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Acetylcholinesterase Inhibition in the Threeridge Mussel (*Amblema plicata*) by Chlorpyrifos: Implications for Biomonitoring

Wendi J. Doran,*[,1] W. Gregory Cope,†[,2] Ronald G. Rada,* and Mark B. Sandheinrich*

** River Studies Center, Department of Biology, University of Wisconsin-La Crosse, La Crosse, Wisconsin 54601; and † Department of Toxicology, North Carolina State University, Box 7633, Raleigh, North Carolina 27695-7633*

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The effects of chlorpyrifos, an organophosphorus insecticide, were examined on the activity of the nervous system enzyme acetylcholinesterase (AChE) in the threeridge mussel *Amblema plicata* in a 24-day laboratory test. Thirty-six mussels in each of seven treatments (18 mussels per duplicate) were exposed to chlorpyrifos (0.1, 0.2, 0.3, 0.6, and 1.2 mg/L), a solvent (acetone), and a solvent-free (well water) control for 12, 24, or 96 h. The activity of AChE was measured in the anterior adductor muscle of eight mussels from each treatment after exposure. To assess potential latent effects, six mussels from each treatment were removed after 24 h of exposure and transferred to untreated water for a 21-day holding period; AChE activity was measured on three mussels from each treatment at 7 and 21 days of the holding period. The activity of AChE in chlorpyrifos-exposed mussels did not differ from controls after 12 or 24 h of exposure (*t*- test, $P > 0.05$), but was significantly less than controls after 96 h (*t*- test, $P = 0.01$). AChE activity did not vary among mussels at 24 h of exposure (i.e., Day 0 of holding period) and those at Day 7 and Day 21 of the holding period. Overall changes in AChE activity of mussels during the test were unrelated to individual chlorpyrifos concentrations and exposure times (repeated measure ANOVA; ($P = 0.06$). A power analysis revealed that the sample size must be increased from 2 to 5 replicates (8 to 20 mussels per time interval and test concentration) to increase the probability of detecting significant differences in AChE activity. This calculated increase in sample size has potential implications for future biomonitoring studies with chlorpyrifos and unionid mussels. © 2001 Academic Press

Key Words: **acetylcholinesterase; chlorpyrifos; mussel; unionidae; biomonitoring.**

INTRODUCTION

The upper Mississippi River (UMR) and its tributaries have historically contained abundant populations of freshwater unionid mussels (van der Schalie and van der Schalie, 1950); however, relatively recent surveys have indicated declining diversity and density of mussels in the UMR (Thiel, 1981; Havlik, 1983). Fuller (1980) reported that 35 of 50 species originally in the river were no longer abundant, and some like the Higgins' Eye *Lampsilis higginsi* have been placed on the federal list of endangered species (Code of Federal Regulations, 1993). The decline in mussel fauna has been attributed to dams, channel modification, sedimentation, navigation, industrial pollution, agricultural runoff, and introduction of nonindigenous species such as the zebra mussel *Dreissena polymorpha* (Fuller, 1974; Williams *et al.*, 1993; Cope *et al.*, 1997).

Potentially toxic substances, such as pesticides, that enter aquatic systems through runoff, leaching, erosion, or aerial deposition can also negatively effect freshwater mussels (Varela and Augspurger, 1996). For example, Fleming *et al.* (1995) attributed the mortality of approximately 1000 freshwater mussels (*Elliptio* spp.) in Swift Creek, North Carolina, to organophosphorus (OP) and carbamate insecticides that entered the stream. These insecticides presumably caused mortality by inhibiting the mussels' cholinesterases (ChE), a group of enzymes important in transmission of nervous system impulses. Fuller (1980) speculated that depletion of mussel populations in the UMR may have been partly caused by increased use of OP insecticides; however, the effects of these and other types of pesticides on native species of unionid mussels in the UMR and its tributaries are unknown.

