Development of a cholinesterase inhibition assay for monitoring organophosphorus insecticide exposure and effects on bluegill (*Lepomis macrochirus*)

by

Kevin James Cole

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GENERAL INTRODUCTION

Organophosphorous insecticides (OPs) are used extensively in midwestern crop lands. According to a 1990 survey of pesticides used in Iowa crop production, state-wide 32.3% of corn acres were treated with organophosphorous insecticides out of the total of 35.2% of corn acres which were treated with insecticides (Hartzler and Wintersteen 1991). Nationwide in 1990, the percentages of OP insecticides used on insecticide treated corn fields were as follows: terbufos (Counter) 43%, chlorpyrifos (Lorsban) 21%, fonofos (Dyfonate) 12%, and phorate (Thimet) 8% (Fairchild et al. 1992). In addition, OPs represented 40% of the global insecticide market for 1989 (Racke 1993).

Their broad and extensive use makes OPs a probable non-point source contaminant. Runoff levels of the OP fonofos were measured in the range of 5-60 $\mu g \cdot L^{-1}$ on the edge of an experimental plot after a severe storm (Fairchild et al. 1992). The effects of OPs on aquatic communities have not been well defined because OPs are difficult to monitor due to many factors. Aquatic exposure to OPs depends on application rates and methods, the OP's physicochemical properties and factors controlling runoff (Fairchild et al. 1992). These insecticides are relatively easily hydrolyzed and, therefore, have low to moderate persistence in aquatic systems. For

chlorpyrifos, reported half-lives ranged from 5.3 hours for pasture water under field conditions to 28 days for canal water incubated in darkness (Racke 1993). Fonofos was shown to have a half-life of less than 2 days in simulated wetlands (Huckins et al. 1986)

In addition to being rapidly hydrolyzed in natural waters, OPs are easily metabolized by aquatic organisms, volatilized from water, and sorbed to sediments (Racke 1993). This rapid dissipation of the parent compound often results in concentrations below detection limits (0.1 $\mu g \cdot L^{-1}$ for fonofos, terbufos and chlorpyrifos: Wnuk et al. 1987), making direct monitoring of the insecticides very difficult. Effects of OPs on aquatic systems are seldom determined because the actual existence of OPs in those systems is seldom varified.

Aquatic invertebrates and fish are very sensitive to exposure to OPs. Mayer and Ellersieck (1986) showed that OPs are very toxic to fish, especially bluegill (*Lepomis macrochirus*) with a 96-h LC_{50} as low as 1.1 $\mu g \cdot L^{-1}$. The sensitivity to specific OPs varies greatly between species, and specific species' sensitivity to OPs is very different between chemicals (Weiss 1958; Weiss 1961; Post and Leasure 1974; Wang and Murphy 1982; Johnson and Wallace 1987).

The effects of OP exposure on fish have been evaluated in many ways. Macek et al. (1972) showed in a 63-d pond study that chlorpyrifos applied at 0.05 lb/acre (after 1 d, 2.39

$\mu g \cdot L^{-1}$) produced 55% mortality in bluegill and 46% mortality for largemouth bass (*Micropterus salmoides*). Sublethal effects have been studied looking at behavior (Symons 1973; Little et al. 1990; Pavlov et al. 1992) and development (Klaverkamp et al. 1977; Jarvinen et al. 1982).

One of the toxic effects of OPs on organisms is the inhibition of cholinesterase (ChE), an essential enzyme in the central and peripheral nervous systems (Matsumura 1985; Habig and Di Giulio 1991; Ware 1994). ChE hydrolyzes acetylcholine, a primary neurotransmitter. Because direct monitoring of OPs is difficult, due to their short half-lives, it has been proposed to use cholinesterase inhibition to detect environmental contamination with OPs (Habig and Di Giulio 1991). In addition, the use of a bio-indicator such as cholinesterase inhibition gives insight into how OPs affect the aquatic organisms of interest. Such a bio-indicator can detect sublethal effects of contamination which can change aquatic community composition and cause population declines.

A colorimetric assay has been developed (Ellman et al. 1961) to quantify cholinesterase activity in the tissues of organisms. ChE activity is determined in the assay by allowing the hydrolysis of acetylthiocholine by cholinesterase, producing thiocholine and acetate. Thiocholine reacts with dithiodinitrobenzoic acid to form thionitrobenzoic acid, which has a yellow color and can be

measured spectrophotometrically at 405 nm. The rate of color formation represents the cholinesterase activity (Ellman et al. 1961). The amount of ChE activity in control organisms is determined and compared with that in organisms exposed to OPs. This method has been used by several researchers to monitor OP exposure and effects (Finlayson and Rudnicki 1985; Johnson and Wallace 1987; Van Der Wel and Welling 1989, Marden et al. 1994) and is a primary method for most wildlife-related applications in the United States and the United Kingdom (Fairbrother et al. 1991). However, there are many unresolved questions in the use of ChE inhibition to monitor OP exposure to fish because little is known about how environmental and biological factors affect the levels of ChE activity. Those factors may confound or limit the technique's ability to assess OP exposure.

Cholinesterase activity tends to be quite variable among and within species (Habig and Di Giulio 1991), thus further background work is needed to account for this natural variability. In addition, Hogan (1970) determined that water temperature and cholinesterase activity in adult bluegills were significantly correlated. Researchers have studied species-related differences in ChE inhibition (Johnson and Wallace 1987; Wang and Murphy 1982), and sample storage and handling as sources of error in measuring ChE (Finlayson and Rudnicki 1985). These and other sources of variability must

be quantified and controlled to improve the assay and to make ChE inhibition a reliable monitoring tool.

The objectives of this research include refining the Ellman cholinesterase assay for fish brain tissue, evaluating the factors of water temperature and fish size on ChE variability in bluegill and determining effective tissue storage techniques for maintaining stable ChE activities in lab and field studies. The final objective is to assess the effects of exposure to the OP insecticide chlorpyrifos on brain ChE activity in juvenile blugill.

