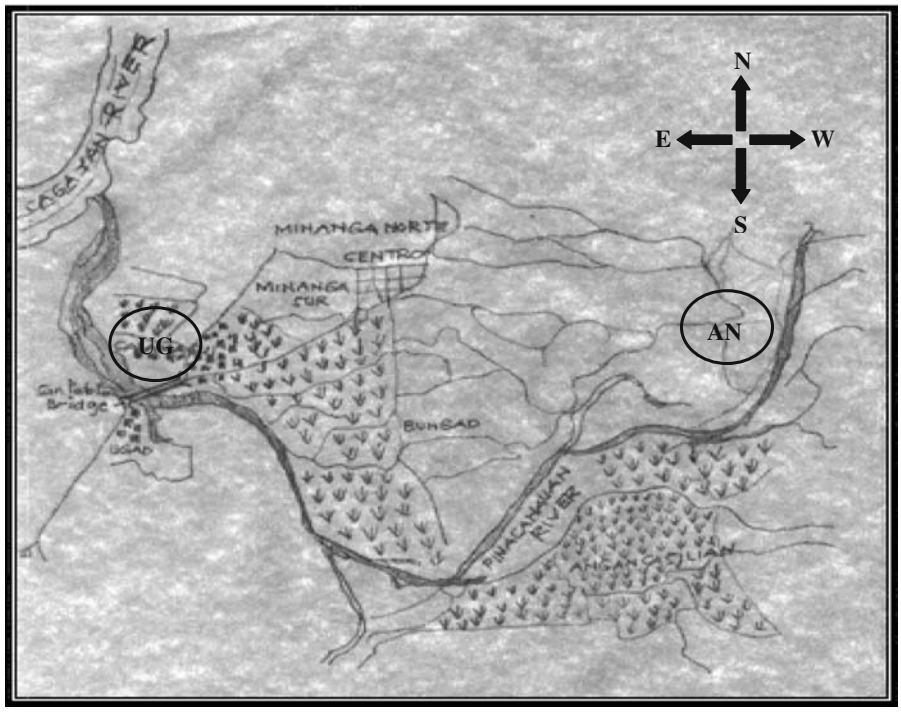

Fig. 1 Map of the Pinacanauan River showing the two sampling sites. It is located in Northern Isabela between 17°27′30″ North and 121°50′00″ East (*UG* Ugad site, *AN* Annanuman site). *Source: UP Diliman Main Library*

Pablo, and Cabagan where rice and corn are well-established crops and use of OP pesticides is rampant. This river provides numerous benefits to the people in Northern Philippines as a source of drinking water, home for fisheries, transportation route, source of irrigation water, and for recreation. The water body is essential not only for its physical, ecological, and biological aspects but also for its economic importance. Two sites were selected namely: Ugad (UG) and Annanuman (AN). Selection of sites was based on the presence of *C. fluminea* as well as the level of anthropogenic activities in the locality. Land alongside the river in Ugad is intensively used for agricultural crops like corn, rice, and tobacco. Annanuman, on the other hand, is sparsely populated; the land uncultivated, thus, was used as a reference or control site in this study.

Hydrological parameters

The physico-chemical quality of the two study areas was monitored in situ every time samples were collected. The surface temperature, pH, salinity, and dissolved oxygen were obtained using, respectively, an ordinary mercury thermometer, hand-held pH meter, salinometer, and by standard Winkler method (Adams 1990). At each collection, measurements were done on three consecutive days at 0900 hr in the morning.

Chemical analysis of water samples

During each collection from the study sites (specifically the Ugad site), a water sample was collected in 500 mL acetone–hexane-rinsed amber glass bottles and stored at 4°C on ice and transported (within 10 h) to the National Pesticide Analytical Laboratory (NPAL) in Quezon City for organophosphate analysis with the use of GC-NPD Analysis (NPAL 1990).

Sample collection and preparation

C. fluminea with a shell length ranging from 2.5 to 3.0 cm (average adult size) were collected in three batches each made up of six individuals in June 2005 of the hot-dry season, September 2005 of the wet season and December 2005 of the cool-dry season. Individual clams were picked from the streambeds by hand and placed immediately inside a plastic bag filled with ice. The clams were dissected over ice within 1 to 2 h after collection. The total soft tissues were dissected out, weighed, wrapped in aluminum foil, labeled, and stored in liquid nitrogen.

Biochemical determination of acetylcholinesterase activity

The method used combined features of the modified Ellman et al. (1961) and the method by Dellali et al. (2001) and Bonacci et al. (2004).

Total soft tissues were homogenized at 4°C in a 1:5 ratio (w/v) of 0.1 M phosphate buffer optimized at pH 7.5. Centrifugation of the homogenates was done at $12,000 \times g$ for a period of 30 min, after which supernatants were obtained and immediately used for assay of AChE activity.