Because of the relatively short half-life of OPs in the environment, their detection and quantification in the water column are difficult, and they are rapidly metabolized by nontarget organisms (Barron and Woodburn, 1995). However, determining the effects of OPs on the ChEs in nontarget organisms has been suggested as a tool for monitoring environmental contamination and organismal exposure to these compounds. Cholinesterase assays have previously been conducted on tissues of birds (Hill and Fleming, 1982; McInnes *et al.*, 1996), fish (Johnson and Wallace, 1987), and invertebrates (Day and Scott, 1990; Galgani and Bocquene,

[1] Present address: U.S. Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI 54603.

[2] To whom correspondence should be addressed at Department of Toxicology, North Carolina State University, Box 7633, Raleigh, NC 27695-7633. Fax: (919) 515-7169. E-mail: greg_cope@ncsu.edu.

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1990). Procedures have recently been developed for the quantification of ChE activity in unionid mussel tissue (Moulton *et al.*, 1996; Verela and Augspurger, 1996).

Chlorpyrifos (phosphorothionic acid *O*, *O*-diethyl *O*-[3,5,6-trichloro-2-pyridyl] ester) is a nonsystemic OP insecticide used extensively on corn in the upper midwestern United States to control corn rootworm, cutworm, and the European corn borer (Doersch *et al.*, 1993). Chlorpyrifos is considered by the U.S. Environmental Protection Agency to be moderately toxic or slightly toxic, depending on the formulation. Chlorpyrifos is acutely lethal (96-h LC_{50}) to bluegill *Lepomis macrochirus* at 2.4 μg/L and to channel catfish *Ictalurus punctatus* at 280 μg/L (Johnson and Finley, 1980). The acute lethality (96-h LC_{50}) of chlorpyrifos to aquatic invertebrates ranges from 0.25 μg/L for the mayfly *Cloen dipterum* to more than 806 μg/L for the snail *Aplexa hypnorum* (Barron and Woodburn, 1995). The lethal and sublethal effects of chlorpyrifos on freshwater mussels are largely unknown.

The purpose of this study was to evaluate acetylcholinesterase (AChE) activity in the threeridge mussel *Amblema plicata* (nomenclature according to Turgeon *et al.*, 1998) as a biomarker of exposure to chlorpyrifos. The threeridge was selected as the test organism because it is widespread and abundant in the UMR (Fuller, 1974); it shares critical habitat with threatened and endangered species, such as the Higgins' Eye, and thus may serve as a suitable surrogate species in toxicological studies.

MATERIALS AND METHODS

Collection, Transport, and Holding of Mussels

Threeridge mussels (approximately 300) with shell lengths ranging from 65 to 105 mm were collected by SCUBA divers from the lower St. Croix River near Lakeland, Minnesota. Mussels were promptly placed on damp burlap in ice chests and transported to an earthen pond at the Upper Midwest Environmental Sciences Center (U.S. Geological Survey) in La Crosse, Wisconsin, where they were held until testing. At the time of test initiation, 270 mussels were placed on damp burlap in ice chests and transported to the River Studies Center at the University of Wisconsin-La Crosse where they were placed in a fiberglass holding tank (0.61 m wide × 8.6 m long × 0.56 m deep). Mussels were fed a diet of algae (Algae Diet C-5, Coast Seafoods Co., Bellevue, WA) that was delivered by peristaltic pump at a rate equivalent to 1.5% of the dry tissue mass of the mussels in the holding tank per day (Daniel J. Hornbach, Macalester College, St. Paul, MN, personal communication).

Experimental Procedures

The experimental design consisted of 14 glass aquaria (experimental units) that received one of five exposure concentrations of chlorpyrifos (target concentrations, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L), a solvent (acetone) control, and a solvent-free (well water only) control with two aquaria per treatment. Each flow through aquarium (30 cm wide × 60 cm long × 36 cm deep) had a water depth of 16 cm and a sand substrate depth of 3 cm. The photoperiod was 16 h light and 8 h dark.