Thesis organization

This thesis contains two papers, each representing a manuscript to be submitted for publication in a scientific journal. The papers are formatted for the requirements of the target journal, *Canadian Journal of Fisheries and Aquatic Sciences*. Authorship of each manuscript will be as follows: Kevin J. Cole and Gary J. Atchison.

In addition to the two main papers, there is a General Introduction and a General Summary, with an accompanying References for both following the General Summary.

DEVELOPMENT AND EVALUATION OF A CHOLINESTERASE INHIBITION ASSAY AS AN INDICATOR OF ORGANOPHOSPHORUS INSECTICIDE EXPOSURE IN BLUEGILL *LEPOMIS MACROCHIRUS*

A paper to be submitted to the Canadian Journal of Fisheries and Aquatic Sciences

Kevin J. Cole and Gary J. Atchison

INTRODUCTION

Organophosphorous insecticides (OPs) are used extensively in midwestern crop lands(Hartzler and Wintersteen 1991). OPs represented 40% of the global insecticide market for 1989 (Racke 1993). Their broad and extensive use makes OPs a potential non-point source contaminant. The effects of OPs on aquatic communities have not been well defined, in part because OPs are difficult to monitor due to their short persistence in water and intermittent transport (usually runoff events) to aquatic systems (Fairchild et al. 1992).

Aquatic invertebrates and fish are very sensitive to exposure to OPs (Mayer and Ellersieck 1986) with bluegill (*Lepomis macrochirus*) having a 96-h LC_{50} as low as 1.1 $\mu g \cdot L^{-1}$. Sensitivity to specific OPs varies greatly between species, and a species' sensitivity to different OPs is also quite variable (Weiss 1958; Weiss 1961; Post and Leasure 1974; Wang and Murphy 1982; Mayer and Ellersieck 1986; Johnson and

Wallace 1987).

The toxic mode of action of OPs in organisms is inhibition of cholinesterase (ChE), an essential enzyme in the central and peripheral nervous systems (Kozlovskaya et al. 1993). ChE hydrolyzes acetylcholine, a primary neurotransmitter. Because direct monitoring of OPs is difficult, measuring irreversible inhibition of ChE to detect environmental contamination with OPs has been proposed (Habig and Di Giulio 1991). Bio-indicators such as ChE inhibition may give insights into how OPs affect aquatic organisms and may permit detection of sublethal effects of contamination which cause population declines and changes in aquatic community composition.

Ellman et al. (1961) developed a colorimetric assay to quantify cholinesterase activity in tissues of organisms. This method has been used to monitor OP exposure and effects in fish (Finlayson and Rudnicki 1985; Johnson and Wallace 1987; Van Der Wel and Welling 1989) and is the primary method for most terrestrial wildlife-related applications in the United States and the United Kingdom (Fairbrother et al. 1991). However, there are many unresolved questions in the use of ChE inhibition to monitor OP exposure to fish because little is known about how environmental and biological factors affect the levels of ChE activity and those factors may confound or limit the technique's ability to assess OP

exposure.

The objectives of this study were: 1) to evaluate and optimize the Ellman ChE assay for use with fish; 2) to determine if fish size affects ChE activity; 3) to determine if water temperature affects ChE activity; and 4) to evaluate the effects of various storage conditions on ChE activity.

MATERIALS AND METHODS

Test organisms

Bluegill were obtained from a private hatchery or collected from a local lake. They were maintained for 3 to 4 months in the laboratory in either a recirculating system with a bio-filter or a flow-through system receiving carbon filtered tap water. Test water had the same characteristics as acclimation water. Test fish were fed daily with Nelson's Sterling Silver Cup® crumbles fish pellets (Murry Elevators, Murry, UT).

Water chemistry analysis

Water chemistry characteristics (mean ± standard deviation) were as follows: temperature, 18.8 ± 2.8°C (n=47); dissolved oxygen, 8.08 ± 0.29 $mg \cdot L^{-1}$; pH, 7.78 ± 0.13; conductivity, 462 ± 24 $\mu mhos \cdot cm^{-1}$; alkalinity, 31 ± 5 $mg \cdot L^{-1}$ as

$CaCO_3$; hardness, 183 ± 6 $mg \cdot L^{-1}$ as $CaCO_3$ (n=5). Water chemistry analyses were conducted with standard procedures (APHA 1989; ASTM 1990). Temperature was measured daily with a Celsius thermometer. Conductivity and pH of treatment water were measured bi-weekly with a Cole Parmer model 1481-50 conductivity meter and an Orion model 407A ion meter, respectively. The conductivity meter and pH meter were calibrated before each use. Dissolved oxygen (Winkler method), total hardness and total alkalinity were measured bi-weekly. Monthly, externally supplied qualitity assurance samples (Hardness and Minerals, Environmental Resource Associates, Arvada, CO) were run with the regular water quality batches. All quality assurance sample results were within Environmental Resource Associates' acceptable ranges.

Cholinesterase determination

Cholinesterase activity was determined using modifications from Ellman et al. (1961), Hill and Flemming (1982) and Hooper et al. (1989). All chemical reagents were purchased from Sigma Chemical, St. Louis, MO. The analysis measured the brain ChE activity of individual fish, and there was no difficulty in acquiring sufficient tissue for analysis. After fish were euthanized by severing the spinal column, brain tissue was removed by cutting away the top of the skull, severing the optic nerves, and then lifting out the tissue.

The brain tissue was kept in iced pH 7.4 Trizma buffer until analysis. Brain tissue was weighed and then homogenized in pH 7.4 Trizma buffer with a motorized teflon pestle and glass tube. Appropriate dilutions of brain tissue were prepared for use in the analysis (usually 200 fold). Acetylthiocholine iodide was used as the substrate at a concentration of 0.001 M, and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 0.000323 M was used as the source of the colored anion product. Prior to analysis, pH 8.0 Trizma buffer, DTNB, diluted enzyme sample and acetylthiocholine iodide were added to the sample wells of a 96-well microtiter plate (Dynatech Microtiter). The samples were analyzed using a 96-well kinetic plate reader with an internal incubator (Molecular Devices Corp., THERMO max) which was controlled by software (Molecular Devices Corp. Softmax) via a Zenith Z386/20 computer. The increase in optical density at 405 nm was monitored for 2 minutes at 25°C with readings every 7 s. The change in optical density per minute was recorded for each well, and the enzyme activity, expressed in micromoles of substrate (acetylthiocholine) hydrolyzed per minute per gram of tissue, was determined (Ellman et al. 1961).