AChE activity was measured using acetylthiocholine iodide (ASChI) as a substrate. ASChI is hydrolyzed by AChE, producing thiocholine and acetic acid. The thiocholine released from the hydrolysis is made to react with 5,5′-di-thio-bis-2-nitobenzoic acid (DTNB) yielding the yellow compound 5-thio-2-nitrobenzoic acid (TNB) which absorbs at 412 nm. In this study, 50 μl of supernatant was placed into a reaction mixture consisting of 850 μl phosphate buffer (0.1 M, pH 7.5), 1.875 mM DTNB, and 50 μl of 2 mM ASChI to start the enzymatic reaction. Within a 30-min period, change in optical density per minute was noted using a Beckman/Spectronic spectrophotometer set at 412 nm for measurement. The temperature of the medium was maintained at 25°C. AChE activity was expressed in nmol min^{-1} mg $protein^{-1}$. The quantity of proteins present in the supernatant was determined using the Bradford Method (Bradford 1976) with Coomasie Brilliant Blue G-25 as a reagent and bovine serum albumin as a standard. Absorbance for protein determination was read at 595 nm.

In vitro exposure experiments

Thirty clams were collected in three batches during the month of December from the reference site and were brought to the laboratory for

depuration in aged tap water within a period of 24 h. Afterwards, the clams were dissected for their total soft tissues, gills, adductor muscles, and digestive glands. The different tissues were separately pooled, weighed, minced, and centrifuged at a rate of $12,000 \times g$. The supernatants were incubated in solutions of malathion (Bayer Company) for 5, 10, and 15 min at 25°C in a shaking water bath. The following concentrations were used: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. For the assay, a volume of 50 µl of the incubated supernatant was added to 850 µl phosphate buffer, 50 µl DTNB, and 50 µl ASChI. Samples from the reaction mixtures were tested for hydrolytic activity at 1-min intervals. Likewise, as in the in vivo assessment, AChE activity was expressed in nmol min^{-1} mg $protein^{-1}$. The experiments were conducted alongside negative controls that were not exposed to the pesticide.

Statistical analysis

All determinations were performed in triplicate for each single sample (in vivo) or pooled supernatant (in vitro) and results were expressed as mean value ± SEM. The data were tested for homogeneity of variance. After normal distribution was established, analysis of variance (ANOVA) was run to test for significant differences between treatments. The multiple comparisons test (Tukey's post hoc) was done to determine which values differed significantly. The entire statistical analysis was carried out using SPSS Software version 10.0. Level of significance was set at $p < 0.005$.

Results

In vivo and in situ AChE activity

Figure 2 shows the mean ± SEM AChE activity values of *C. fluminea* from the two sampling sites at different times of the year. The Ugad site exhibited AChE activity of 43.39 ± 4.16 nmol min^{-1} mg $protein^{-1}$ in June, 9.11 ± 1.06 nmol min^{-1} mg $protein^{-1}$ in September, and 13.62 ± 0.65 nmol min^{-1} mg $protein^{-1}$ in December. On the other hand, Annanuman displayed an average AChE activity of 50.55 ± 0.49 nmol min^{-1} mg $protein^{-1}$ in June, 31.97 ± 0.86 nmol min^{-1} mg $protein^{-1}$ in September, and 37.19 ± 0.47 nmol min^{-1} mg $protein^{-1}$ in December. Similar trends of varying AChE activity was exhibited in both sites. Generally, the lowest enzymatic activity was seen in September, the highest activity in June. Statistical analysis revealed that clams collected from Annanuman site had significantly higher activity than those from Ugad site in September and December but not in June.

The hydrological characteristics of the river body registered a normal range in pH, temperature, salinity, and DO in all represented seasons