A chlorpyrifos stock solution was prepared by dissolving technical-grade chlorpyrifos (99.8% purity, DowElanco, Midland, MI) in acetone (SpectraAnalyzed, Fischer Scientific Co., Pittsburgh, PA). A proportional diluter was used to deliver the chlorpyrifos-acetone stock solution (with well water as the diluent) and solvent to 12 of the experimental units (i.e., the five chlorpyrifos treatments plus the solvent-only control). The delivery rate of each flowthrough aquarium was 0.3 L/min. The chlorpyrifos stock solution and solvent were delivered to the diluter with an automatic pipette (Micromedic, Model 25000).

Prior to exposure with chlorpyrifos, 18 mussels were randomly removed from the holding tank and placed into each of the 14 aquaria and acclimated to the test temperature (15 ± 2°C) for 6 days. The mussels were not fed during the acclimation period or the exposure portion of the test. Before beginning toxicant delivery with the diluter at the initiation of the test, each exposure and solvent-only control aquarium was spiked with an appropriate amount of stock chlorpyrifos solution or solvent to achieve the target concentrations in each replicate. Four mussels chosen at random were removed from each aquarium at 12, 24, and 96 h of exposure and their shell length and wet weight were measured. The anterior adductor muscle of each organism was removed, placed in a cryovial, and stored at −84°C until analysis for AChE activity and protein concentration.

Temperature, dissolved oxygen, and pH were measured daily in each aquarium. Total alkalinity and total hardness were measured on samples taken from each aquarium on Day 4 and Day 9 during the acclimation and exposure periods (APHA *et al.*, 1995). Physicochemical characteristics of water in all aquaria during the acclimation and exposure phases of the test averaged 15.3°C (range 14.3–16.7) for temperature, dissolved oxygen 9.3 mg/L (range 7.6–10.4), pH 7.7 (range 7.3–8.1), alkalinity 334 mg/L $CaCO_3$ (range 323–352), and hardness 390 mg/L as $CaCO_3$ (range 313–404). Water samples were also taken from each aquarium at 0 and 48 h of the exposure period and analyzed for chlorpyrifos concentration.

To assess the potential latent effects of a brief exposure to chlorpyrifos (the type that would likely occur in a natural stream system) on AChE activity, six randomly chosen mussels were removed from each aquarium after 24 h of exposure and held for 21 days in untreated water. Each mussel was uniquely marked on the periostracum with a motorized Dremel tool and transferred to a flowthrough

fiberglass tank (0.61 m wide × 8.6 m long × 0.56 m deep) containing 3 cm of sand substrate and supplied with fresh well water at a rate of 1 L/min. During the 21-day holding period, mussels were fed a diet of algae (Algae Diet C-5, Coast Seafoods Co.) that was delivered by peristaltic pump at a rate equivalent to 1.5% of the dry tissue mass of the mussels in the holding tank per day. Three mussels from each replicate exposure concentration were randomly removed from the holding tank on Day 7 and Day 21. The anterior adductor muscle of each organism was removed, placed in a cryovial, and stored at −84°C until analysis for AChE activity and protein concentration.

Temperature, dissolved oxygen, and pH in the holding tank were measured daily. Total alkalinity and total hardness were measured on samples taken from the holding tank on Day 7 and Day 21 (APHA *et al.*, 1995). Physicochemical characteristics of water in the holding tank averaged 15.7°C (range 14.4–16.9) for temperature, dissolved oxygen 9.8 mg/L (range 8.8–12.3), pH 7.5 (range 7.0–8.0), alkalinity 335 mg/L as $CaCO_3$ (range 333–336), and hardness 390 mg/L as $CaCO_3$ (range 385–395).