Quality assurance

All samples were run in triplicate on the plate, and if the coefficient of variance between the triplicates was >0.10

those samples were rerun. A set of pooled bluegill brains, prepared as normal (200-fold dilution in pH 7.4 trizma buffer), was divided into 1-ml aliquots in 2.0-ml Corning cryovials and placed in a liquid nitrogen freezer (-198°C). These aliquots were used as check standards and analyzed in triplicate with each plate of samples. The values were monitored using a control chart to identify errors in each assay. Rejection criteria were any point outside 3 standard deviations (SD) of the mean, two in a row outside of 2 SD, four in a row outside of 1 SD, two in a row with range >4 SD or ten in a row on same side of the mean. If a check standard was outside of control, the entire plate was rerun with new samples and check standards until it was back in control. During the experiments none of the check standards were found to be out of control. A commercial cholinesterase standard of electric eel serum (Sigma) was tried, but our analysis produced values which were variable and different from the reported values from the company.

Optimization

To increase precision and increase sensitivity, steps were taken to determine the optimal concentration of substrate, acetylthiocholine iodide, which would produce the greatest response in the enzyme sample from bluegill. A series of assays was performed using a range of concentrations

of acetylthiocholine iodide (0.01 - 0.0001 M) and set concentrations of the remaining reagents. Acetylthiocholine iodide at 0.001 M gave the greatest response.

Size experiment

Four size-classes of bluegill (collected from a local lake) were acclimated for at least 45 d in separate 100 L recirculating tanks. Each tank received the same source water (conditions described above) at 19°C and held 9-11 bluegill. After acclimation, total length and weight were recorded for each fish, then fish were euthanized, brain tissue removed and prepared for ChE analysis to test the effect of fish weight on ChE activity. The null hypothesis was fish weight does not have a linear relationship with ChE activity.

Temperature experiment

To determine the effect of temperature on ChE activity, five juvenile bluegill (from a private hatchery) per test tank (50-L) were exposed to five temperatures (20, 23, 26, 29, and 31°C) in duplicate for at least 14 d. Test water chemical characteristics were the same as described above; each of the ten tanks had two submersible automatic aquarium heaters (300 W Visitherm, Aquarium Systems, Mentor, OH) calibrated to the target temperature. Mean weight of bluegill (± standard deviation) was 8.6 ± 4.1 g with mean total length of 77.4 ±

9.9 mm (n=50). After acclimation, brains were removed and analyzed for ChE activity. The null hypothesis was temperature does not have a linear relationship with ChE activity.

Storage experiment

Long-term storage. Two sets of pooled bluegill brains (3 bluegill brains each), prepared as normal (200-fold dilution in pH 7.4 trizma buffer), were divided into 1-ml aliquots in 2.0-ml Corning cryovials and placed in a liquid nitrogen freezer (-198°C). One aliquot from each set was analyzed initially and after 1 d, 7 d, 30 d, 60 d and 90 d.

Short-term storage. Fifty juvenile bluegill, weight (± standard deviation) 5.0 ± 2.0 g and length 67.3 ± 8.4 mm, were euthanized, and 5 fish were immediately analyzed in duplicate (10 fish) for time 0 cholinesterase activity. Twenty of the bluegill were stored on ice in coolers (10 fish per cooler); the remaining twenty bluegill were kept in 19°C water (10 fish per tank). At 4 and 8 h five fish from each replicate were analyzed for ChE activity. The null hypothesis was that storage at -198°C, on ice or in 19°C water does not affect ChE activity.

Statistical methods

Analysis of variance (ANOVA) was done with Statistical

Analysis System (SAS) ANOVA procedure (SAS Institute, Inc. 1985) to determine if there were treatment effects on cholinesterase activity. All ANOVA tables are found in Appendix B. Regression analysis was also done for the size and temperature experiments to determine if there was a linear relationship between treatment and cholinesterase activity. A $P < 0.05$ was used to determine significance.

RESULTS

Fish size experiment

Cholinesterase activity of four bluegill size classes is shown in Table 1. ANOVA indicated a significant treatment effect of fish weight on ChE activity ($P < 0.003$; 3,36 df). Regression analysis of bluegill weight compared to ChE activity resulted in an $r^2=0.11$ (3 df) indicating no linear relationship between fish weight and ChE activity.

Temperature experiment

Cholinesterase activity at different acclimation water temperatures is shown in Table 2. Temperature significantly affected ChE activity (ANOVA; $P < 0.024$; 4,5 df; n=2). Fish in >26°C water tended to have higher ChE activities than the fish in <26°C water. Regression analysis of water temperature

Table 1. Effect of bluegill size on brain ChE activities, expressed in (μmoles AThCh hydrolyzed/min)/g of tissue, for each bluegill size class. N = number of fish in each size group.

Size Class - Length (cm) (Mean; SD)	Weight (g) Mean (SD)	N	ChE Activity Mean (SEM[a])
4 cm (3.9; 0.2)	0.7 (0.2)	10	8.90 (0.35)
9 cm (9.2; 1.1)	14.0 (5.6)	10	9.83 (0.35)
13 cm (13.5; 0.8)	44.5 (13.7)	9	7.80 (0.37)
18 cm (17.9; 1.6)	125.7 (65.5)	11	9.95 (0.33)

[a]SEM = standard error of the mean.

Table 2. Temperature effects on bluegill based on 10 fish per temperature treatment (5 fish per tank). ChE activities are expressed in (μmoles AThCh hydrolyzed/min)/g of bluegill brain tissue.