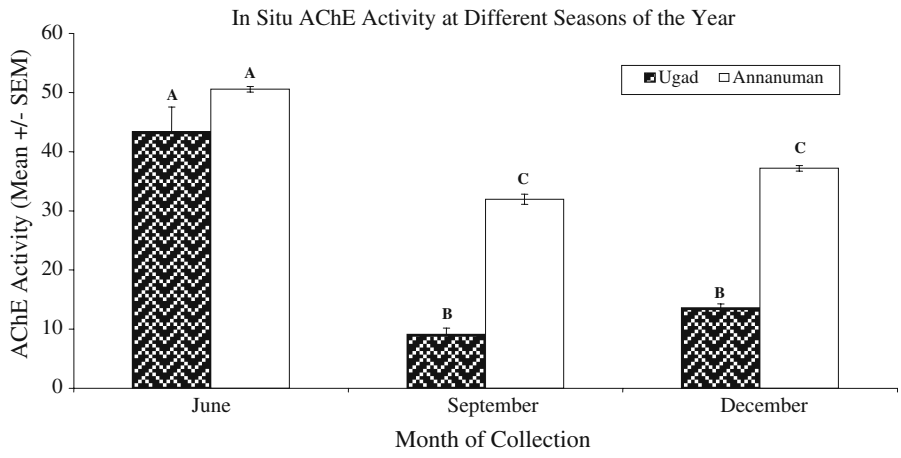

Fig. 2 Variation in AChE activities (mean ± SEM) in the total soft tissue of *C. fluminea* collected from Ugad and Annanuman during hot-dry (June), wet (September), and cool-dry (December) seasons. *Means with different letters on the bar* indicate significant difference in AChE activity based on the Tukey's test at $p < 0.005$

of the year. The pH level varied from 7.4 to 7.5 in both sampling sites. Both sites also exhibited the same surface water temperature of 28°C in June and September. A noticeable decrease to 24 and 25°C in sites 1 and 2, respectively, was recorded during the month of December. Dissolved oxygen (DO) ranged from 9.10 to 9.35 mg/L in site 1 and 9.20–9.45 mg/L in site 2 while salinity ranged from 0.5 to 0.85 ppt in site 1 and 0.55–0.75 in site 2. The lowest level was recorded from site 2 in June although this did not differ much while the highest salinity was recorded from site 2 in September. The National Pesticide Analytical Laboratory reports no traces of OPs in the water samples from June to December 2005 which only emphasizes the short life of OP in the water, hence, the relevance of assessment of biological impact.

In vitro effects of OP pesticides

The in vitro experiment was primarily utilized in order to provide a reference value with which relative inhibition can be estimated. Furthermore, in this part, the amount of AChE present in selected tissues was investigated. The digestive glands, gills, and adductor muscles were chosen because they have been reported to contain significant amounts of AChE (Escartin and Porte 1997). Results of the in vitro experiments are shown in Figs. 3 and 4, which depicted only the values obtained within 15-min exposures in as much as these did not vary significantly from those obtained during 5- and 10-min exposures.

In the control set-up, it was demonstrated that the adductor muscles contained the highest amounts of the enzyme at activity levels of 154.77 ± 0.20 nmol min^{-1} mg $protein^{-1}$. Next were the gill tissues, which displayed a mean enzyme activity of 27.49 ± 0.14 nmol min^{-1} mg $protein^{-1}$ and the digestive gland tissue rendering the lowest activity of 17.52 ± 0.30 nmol min^{-1} mg $protein^{-1}$. Levels of AChE activity in the tissues significantly differ from each other. The total soft tissues registered a mean AChE activity of 40.39 ± 0.57 nmol min^{-1} mg $protein^{-1}$. Furthermore, a dose-dependent response in all tissue AChE was observed in Fig. 3. The adductor muscles which displayed a mean enzymatic activity of 154.77 ± 0.20 nmol min^{-1} mg $protein^{-1}$ were reduced to 84.91 ± 0.25 nmol min^{-1} mg $protein^{-1}$ when exposed to the lowest malathion concentration which is 10^{-6} M. This was further lessened to 19.35 ± 0.38 nmol min^{-1} mg $protein^{-1}$ upon exposure to 10^{-2} M which happens to be the highest