Analytical Procedures

Concentrations of chlorpyrifos in samples of test water were analyzed with an enzyme-linked immunosorbant assay (ELISA) with a RaPID assay kit (Strategic Diagnostics, Inc., Newark, DE). The absorbance of solution was measured at 450 nm with the RPA-I™ RaPID photometric analyzer (Strategic Diagnostics, Inc.). Samples were analyzed in duplicate to estimate precision; percentage difference averaged 2.4% and ranged from 0.5 to 8.5%. Bias was evaluated through recovery of known additions on one sample (in duplicate) per batch; percentage recovery averaged 56% and ranged from 45 to 65%. The least detectable concentration was 0.1 μg/L, and the limit of quantification was 0.22 μg/L (Strategic Diagnostics, Inc.).

Activity of AChE in the anterior adductor muscle (Fleming *et al.*, 1995; Mohan *et al.*, 1987) of *A. plicata* was analyzed based on the modified method of Ellman *et al.* (1961). Tissues were placed in Trizma buffer (1% Triton X-100) at a ratio of 1 : 25 (w : v) and homogenized with a metal-bladed homogenizer. Change in absorbance per minute was determined at 405 nm with a microplate reader (Molecular Devices Co., Model Kinetic V-max). All assays were conducted at a temperature of 23–25°C. Samples were analyzed in triplicate to evaluate precision; the relative standard deviation (RSD) averaged 4.3% and ranged from 0.4 to 13.7%. Procedural blanks and triplicate subsamples of an internal reference material (IRM, prepared from a composite sample of anterior adductor muscle tissue taken from six mussels at the time they were collected from the St. Croix River) were analyzed with each batch of 5 to 6 samples. The mean (± 95% confidence interval) AChE activity for IRM analyses was 737 (± 46) μmol substrate hydrolyzed/min · g protein.

Protein content of each homogenate was determined (BioRad DC protein assay, BioRad Laboratories, Inc., Hercules, CA) and enzyme activity expressed as micromoles substrate hydrolyzed per minute per gram protein. Accuracy of protein determinations was assessed by analysis of triplicate samples, IRM, recovery of known additions, and calibration standards. The RSD for the protein assay averaged 5.0% (range 0.3–19.6). The mean (± 95% confidence interval) protein concentration in the IRM was 0.69 ± 0.03 mg protein/mL homogenate. Recovery of known additions averaged 113% and ranged from 90 to 135%. The method detection limit (0.12 mg/mL) and limit of quantification (0.31 mg/mL) for analysis of protein were determined according to APHA *et al.* (1995).

Statistical Analysis

Data were analyzed with Statistical Product and Service Solutions (SPSS, 1996) computer software. If necessary, the data were transformed ($\log_{10}$) to achieve normality and homogeneity of variance. Variation in mean AChE activity between the solvent and the solvent-free control was analyzed with a Student's *t* test. Variation in AChE activity among treatments was analyzed by repeated measures analysis of variance (ANOVA). Because of the relatively large variation in AChE activity among individual treatments (range for coefficient of variation; CV, 1–36%), the grand mean of treatment responses was compared to that of the control with a Student's *t* test. A Type I error (α) of 0.05 was used to judge the significance of statistical tests.

RESULTS

Measured concentrations of chlorpyrifos in test water ranged from 0.09 to 1.4 mg/L at the beginning of the test (0 h) and from 0.08 to 0.96 mg/L at 48 h of exposure. Average concentrations of chlorpyrifos measured during the exposure phase of the test ranged from 0.09 to 1.2 mg/L and were 58–90% of the target concentrations (Table 1). Chlorpyrifos was not lethal to *A. plicata* in these exposures. Two mussels died during the postexposure holding period, but this mortality was unrelated to chlorpyrifos concentration.