Temperature (°C) Target	Mean (SD)	Brain ChE Activity Mean (SEM[a]=0.29)
20	20.1 (0.2)	8.27
23	23.0 (0.1)	8.05
26	26.0 (0.2)	10.06
29	28.9 (0.2)	8.85
31	30.9 (0.1)	9.24

[a]SEM = standard error of the mean.

compared to ChE activity resulted in a r^2=0.30 (3 df) indicating no linear relationship between the two factors.

Long-term storage

The stability of ChE samples over time is shown in Figure

1. ANOVA showed no effect of storage time on ChE activity ($P < 0.95$; 5,6 df; n=2). The overall mean (± SEM) for these samples was 12.77 (2.13).

Short-term storage

The stability of ChE samples for field sampling techniques showed no effect for storage methods over the 4 and 8-h time intervals between treatments and time 0 (ANOVA; $P < 0.192$; 4,4 df; n=2), and therefore no treatment effect was shown (Figure 2). The effect of storage condition (ice vs. 19°C water) on ChE activity had the strongest significance (ANOVA; $P < 0.060$; 1,4 df; n=2).

DISCUSSION

Biological and environmental sources of variation for cholinesterase activity could include fish size and water temperature. Concern for the effects of size and acclimation temperature on ChE activity is two-fold. First, those variables could have a direct effect on ChE activity itself. Secondly, they could increase the variance in a sample, thus making it more difficult to statistically discern differences in samples collected from different sites. Information on the effects of fish size and water temperature acclimation can

Figure 1. Mean cholinesterase activity from two sets of pooled bluegill brain tissue stored in liquid nitrogen and analyzed at 0, 1, 7, 30, 60, and 90 d. The verticle lines represent ± SEM.

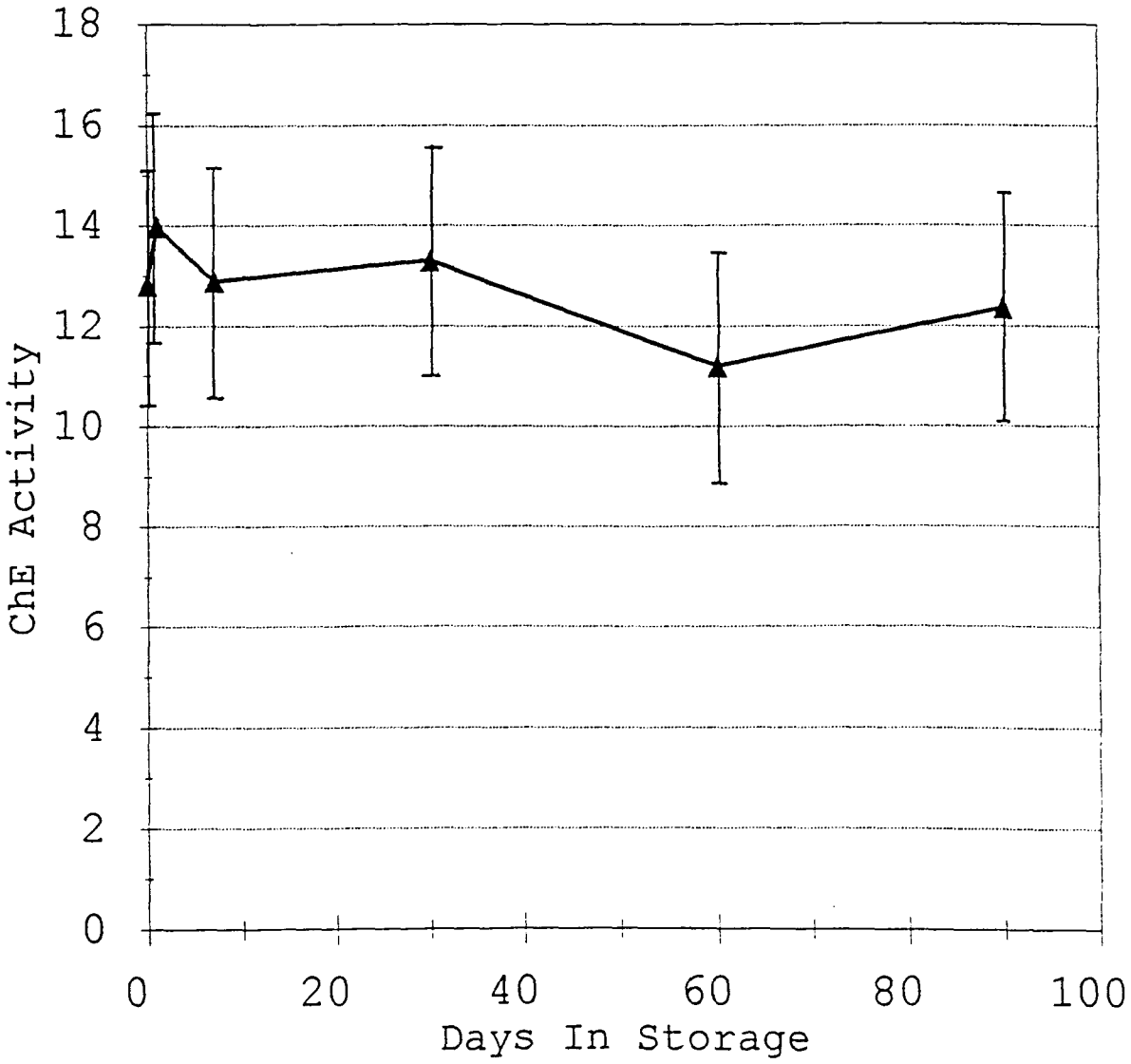
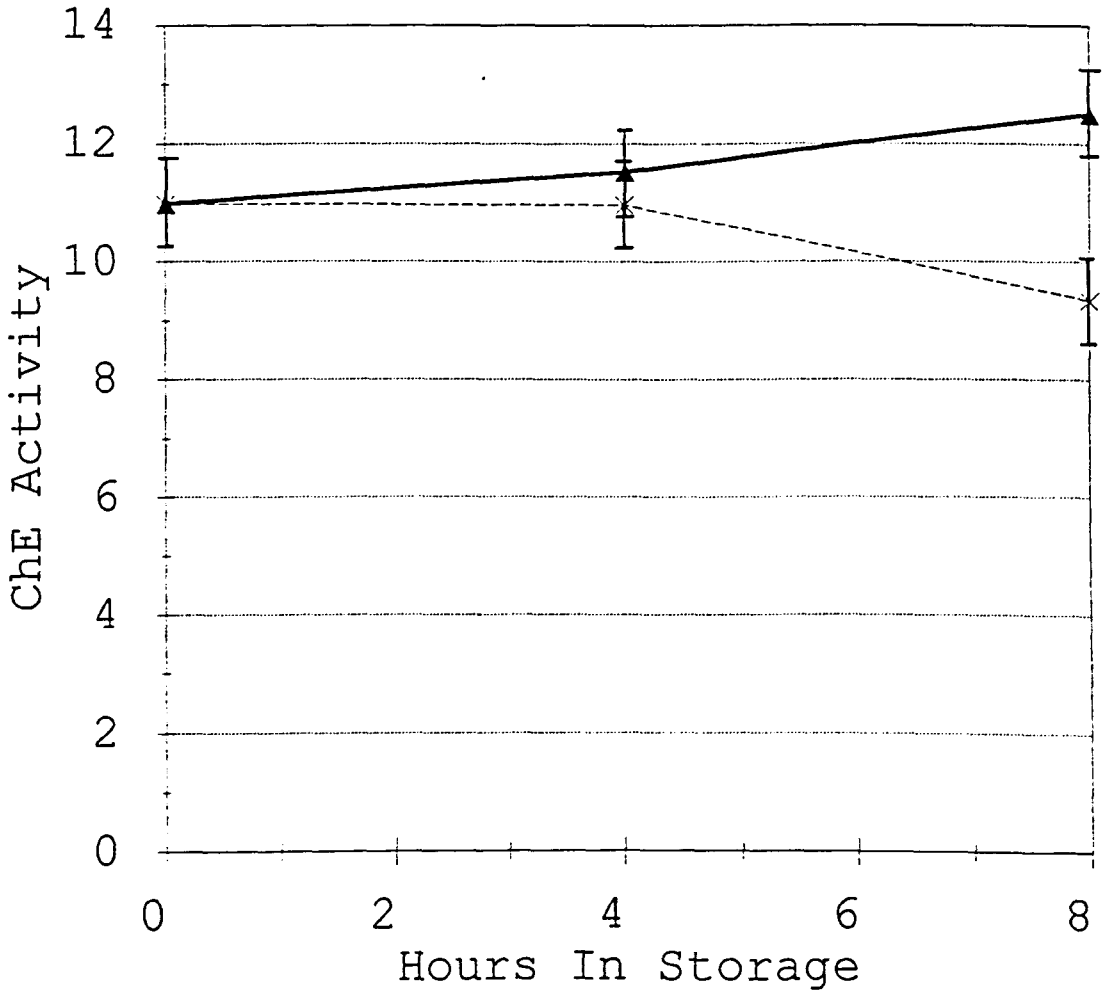