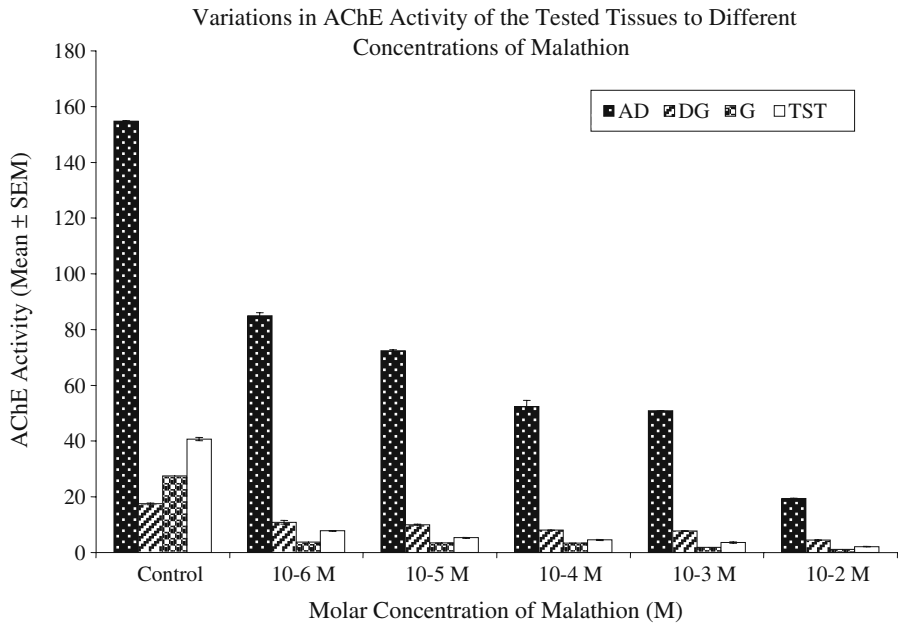

Fig. 3 Variations in the AChE activity of the tested tissues during a 15-min exposure to different concentrations of malathion. All tissues revealed a highly significant difference in AChE content and activity with $p < 0.005$ (*DG* digestive gland, *AD* adductor muscle, *G* gill, *TST* total soft tissue)

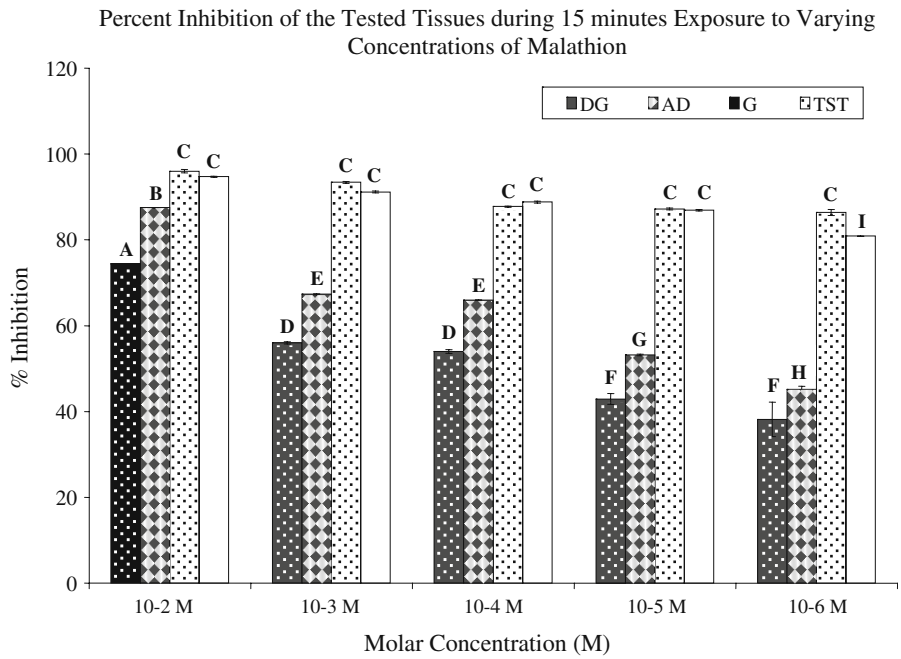

Fig. 4 Percent inhibition of the tested tissues during 15-min exposure to varying concentrations of malathion. *Means with different letters on the bar* indicate significant difference in AChE activity based on the Tukey's test at $p < 0.005$ (*DG* digestive gland, *AD* adductor muscle, *G* gill, *TST* total soft tissue)

concentration of malathion used in this study. Similarly, the digestive gland showed the same pattern. It registered a mean AChE activity of only 10.84 ± 0.29 nmol min^{-1} mg $protein^{-1}$ under 10^{-6} M and 4.49 ± 0.20 nmol min^{-1} mg $protein^{-1}$ under 10^{-2} M. The gill and total soft tissue were the most vulnerable since their enzyme activity went down to 3.75 ± 0.21 nmol min^{-1} mg $protein^{-1}$ and 7.78 ± 0.15 nmol min^{-1} mg $protein^{-1}$ upon exposure to the lowest concentration. The enzyme activity was further reduced upon exposure to highest concentration registering an enzyme activity of only 1.1 ± 0.10 and 2.16 ± 0.17 nmol min^{-1} mg $protein^{-1}$, respectively.

Results of the exposures to varying concentrations of malathion, however, did not parallel the results obtained in the control set-up. Figure 4 shows the percent inhibition of the enzyme in different tissues as a result of the exposure. Gill AChE was found to be most susceptible. A marked percent inhibition of 96.00 ± 0.37% upon 15-min incubation of the enzyme to the same pesticide concentration was noted. Gill AChE has an IC_{50} of 0.0000075 mM. This pattern of response was also observed in the case of the total soft tissue AChE since its percent inhibition went up to 94.69% during a 15-min exposure to the highest pesticide concentration (10^{-2} M) and at the lowest dose of pesticide exhibited 80.88% inhibition at the maximum duration of exposure for the assay. It revealed an IC_{50} of 0.000007 mM. Digestive gland AChE, on the other hand, although occurring at lowest activity levels in the unexposed condition, was found to be quite tolerant. A 15-min exposure to the highest pesticide concentration yielded a significantly lower inhibition of 74.37%. Calculated IC_{50} of digestive gland AChE is 0.33 mM. On the other hand, the adductor muscle AChE was inhibited by 87.48 ± 0.06% at 10^{-2} M within the same period of exposure. Calculated IC_{50} of the adductor muscle is 0.0011 mM.