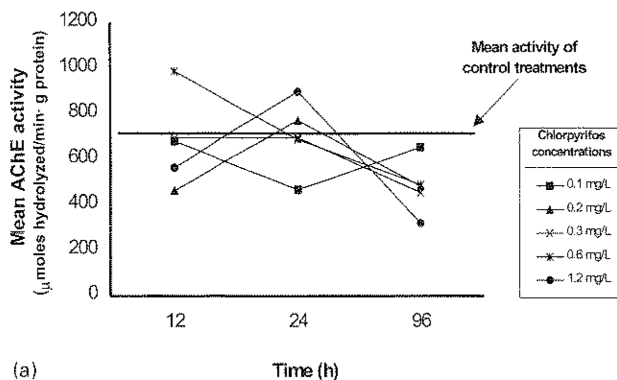
Activity of AChE in *A. plicata* from the solvent and solvent-free controls did not differ significantly (*t* test, $P \geq 0.05$) during the exposure and holding phases of the test. Therefore, data for AChE activity of *A. plicata* from the solvent and solvent-free controls were combined for statistical analysis. Mean AChE activity (μmol substrate hydrolyzed/min · g protein) in *A. plicata* exposed to chlorpyrifos ranged from 464 to 988 at 12 h, from 467 to 897 at 24 h, and from 319 to 653 at 96 h (Table 2). Mean AChE activities of mussels at 12 or 24 h (all exposure concentra-

TABLE 1
Mean (SD) Concentrations of Chlorpyrifos in Water Measured at 0 and 48 h of the Exposure Phase of the 96-h Toxicity Test with *Amblema plicata*

Target concentration (mg/L)	Measured concentration (mg/L) 0 h	48 h	Mean
0.0	ND[a]	ND[a]	ND[a]
0.1	0.09 (0.01)	0.08 (0.01)	0.09 (0.01)
0.2	0.17 (0.03)	0.14 (0.01)	0.16 (0.02)
0.5	0.32 (0.03)	0.26 (0.01)	0.29 (0.04)
1.0	0.70 (0.06)	0.57 (0.03)	0.64 (0.09)
2.0	1.4 (0.1)	0.96 (0.02)	1.2 (0.31)

[a] ND, not detected.

tions combined) did not vary from those of the controls (t test; $P > 0.05$). However, activities of AChE in mussels exposed to chlorpyrifos for 96 h were significantly less than those in mussels of the control treatment (t test, $P = 0.01$; Table 2, Fig. 1a). Overall changes in AChE activity in *A. plicata* during the exposure phase of the test were unrelated to individual chlorpyrifos concentrations and exposure times (repeated measures ANOVA, $P = 0.06$).



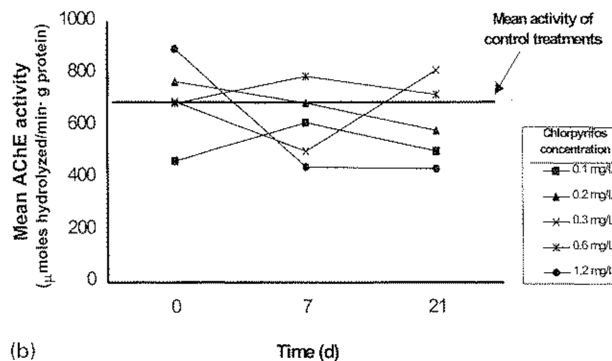


FIG. 1. Mean acetylcholinesterase (AChE) activity in adductor muscle of *Amblema plicata* (a) after exposure to chlorpyrifos for 12, 24, and 96 h, and (b) after exposure to chlorpyrifos for 24 h and then placed in untreated water for 21 days.

TABLE 2
Mean (SD) Acetylcholinesterase Activity in Adductor Muscle *Amblema plicata* after Exposure to Chloropyrifos

Mean measured chlorpyrifos concentration (mg/L)	Mean acetylcholinesterase activity (μmol substrate hydrolyzed/min · g protein) Exposure phase (h) 12	24	96	Holding phase (days) 7	21
0.0	793 (75)	703 (147)	828 (324)	587 (175)	643 (87)
0.1	680 (223)	467 (67)	653 (86)	614 (37)	505 (113)
0.2	464 (29)	772 (141)	483 (71)	689 (103)	584 (125)
0.3	695 (37)	695 (217)	453 (39)	504 (14)	815 (259)
0.6	988 (31)	688 (50)	489 (44)	792 (307)	721 (304)
1.2	565 (3)	897 (263)	319 (69)	444 (189)	437 (205)

During the holding phase of the test, mean AChE activity (μmol substrate hydrolyzed/min · g protein) in *A. plicata* that had been exposed to chlorpyrifos for 24 h ranged from 444 to 792 on Day 7 and from 437 to 815 on Day 21 (Table 2). There were no differences among AChE activities at 24 h of exposure (i.e., Day 0 of holding period) and those at Day 7 and Day 21 of holding (Fig. 1b).