18
16
14
12
10
8
6
4
2
0
ChE Activity
0
20
40
60
80
100
Days In Storage

Figure 2. Cholinesterase activity of euthanized bluegills stored in ice or 19°C water for 0, 4 and 8 h. The solid line is ice treatment and the dashed line is 19°C water treatment. The verticle lines represent ± SEM.


14
12
10
8
6
4
2
0
ChE Activity
0
2
4
6
8
Hours In Storage

help establish monitoring protocol for assessing field exposure to OPs.

The weight of bluegill did not show a linear relationship with ChE activity. Previous research by Zinkle et al. (1987) on rainbow trout (*Oncorhynchus mykiss*) and Rath and Misra (1981) on tilapia (*Tilapia mossabica*) determined that lower ChE activities were associated with larger fish.

The effect of temperature on cholinesterase activity was also determined to be significant, but again there was not a linear relationship between the two variables. In general, higher water temperatures produced higher activities. Hogan (1970) showed in a pond study that water temperature had a direct positive relationship on cholinesterase activity in bluegill. The span of temperatures in Hogan's study was from 2°C to 28°C. A study using rainbow trout found no relationship between fish maintained between 9 and 20°C and ChE activity (Zinkle et al. 1987).

The ability to store enzyme samples is very critical for field monitoring and maintaining quality assurance. Previous research showed that rainbow trout stored at -20°C and -68°C produced stable ChE activities for 55 d (Zinkle et al. 1987). The long-term storage experiment produced activity readings with a high standard deviation (1.5) for the check standards but the trend was consistent around a specific mean. Such consistency shows that storage of enzyme samples in liquid

nitrogen can be done with confidence at least up to 90 d.

The handling of fish for short periods prior to analysis may be a source of error for measuring ChE activity. Finlayson and Rudnicki (1985) found that euthanized whole fish, when stored in the refrigerator or freezer, had decreased ChE activity after 1 and 2 d. Zinkle et al. (1987) found that fish stored at 4°C (simulating ice) had ChE activities not significantly different from controls ($p<.001$) until 7 d in storage.

Zinkle et al. (1987) showed that rainbow trout exposed to an OP, euthanized and then left in 4 - 20°C water for 24 h before analysis did not exhibit any changes in ChE activity. A study by Stansley (1992) determined that time after death at 19-22°C did not significantly affect ChE activity for 24 h for fathead minnow (*Pimephales promelas*). In this study, part of the short-term storage test looked at how time after death in 19°C water affected ChE activity, to mimic field mortality. No treatment effect of the 19°C water over the 4 and 8 h intervals was found. For longer storage, other storage techniques should be considered, such as freezing on dry ice or in liquid nitrogen.

The use of check standards proved to work well to assure quality in the ChE measurements. In addition, we used a 96-well microplate reader with a built-in incubator which improved our ability to run consistent analyses. The plate

reader allowed us to run each sample in triplicate under the same conditions and to have tight control over the reaction temperature. The ChE assay is very sensitive to assay conditions such as fresh reagents, calibrated pipettes and control over reagent temperatures.