Discussion

The present study provides evidence that AChE activity of *C. fluminea* may be correlated to the characteristics of ambient water. Ugad is a highly populated area, at the same time surrounded by large rice and cornfields treated with OPs. The

heavy downpour of rains due to successive storms experienced between and during the months of September and December was likely responsible for the decreased AChE activity observed in the clams from the area during the two seasons. The heavy rain caused agricultural run-offs, which brought about influxes of pesticides into the river.

The data on the physico-chemical characteristics of the water system rendered no relationship with that of the AChE activity of the clams since the obtained values were all within normal range. A slight change in salinity observed in Ugad and Annanuman site especially during the wet season (September), which paralleled the lowering of the AChE activity during this month, could have some effect though very minor or none at all. Pfeifer et al. (2005) has reported that salinity can affect the activity of AChE but this was observed in marine water. In this study, salinity may have only very slight influence since the observed range was normal for freshwater systems.

The chemical analysis of water samples derived from the Ugad site gave no trace of OP pesticides. This is because OPs like malathion relatively have a short half-life of about 10–20 days in the water column (Eto 1974). These synthetic chemicals are also subject to degradation by air, UV light, chemical hydrolysis, and soil microbes; the very reason why the organochlorine compounds previously used as pesticide were removed and replaced by OPs (Galloway et al. 2002; Cooper and Bidwell 2006). The Pinacanauan River is a lotic system, with constant water flow. The water currents might have immediately carried away the leached OPs from the surrounding farmlands before the collection of the water samples. Various authors like Bedford et al. (1968), Muncaster et al. (1990), and Cairns et al. (1991) have alleged that chemical analysis may not effectively predict the potential for impact on aquatic system since total contaminant loads may not always match those concentrations that are bioavailable. A study by Pereira et al. (1996) assessing the occurrence and accumulation of pesticides and organic contaminants in river sediments and *C. fluminea* tissue has also shown that chlorpyrifos was present in clam tissue but not in dissolved and particular phases at the same location. These results suggest that chemical analysis alone may not accurately indicate the compounds that have entered the water at the particular location, although its use as a method of detection is inevitable since data derived from this analysis may also be useful. This phenomenon only emphasizes the importance of the use of biomarkers.

Clams from Annanuman consistently showed high AChE activities. The relative reduction of AChE activity observed during the second season of collection may be attributed to the flooding caused by the heavy downpour of rain during these months. Other works had suggested the probable importance of other factors. Varela and Ausperger (1996) reported that AChE activity of *Elliptio complanata* did not vary significantly even upon exposure to pollution. The authors attributed this to the ability of the mussels to close its valves upon exposure thereby isolating itself from the environment. This observation was also confirmed recently by several authors who demonstrated that valve closure in *C. fluminea* can influence exposure profiles and AChE as a biomarker (Cooper and Bidwell 2006; Simon and Laplace 2004; Liao et al. 2005; Mora et al. 1999). However, these prepositions were based on experiments seen in laboratory organisms acutely exposed to environmentally relevant concentrations of OPs and this might not be the same in chronically exposed organisms like those reared in their natural environments.

The physiological demand of reproduction is another factor influencing lowering of AChE activity according to Varela and Ausperger (1996), but this preposition may not apply in the present case. McMahon and Williams (1986), Cataldo et al. (2001), and McMahon (2001) had reported that reproduction of the *C. fluminea* usually takes place twice a year, in the months of May–July and September to December. The observed levels of activities of AChE in the present study do not synchronize with this cycle. Owen et al. (2002) noted that *Euvola ziczac*, a scallop species, showed no reduction of AChE activity immediately prior to spawning. Nevertheless, further studies can elucidate on the role of these factors.