DISCUSSION

The solubility of chlorpyrifos ranges form 0.94 to 2.0 mg/L at 25°C (Racke, 1993) and decreases to 0.45 mg/L at 10°C (Bowman and Sans, 1985). Mean concentrations of chlorpyrifos measured during the exposure phase of this test with *A. plicata* were 58–90% of target concentrations. Differences between target and measured concentrations of chlorpyrifos in this study, especially at the higher target concentrations (0.5, 1.0, and 2.0 mg/L), may be due to reduced solubility of chlorpyrifos at the test temperature (15°C). Similar studies with chlorpyrifos have reported that

measured concentrations were lower than target concentrations and have attributed the differences to adsorption onto particulate matter and exposed glass surfaces (Bowman and Sans, 1985; Macalady and Wolfe, 1985). In this study, chlorpyrifos may also have adsorbed onto the glass walls of the aquaria and tubing, thus contributing to the lesser measured concentrations.

The lack of chlorpyrifos-induced mortality of *A. plicata* allowed assessment of the sublethal effects of chlorpyrifos. However, the results suggest that *A. plicata* may be relatively insensitive to chlorpyrifos. For example, the greatest average exposure concentration in this test (1.2 mg/L) was near the LC_{50} of 2.0 mg/L for the eastern oyster *Crassostrea virginica* (Borthwick and Walsh, 1981), but did not produce mortality in *A. plicata*.

Moreover, the overall variation in AChE activity in *A. plicata* observed during the exposure phase of the test was unrelated to chlorpyrifos concentrations and exposure times. However, the activity of AChE was significantly less in *A. plicata* exposed to chlorpyrifos for 96 h than in control mussels. A 20% reduction in AChE activity is generally considered indicative of OP overexposure in birds (Ludke *et al.*, 1975). However, Moulton *et al.* (1996) recommended a 30% decrease in AChE activity in the anterior adductor muscle of freshwater mussels to indicate overexposure to OPs. Relative to control mussels, a 40–61% decrease in AChE activity was observed in *A. plicata* exposed to chlorpyrifos for 96 h in all but the 0.1 mg/L treatment (Fig. 1a). Mussels exposed to the lowest chlorpyrifos concentration of 0.1 mg/L had an AChE activity 21% less than that of control mussels at 96 h—a value near the recommended 30% threshold for critical reduction in AChE activity for mussels.

There were no differences in AChE activities at 24 h of exposure relative to those at Day 7 and Day 21 of the holding period (Fig. 1b). The AChE activities in mussels from all treatments after Day 21 of the holding period were at least 68% of the activities in control mussels. Therefore, there were no detectable long-term adverse effects of a brief (24 h) exposure to chlorpyrifos in *A. plicata*.

Significant differences in AChE activity among mussels exposed to chlorpyrifos were likely not detected by repeated measures ANOVA because of the relatively small sample sizes for each time interval (e.g., 2 replicate aquaria with 3–4 mussels for each time interval) and relatively high variation (mean CV, 39%) in AChE activity among individual mussels. Other researchers have reported similar variation in AChE activity of unionid mussels within certain treatments and sample sets. For example, Varela and Augspurger (1996) studied AChE activity in anterior adductor muscle of eastern elliptio (*Elliptio complanata*) from streams in North Carolina and found that relatively large variation (CV, 39–49%) within sets of samples did not allow them to detect significant differences between normal and depressed AChE activity. Moreover, they determined that minor improvements in the assay for AChE activity (based on Ellman *et al.*, 1961) did not significantly reduce the variation (CV, 24–65%) in mussel samples collected from the field.