Other possible sources of ChE variability could include reproductive condition, seasonal variation and developmental changes (Fairbrother et al. 1991, Zinkle et al. 1991, Kozlovskaya et al. 1993). In developing a monitoring plan, it would be prudent to select fish of similar size, condition and from waters with similar temperatures, if possible, to reduce variability.

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ACUTE TOXICITY AND RECOVERY OF CHOLINESTERASE INHIBITED BLUEGILL, *LEPOMIS MACROCHIRUS*, EXPOSED TO THE ORGANOPHOSPHORUS INSECTICIDE CHLORPYRIFOS

A paper to be submitted to the Canadian Journal of Fisheries and Aquatic Sciences

Kevin J. Cole and Gary J. Atchison

INTRODUCTION

Organophosphorous insecticides (OPs) are used extensively in midwestern crop lands. According to a 1990 survey of pesticides used in Iowa crop production, state-wide 32.3% of corn acres were treated with organophosphorous insecticides out of the total of 35.2% of corn acres which were treated with insecticides (Hartzler and Wintersteen, 1991). In addition, OPs represented 40% of the global insecticide market for 1989 (Racke 1993).

Their broad and extensive use makes OPs a probable non-point source contaminant. Runoff levels of the OP fonofos were measured in the range of 5-60 $\mu g \bullet L^{-1}$ on the edge of an experimental plot after a severe storm (Fairchild et al. 1992). The effects of OPs on aquatic communities have not been well defined because OPs are difficult to monitor due to many factors. Aquatic exposure to OPs depends on application rates and methods, the OP's physicochemical properties and

factors controlling runoff (Fairchild et al. 1992). For the OP chlorpyrifos, reported half-lives ranged from 5.3 hours for pasture water under field conditions to 28 days for canal water incubated in darkness (Racke 1993).

In addition to being rapidly hydrolyzed in natural waters, OPs are easily metabolized by aquatic organisms (Racke 1993). This rapid breakdown of the parent compound results in concentrations below detection limits (0.1 $\mu g \cdot L^{-1}$ for fonofos, terbufos and chlorpyrifos: Wnuk et al. 1987), making direct monitoring of the insecticides very difficult. Effects of OPs on aquatic systems are seldom determined because the actual existence of OPs in those systems is seldom varified.

Aquatic invertebrates and fish are very sensitive to exposure to OPs (U.S. EPA 1986). Mayer and Ellersieck (1986) showed that among fish species OPs are especially toxic to bluegill (*Lepomis macrochirus*) with a 96-h LC_{50} as low as 1.8 $\mu g \bullet L^{-1}$ for terbufos and 2.4 $\mu g \bullet L^{-1}$ for chlorpyrifos. The sensitivity of specific OPs varies greatly between species, and specific species' sensitivity to OPs is very different between chemicals (Weiss 1958; Weiss 1961; Post and Leasure 1974; Wang and Murphy 1982; Johnson and Wallace 1987).

One of the toxic effects of OPs on organisms is the inhibition of cholinesterase (ChE), an essential enzyme in the central and peripheral nervous systems (Matsumura 1985). ChE hydrolyzes acetylcholine, a primary neurotransmitter. Because

direct monitoring of OPs is difficult, due to their short half-lives, it has been proposed to use cholinesterase inhibition (which can be exhibited for longer time periods) to detect environmental contamination with OPs (Habig and Di Giulio 1991). In addition, the use of a bio-indicator such as cholinesterase inhibition gives insight into how OPs affect the aquatic organisms of interest. Such a bio-indicator can detect sublethal effects of contamination which can change aquatic community composition and cause population declines.

A colorimetric assay has been developed (Ellman et al. 1961) to quantify cholinesterase activity in the tissues of organisms. This method has been used by several researchers to monitor OP exposure and effects in fish(Finlayson and Rudnicki 1985; Johnson and Wallace 1986; Van Der Wel and Welling 1989).

The characteristics of test organisms and their responses to OP exposure are informative in determining the best monitoring system. The OP chlorpyrifos was selected for this study because it is one of the most commonly used OPs in Iowa (Hartzler and Wintersteen 1991), and it has been widely investigated for effects on aquatic systems (Macek et al. 1972; Eaton et al. 1985; U.S. EPA 1986; Racke 1993). The response of cholinesterase activity to exposure can give insight into the sensitivity of an organism to exposure and give baseline data to compare to field samples. The levels of

ChE activity of fish which have been killed by organophosphorus insecticides can help in determining if dead fish found in the field have been exposed to OPs. The recovery of ChE inhibited fish can also help in determining the window of opportunity which exists for detection of exposure in field fish.

The objectives of this study were: 1) to determine the concentration of chlorpyrifos which produces a 50% reduction in ChE activity in bluegill; 2) to determine the concentration of chlorpyrifos required to produce 50% mortality in bluegill; and 3) to evaluate the changes of ChE levels in blugill with depressed ChE levels (from exposure to chlorpyrifos) when placed in chlorpyrifos-free water over a two-week period.

MATERIALS AND METHODS

Test organisms

Juvenile bluegill, weighing (mean ± standard deviation (SD); n=189) 5.6 ± 3.6 g and 68.7 ± 12.6 mm in total length, used in these experiments were obtained from a private hatchery. They were maintained for 6 to 9 months in a flow-through system receiving carbon filtered tap water . Water chemistry characteristics (mean ± SD) were as follows: temperature, 18.8 ± 2.8°C (n=47); dissolved oxygen, 8.08 ±

0.29 $mg \cdot L^{-1}$; pH, 7.78 ± 0.13; conductivity, 462 ± 24 $\mu mhos \cdot cm^{-1}$; alkalinity, 31 ± 5 $mg \cdot L^{-1}$ as $CaCO_3$; hardness, 183 ± 6 $mg \cdot L^{-1}$ as $CaCO_3$ (n=5). Test fish were fed daily with Nelson's Sterling Silver Cup® crumbles fish pellets (Murry Elevators, Murry, UT).