The high AChE activity of the adductor muscles was also seen in various experiments in marine bivalves. As compared to a marine species like *Mytilus* sp., however, *C. fluminea* has

relatively lower AChE activity. The hemolymph of marine bivalves usually drawn from the adductor muscles of the organisms yields an AChE activity ranging from 300 to 1,500 nm min^{-1} mg $protein^{-1}$. These differences seen between the AChE activity of mussels and clams can be attributed to species and habitat differences. In terms of osmolarity, marine organisms tend to store more proteins in their adductor muscles to maintain osmotic balance in a highly saline environment (Pfeifer et al. 2005). In contrast with other tissues tested in *C. fluminea*, the increased activity noted in AChE of adductor muscles may be explained essentially as related to its function. By a neuromuscular mechanism, the tissue regulates the opening of the shell of the organisms. By its contained hemolymph, it functions for osmoregulation as well as helps in preventing the initial entrance of pollutants inside the body. In addition, the adductor muscle contains the hemolymph found to be essential in defense system of aquatic invertebrates at the same time reported to be one of the richest source of AChE (Smith 1991; Galloway and Depledge 2001; Galloway et al. 2002).

The sensitivity to OP of gills has been observed in previous works on both marine and freshwater bivalves (Escartin and Porte 1997; McHenery et al. 1997; Mora et al. 1999; Lehtonen and Leinio 2003; Bonacci et al. 2004; Brown et al. 2004). This property has been explained as an aspect of the role of the gills as a first line of defense against contaminants and pollutants. Resultant inactivity of the gills as a result of exposure would prevent further entry of harmful substances through the filter-feeding process (Hawkins et al. 1998; Lau and Wong 2004).

In contrast, the digestive glands showed a greater tolerance for the pesticide. Escartin and Porte (1997) had demonstrated that the gland possesses not only cholinesterase but also carboxylesterases, which can remove significant amounts of activated metabolites from parent organophosphate compounds, thus preventing them from reaching the main target AChE. These esterases hydrolyze a wide range of xenobiotic compounds and are believed to play a role in detoxification and tolerance for some OP pesticides (Maxwell 1992; Parkinson 1996), providing protection against neurotoxic compound poisoning (Gupta et al. 1985; Jokanovic et al. 1996; Parkinson 1996; Escartin and Porte 1997).

The results of the in vitro experiments utilizing the total soft tissues of the clams showed that the concentration of pesticide rendering a 79% inhibition might be around 10^{-6} M since this inhibition was obtained during a 10–15-min exposure to this concentration of the pesticide. According to the results of the in vitro experiments, this level of pesticide would cause an inhibition of AChE activity of 45.14% in the adductor muscle, 38.13% in the digestive gland, and 86.36% in the gill. To determine a more accurate assessment of river systems, it might be worthwhile also to have a complete profile of various in vitro test systems. Apparently, the extreme sensitivity of the gill AChE would limit its use to detect low concentrations of pesticide since no clear delineation of its activity can be drawn at the concentration range (10^{-2} to 10^{-1} M). On the other hand, among the four test systems, the adductor muscle, which exhibited the highest level of activity, would be useful in the detection within the higher ranges of pesticide concentration in the water.

In conclusion, *C. fluminea* is a good sentinel organism for water monitoring. In vivo experiments using the total soft tissues showed that AChE activity is largely affected by the season and site of collection. The lack of interference of the majority of the monitored environmental parameters, specifically the temperature, pH, and DO in its activity, makes it more suitable as a specific biomarker of OP pollution. The in vitro experiments on the selected tissues, in particular, clearly showed that the four tested tissues (gills, adductor muscles, digestive glands, and total soft tissues) contain significantly varying levels of AChE activity which can be a basis for the development of test systems working on limited ranges of OP concentration. The adductor muscles and digestive gland AChE are able to respond and tolerate higher concentrations of pesticide, the total soft tissue AChE of intermediate range, while the gill AChE would be exceptional in sensing lower concentrations. It is recommended that further studies on in vitro test systems be conducted on a wider range of pesticide concentration for more effective evaluation.

References

Adams, V. D. (1990). *Water and wastewater examination manual*. Michigan: Lewis.

Barnes, S., & Riggert, C. (2000). Tracking Missouri's exotics. *Missouri Conservationist, 61*, 8–13.

Bedford, J. W., Reloffs, E. W., & Zabik, M. J. (1968). The freshwater mussel as a biological monitor of pesticide concentration in lotic environment. *Limnology and Oceanography, 13*, 118–136.

Bonacci, S., Browne, M. A., Dissanayake, A., Hagger, J. A., Corsi, I., Focardi, S., et al. (2004). Esterase activities in the bivalve mollusk *Adamussium colbecki* as a biomarker for pollution monitoring in the Antarctic marine environment. *Marine Pollution Bulletin, 49*, 445–455. doi:10.1016/j.marpolbul.2004.02.033.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*, 258–254. doi:10.1016/0003-2697(76)90527-3.