Variation in AChE activity in mussels may be related to several factors, including differences in sex, age, size (length and weight), or physiological condition (Varela and Augspurger, 1996; Fairbrother *et al.*, 1989). Mussels often close their shells and cease filtering in the presence of toxicants, thereby reducing exposure to pollutants from the surrounding environment (Epifanio and Srna, 1975; Bedford *et al.*, 1968). During this study, it was observed that some *A. plicata* often had shells open and siphoning, whereas others did not. This indicates that mussels may not have been uniformly exposed to chlorpyrifos, thus contributing to the variation observed among AChE activities of individual mussels.

Detoxification mechanisms and individual differences in metabolism of the mussels may have also influenced the effects of chlorpyrifos on AChE activity. When exposed to methyl parathion, for example, metabolic adjustments (e.g., ammonia detoxification mechanisms) have aided mussels (*Lamellidens marginalis*) in mitigating pesticide toxicity and in increasing survival (Mohan *et al.*, 1987). In addition, Dauberschmidt *et al.* (1996) found that metabolites of thiometon and disufolton, which are 3- to 4-fold more toxic than their parent compounds, were not detected in the tissues of exposed zebra mussels, indicating that these chemicals were not metabolized by the mussels. Other freshwater bivalves have varying abilities to eliminate pesticides (Leard *et al.*, 1980), perhaps making them more tolerant to the chemicals (Varanka, 1987). The ability of *A. plicata* to metabolize chlorpyrifos and other similar pesticides is unknown.

Alternatively, measured variation in AChE activity may be influenced by the assay technique or the tissues analyzed. For example, Day and Scott (1990) found less variation with the microplate assay technique (CV, 6–26%) than with a modified kit procedure using a spectrophotometer (CV, 35–55%). In this study, the microplate assay technique was used based on the modified method of Ellman *et al.* (1961)—presumably a procedure with reduced variation. The chemical reactivation technique, another procedure that has been used to assess OP exposure in birds and fishes (Martin *et al.*, 1981; Fleming and Bradbury, 1981), offers an alternative to potentially reduce variation in AChE measurements with freshwater mussels. In that assay, AChE activity is reactivated by the nucleophilic reagent, pyridine-2-aldoxime methiodide (2-PAM). Pyridine-2-aldoxime methiodide increases the activity of OP-inhibited ChE and restores the ChE to near normal levels. This technique reduces variation in AChE activity within treatments, because "inhibited" and "control" activities are measured on the same organism.

Similar to the study of Varela and Augspurger (1996) with eastern elliptio, the anterior adductor muscle of *A. plicata* was analyzed for AChE activity. Several studies of marine bivalves have found greater AChE activity in other tissues. For example, Ozretic and Krajnovic-Ozretic (1992) found that the hepatopancreas was the richest source of esterase activity in the mussel *Mytilus galloprovicialis*. Bocquene *et al.* (1990) also found that the AChE activity was greater in mantle tissue than in gill and adductor muscle of the oyster *Crassostrea gigas*. Organophosphates affect esterase isozymes differently—specifically, the carboxylesterases are more sensitive than the acetylesterases to some OPs (Ozretic and Krajnovic-Ozretic, 1992). For *A. plicata*, it is unknown if a different tissue, such as the hepatopancreas, gill, or mantle, would have greater esterase activity than adductor muscle. Moreover, the relative sensitivities of the acetylesterases and the carboxylesterases to chlorpyrifos exposure in *A. plicata* are unknown. Consequently, additional studies on the nature of the enzymes in *A. plicata* are required before AChE activity can be implemented as a useful biomarker of exposure to OPs or carbamates.