Cholinesterase determination

Cholinesterase activity was determined using modifications from Ellman et al. (1961), Hill and Flemming (1982) and Hooper et al. (1989). All chemical reagents were purchased from Sigma Chemical, St. Louis, MO. After euthanizing the fish by severing the spinal column, brain tissue was removed by cutting away the top of the skull, severing the optic nerves, and lifting out the tissue. The brain tissue was kept in iced pH 7.4 trizma buffer until analysis. Brain tissue was weighed and then homogenized in pH 7.4 trizma buffer with a motorized teflon pestle and glass tube. Appropriate dilutions of brain tissue were prepared for use in the analysis (usually 200 fold). Acetylthiocholine iodide was used as the substrate at a concentration of 0.001 M, and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 0.000323 M was used as the source of the colored anion product. Prior to analysis, pH 8.0 trizma buffer, DTNB, diluted enzyme sample and acetylthiocholine iodide were added to sample wells of a 96-well microtiter plate (Dynatech Microtiter). The samples

were analyzed using a 96-well kinetic plate reader with an internal incubator (Molecular Devices Corp. THERMO max) which was controlled by software (Molecular Devices Corp. Softmax) via a Zenith Z386/20 computer. The increase in optical density at 405 nm was monitored for 2 minutes at 25°C with readings every 7 s. The change in optical density per minute was recorded for each well and the enzyme activity, expressed in micromoles of substrate (acetylthiocholine) hydrolyzed per minute per gram of tissue, was determined (Ellman et al. 1961).

Check standards (pooled bluegill brain tissue stored in liquid nitrogen at -198°C) were run with each plate to assess the quality of the measurements. All samples and check standards were analyzed in triplicate on each plate, and if their coefficient of variation was >0.10, those samples were analyzed again.

Cholinesterase inhibition test

A 1 $mg \cdot ml^{-1}$ solution of chlorpyrifos [0,0-diethyl 0-(3,5,6-trichloro-2-pyridyl)phosphorothioate] technical grade (98.7 % purity) in acetone was diluted 1000-fold in nanopure water to obtain a 1.0 $mg \cdot L^{-1}$ stock solution. Four concentrations of chlorpyrifos (1, 1.5, 2 and 3 $\mu g \cdot L^{-1}$) and a control in duplicate were used with 5 fish per tank (experimental unit). Chlorpyrifos stock solution was

delivered to the 44 L treatment tanks by a flow-through proportional diluter. After 96 h, all the fish were removed, and their cholinesterase activities determined. The concentration resulting in 50% inhibition of ChE (IC_{50}) was determined using the probit method (Toxcalc, Stephan 1984).

Acute lethality test

A 1.0 $mg \cdot L^{-1}$ stock solution of chlorpyrifos was used to dose treatment tanks in a static renewal dosing system. The nominal concentrations (4, 8, 14 and 20 $\mu g \cdot L^{-1}$) plus controls in duplicate were delivered to ten 44-L aquaria with 5 fish per tank. Every 24 h, 22 L of treatment water was drained from each tank and fresh water added along with enough chlorpyrifos stock solution to make up 22 L of the appropriate nominal concentration of chlorpyrifos. The treatment tanks were checked three times a day to remove dead fish which were then stored at 4°C. At the end of each day, brains of the dead fish were removed, homogenized and diluted with pH 7.4 trizma buffer; those enzyme samples were then placed in a liquid nitrogen freezer and held for ChE analysis. The test ended after 96 h; the surviving fish and stored samples from the previous 3 d were analyzed for their cholinesterase activity. The LC_{50} value was determined using the probit method (Toxcalc, Stephan 1984).

Cholinesterase recovery test

Thirty bluegill in duplicate (60 total) were exposed to near the IC_{50} of chlorpyrifos (3 $\mu g \cdot L^{-1}$ nominal) for 96 h in a flow-through proportional diluter. At the end of exposure, time 0 cholinesterase levels were determined by analyzing 10 fish from each of the two treatment replicates. With each analysis of chlorpyrifos-treated fish a control group of 10 bluegill from non-treated water was also analyzed for ChE activity as a reference. Each replicate was then placed in a clean water flow-through system. At one week intervals, 10 control fish and 10 of each duplicate were analyzed for cholinesterase activity. Analysis of variance (ANOVA) was done with Statistical Analysis System (SAS) ANOVA procedure (SAS Institute, Inc. 1985) to determine if there were treatment effects on cholinesterase activity at the different recovery times. All ANOVA tables are found in Appendix B.

Chemical analysis

During each of the 96-h chlorpyrifos exposures, 500-mL water samples were taken from the test tanks two times. At each of the sampling times one randomly selected tank was sampled to provide enough water sample to do a triplicate analysis. The samples were collected in acetone cleaned glass jars with teflon cap liners. After collection, the jars were wrapped in aluminum foil and stored at 4°C until analysis (< 1

wk). The water samples were extracted by vacuum filtering the samples through ENV-18 solid phase extraction tubes. The chlorpyrifos was then eluted off the packing with hexane and rotary evaporated to volume for analysis. The samples were then analyzed on a Varian 3740 gas chromatograph (GC) with a ^{63}Ni-electron capture detector to determine the amount of parent compound in the sample. Spike and recovery analysis, procedural blanks, calibration standards and replicate samples were done for QA/QC. The detection limit was 1 $\mu g \cdot L^{-1}$.

RESULTS

Cholinesterase inhibition test

An IC_{50} (concentration of chlorpyrifos which produces 50% ChE inhibition) test was run using target concentrations 0, 1, 1.5, 2, and 3 $\mu g \cdot L^{-1}$ (Table 1). Analysis of variance showed that exposure to chlorpyrifos significantly affected ChE activity ($P < 0.051$; 4,5 df; n=2). The IC_{50} (based on the measured concentrations) was determined to be 2.22 $\mu g \cdot L^{-1}$ (95% confidence limits 1.67 and 16.03 $\mu g \cdot L^{-1}$) using the probit method (Toxcalc, Stephan 1984).

Acute lethality test

An LC_{50} (concentration of chlorpyrifos which produces 50%

Table 1. Chlorpyrifos IC_{50} experiment results based on 10 bluegill per concentration treatment (5 fish per tank). ChE activities are expressed in (µmoles AThCh hydrolyzed/min)/gram of brain tissue.