Brown, M., Davies, I. M., Moffat, C. F., Redshaw, J., & Craft, J. A. (2004). Characterization of choline esterases and their tissue and subcellular distribution in the mussel (*Mytilus edulis*). *Marine Environmental Research, 57*, 155–169. doi:10.1016/S0141-1136(03)00067-9.

Cairns, M. S., Maguire, C. C., Williams, B. A., & Bennett, J. K. (1991). Brain cholinesterase activity of bobwhite acutely exposed to chlorpyrifos. *Environmental Toxicology and Chemistry, 10*, 657–664. doi:10.1897/1552-8618(1991)10[657:BCAOBA]2.0.CO;2.

Cataldo, D. H., Boltovskoy, D., Stripeikis, J., & Pose, M. (2001). Condition index and growth rates of field caged *Corbicula fluminea* (Bivalvia) as biomarkers of pollution gradients in the Paraná River Delta, Argentina. *Aquatic Ecosystem Health & Management, 4*(2), 187–201. doi:10.1080/14634980127712.

Cooper, N. L., & Bidwell, J. (2006). Cholinesterase inhibition and impacts on behavior of the Asian clam, *Corbicula fluminea*, after exposure to an organophosphate insecticide. *Aquatic Toxicology (Amsterdam, Netherlands), 76*(3–4), 258–267. doi:10.1016/j.aquatox.2005.09.012.

Day, K. E., & Scott, I. M. (1990). Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. *Aquatic Toxicology (Amsterdam, Netherlands), 8*, 101–114. doi:10.1016/0166-445X(90)90021-G.

Dellali, M., Barelli, M. G., Romeo, M., & Aissa, P. (2001). The use of cholinesterase activity in *Ruditapes decussates* and *Mytilus galloprovincialis* in the biomonitoring of Bizerta lagoon. *Comparative Biochemistry and Physiology, 130*(Part C), 227–235. doi:10.1016/S1096-4959(01)00426-2.

Ellman, G. L., Courtney, K. O., Anders, V., Jr., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology, 7*, 88–95. doi:10.1016/0006-2952(61)90145-9.

Escartin, E., & Porte, C. (1997). The use of cholinesterase and carboxylase activities from *Mytilus galloprovincialis* in pollution monitoring. *Environmental Toxicology and Chemistry, 16*, 2090–2095. doi:10.1897/1551-5028(1997)016<2090:TUOCAC>2.3.CO;2.

Eto, M. (1974). *Organophosphorus pesticides: Organic and biological chemistry*. Cleveland: CRC.

Galloway, T. S., & Depledge, M. H. (2001). Immunotoxicity in invertebrates: Measurement and ecotoxicological relevance. *Ecotoxicology (London, England), 10*, 5–23. doi:10.1023/A:1008939520263.

Galloway, T. S., Millward, N., Brown, M. A., & Depledge, M. H. (2002). Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusk *Mytilus edulis* using combined esterase activities as biomarkers. *Aquatic Toxicology (Amsterdam, Netherlands), 61*, 169–180. doi:10.1016/S0166-445X(02)00051-6.

Gupta, R. C., Patterson, G. T., & Dettbarn, W. D. (1985). Mechanisms involved in the development of tolerance to DFP toxicity. *Fundamental and Applied Toxicology, 5*, 517–528. doi:10.1016/0272-0590(85)90111-3.

Hartley, D. M., & Johnston, J. B. (1983). Use of the freshwater clam, *Corbicila manilensis* as a monitor for organochlorine pesticide. *Bulletin of Environmental Contamination and Toxicology, 31*, 33–40. doi:10.1007/BF01608763.

Hawkins, A. J. S., Smith, R. F. M., Tam, S. H., & Yasin, Z. B. (1998). Suspension-feeding behavior of tropical bivalve mollusk, *Perna viridis, Crassostrea belcheri, Crassostrea iradelei, Saccostrea cuculata, Pinctada margariferia*. *Marine Ecology Progress Series, 66*, 173–185. doi:10.3354/meps166173.

Jokanovic, M., Kosanovic, M., & Maksimovic, M. (1996). Interaction of the organophosphorous compounds with carboxylesterases in the rat. *Archives of Toxicology, 70*, 444–450. doi:10.1007/s002040050297.

Lau, P. S., & Wong, H. L. (2004). Effects of size, tissue parts and location on six biochemical markers in the green-lipped mussel, *Perna viridis*. *Marine Pollution Bulletin, 46*, 1563–1572. doi:10.1016/S0025-326X(03)00321-7.

Lehtonen, K. K., & Leinio, S. (2003). Effects of exposure to copper and malathion on metallothionien levels and acetylcholinesterase activity of the mussel *Mytilus edulis* and the clam *Macoma baltica* from the Northern Baltic Sea. *Bulletin of Environmental Contamination and Toxicology, 79*, 489–496. doi:10.1007/s00128-003-8853-6.