Field studies that examine the effects of pollutants on freshwater mussels often encounter constraints associated with sampling logistics and experimental design, thus requiring that individual mussels be defined as the experimental unit in statistical analyses (e.g., Varela and Augspurger, 1996). Although the use of AChE activity has potential for use in environmental monitoring programs (e.g., Payne *et al.*, 1996), it was not possible to detect differences in AChE activity of *A. plicata* among chlorpyrifos treatments in the laboratory (with aquaria as experimental units in repeated measures ANOVA). Because there was relatively large variation (mean CV, 39%) associated with individual AChE measurements and relatively small sample sizes ($n = 2$ aquaria with 3–4 mussels per time interval) in the current study, there was interest in determining how many mussels per treatment would be needed to detect a statistically significant difference—information relevant to determining the value of using AChE measurements in future field assessments. Therefore, power analyses were conducted, which estimate the probability of failing to reject the null hypothesis when it is false (Zar, 1982). Two power analyses were conducted with either the aquarium or the mussel as the experimental unit—both with data from mussels in the control and the 0.1 mg/L treatments at the 12-h exposure. With the aquarium as the experimental unit (the preferred statistical analysis), power analysis revealed that 3 additional aquaria (total of 5 aquaria per treatment rather than 2), each with 4 *A. plicata*, would be needed at the 12-h exposure for a 90% probability of detecting a 30% difference in AChE activity. Thus, increasing the sample size within treatments from 2 to 5 aquaria (8 to 20 mussels) would reduce variation enough to detect significant differences (if they exist) in AChE activity between the control and the 0.1 mg/L treatment at the 12-h chlorpyrifos exposure with *A. plicata*. With the experimental design used in this study, the number of mussels needed to detect statistical significance among treatments would have increased to 90 mussels per treatment (rather than 36) and 630 for the experiment (rather than 252). If individual mussels were used as the experimental unit (which would be analogous to the situation with many field studies), 30 *A. plicata* per time interval would be needed for a 90% probability of detecting a 30% difference in AChE activity at a chlorpyrifos concentration of 0.1 mg/L. This needed sample size of 30 mussels per time interval and site has potential implications for future environmental monitoring for the effects of chlorpyrifos because of the increased expense associated with collection and analysis and the potential damage to natural populations by removing large numbers of mussels.

To reduce the calculated number of mussels needed for such future studies, several possible alternatives are suggested for reducing variation within treatments. First, in laboratory studies, the number of treatments could be decreased, thereby leaving a greater number of mussels available per treatment. Second, the use of other analytical methods (e.g., the reactivation technique) or other tissues (e.g., the gill or hepatopancreas) may reduce variability in AChE activity among individual mussels. Alternatively, a mussel species that is more sensitive than *A. plicata* to OP compounds may be used as the test or sentinel organism.

CONCLUSIONS

The activity of AChE in *A. plicata* exposed to chlorpyrifos for 96 h was found to be significantly reduced in all treatments except the lowest concentration (0.1 mg/L) tested. Exposed mussels had AChE activities that were 40–61% less than the AChE activity in control mussels—values greater than the recommended 30% threshold for critical reduction in AChE activity for mussels. However, statistically significant differences in AChE activity in mussels exposed to chlorpyrifos across all concentrations and time intervals were not detected by repeated measures ANOVA in this study because of the relatively small sample size ($n = 2$ aquaria with 3–4 mussels for each time interval) and relatively high variation (mean CV, 39%) in AChE activity among individual mussels. Power analysis revealed that the sample size must be increased from 2 to 5 aquaria (8 to 20 mussels per time interval) per test concentration to increase the probability of detecting significant differences in AChE activity. In analogous field monitoring situations where individual mussels would be classified as the experimental unit, 30 *A. plicata* per time interval would be needed to detect a significant difference in AChE activity at a chlorpyrifos exposure concentration of 0.1 mg/L. This needed

sample size of 30 mussels per time interval and site has potential implications for future environmental monitoring for the effects of chlorpyrifos because of the expense associated with collecting with analyzing additional mussels and the potential damage to natural populations by removing large numbers of resident mussels. More research is needed to determine methods of reducing within and among treatment variation before *A. plicata* can be successfully used as a sentinel of OP exposure.

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