Liao, C. M., Jou, L. J., & Chen, B. C. (2005). Risk-based approach to appraise valve closure in the clam *Corbicula fluminea* in response to waterborne metals. *Environmental Pollution, 135*(1), 41–52. doi:10.1016/j.envpol.2004.10.015.

Maxwell, D. M. (1992). Detoxification of organophosphorous compounds by carboxylesterase. In J. E. Chambers & P. E. Levi (Eds.), *Organophosphates chemistry fate and effects*. San Diego: Academic.

McHenery, J. G., Lindley-Adams, G. E., Moore, D. C., Rodger, G. K., & Davies, I. M. (1997). Experimental and field studies of effects of dichlorvos exposure on acetylcholinesterase activity in the gills of the mussel, *Mytilus edulis* L. *Aquatic Toxicology (Amsterdam, Netherlands), 38*, 125–143. doi:10.1016/S0166-445X(96)00834-X.

McMahon, R. F. (2001). Mollusca: Bivalvia. In J. H. Thorp & A. P. Covich (Eds.), *Ecology and classification of North American freshwater invertebrates* (2nd ed., pp. 331–430, pp. 1056). San Diego: Academic.

McMahon, R. F., & Williams, C. J. (1986). Growth, life cycle, upper thermal limit and downstream colonization rates in a natural population of the freshwater bivalve molluscs, *Corbicula fluminea* (Muller) receiving thermal effluents. *American Malacological Bulletin, Special Ed. No. 2*, 231–239.

Mora, P., Fournier, D., & Narbonne, J. F. (1999). Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *Mytilus edulis* L. and from the freshwater bivalve *Corbicula fluminea* Mull. *Comparative Biochemistry and Physiology, 122*(3), 353–361.

Muncaster, B. W., Hebert, P. D. N., & Lazar, R. (1990). Biological and physiological factors affecting the body burden of organic contaminants in freshwater mussels. *Archives of Environmental Contamination and Toxicology, 19*, 25–34. doi:10.1007/BF01059809.

NPAL Philippines (1990). *In house method on determination of organophosphate pesticides in water by gas chromatography with a nitrogen–phosphorus detector*. Manila: National Pesticide Analytical Laboratory, Bureau of Plant Industry, Department of Agriculture.

Owen, R., Buxton, L., Sarkis, S., Toaspern, N., Knap, A., & Depledge, M. (2002). An evaluation of hemolymph cholinesterase activities in the tropical scallop, *Euvola ziczac*, for the rapid assessment of pesticide exposure. *Marine Pollution Bulletin, 44*, 110–117.

Parkinson, A. (1996). Biotransformation of xenobiotics. In C. D. Klaasen (Ed.), *Casarett and Doull's toxicology. The basic science of poisons* (5th ed., pp. 230–278). New York: McGraw Hill.

Pereira, W. E., Domagalski, J. L., & Hostettler, F. D. (1996). Occurrence and accumulation of pesticides and organic contaminants in river sediments, water and clam tissues from the San Joaquin River and tributaries, California. *Environmental Toxicology and Chemistry, 5*, 172–180. doi:10.1897/1551-5028(1996)015<0172:OAAOPA>2.3.CO;2.

Pfeifer, S., Schiedek, D., & Dippner, J. W. (2005). Effect of temperature and salinity on AChE activity, a common pollution biomarker in *Mytilus* sp. from South Western Baltic Sea. *Journal of Experimental Marine Biology and Ecology, 320*, 93–103. doi:10.1016/j.jembe.2004.12.020.

Simon, O., & Laplace, J. G. (2004). Kinetic analysis of uranium accumulation in the bivalve *Corbicula fluminea*: Effect of pH and direct exposure levels. *Aquatic Toxicology (Amsterdam, Netherlands), 68*(2), 95–108. doi:10.1016/j.aquatox.2004.03.002.

Smith, V. J. (1991). Invertebrate immunology, phylogenetic, ecotoxicological and biomedical implications. *Comparative Haematology International, 1*, 61–76. doi:10.1007/BF00422876.

Soucek, D. J., Schimdt, T. S., & Cherry, D. S. (2001). In situ studies with the Asian clams, *Corbicula fluminea* to detect acid mine drainage and nutrient inputs in low order streams. *Canadian Journal of Fisheries and Aquatic Sciences, 58*, 602–608. doi:10.1139/cjfas-58-3-602.

Varela, V. W., & Ausperger, T. (1996). *Cholinesterase activity as a device for biomonitoring pesticide exposure in the freshwater mussel, Elliptio complanata* (pp. 110–115). Raleigh: US Fish and Wildlife Service.