Concentration (µg•L^{-1}) Target	Measured (SD)	Brain ChE Activity mean SEM[c]=1.04	%inhibition
0	0.3 (0.5)[a]	9.59	----
1	1.0 (0.1)	7.70	19.7
1.5	1.1 (0.1)	10.31	----[b]
2	1.7 (0.2)	6.11	36.3
3	2.3 (0.1)	4.65	51.5

[a]one subsample out of four was contaminated with 1.0 µg•L^{-1}.
[b]not inhibited; above controls.
[c]SEM = standard error of the mean.

mortality) test was run using target concentrations 0, 4, 8, 14, and 20 µg•L^{-1} (Table 2). Analysis of variance showed that exposure to chlorpyrifos significantly affected ChE activity ($P < 0.003$; 4,5 df; n=2). The LC_{50} (based on the measured concentrations) was determined to be 7.74 µg•L^{-1} (95% confidence limits 5.76 and 10.00 µg•L^{-1}) using the probit method (Toxcalc, Stephan 1984).

Cholinesterase recovery test

There was a gradual decrease in cholinesterase inhibition in the bluegill that were exposed to 3 µg•L^{-1} nominal concentration of chlorpyrifos (Figure 1). The variability in the samples (SEM=12.2) was great enough to not produce

Table 2. Chlorpyrifos LC_{50} experiment results based on 10 bluegill per concentration treatment (5 fish per tank). ChE activities are expressed in (μmoles AThCh hydrolyzed/min)/gram of brain tissue.

Concentration ($\mu g \cdot L^{-1}$) Target	Measured (SD)	Mortality	Brain ChE Activity mean SEM[a]=0.67	%inhibition
0	0.8 (0.3)	0	11.91	----
4[a]	4.2 (0.9)	1	2.71	77.2
8[b]	8.1 (2.1)	4	1.05	91.2
14[c]	16.5 (3.6)	10	1.01	91.5
20[d]	19.7 (1.2)	10	0.96	91.9

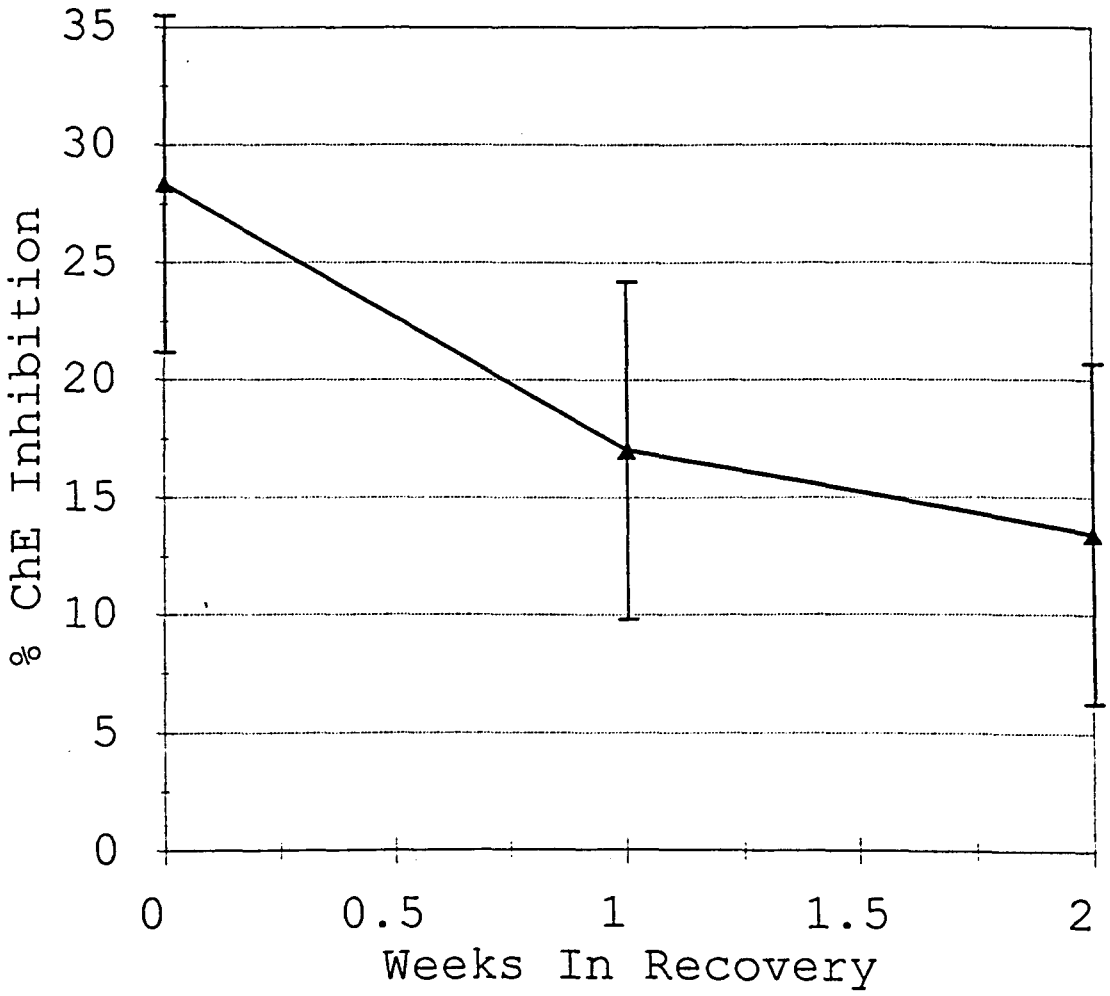
[a]SEM = standard error of the mean.

significant differences between activity and recovery time ($P < 0.41$; 2,3 df; n=2).

DISCUSSION

The relationship between cholinesterase activity and organophosphorus insecticide exposure has been studied by several researchers (Weiss 1961; Post and Leasure 1974; Jarvinen et al. 1983). The sensitivity of cholinesterase inhibition to low concentrations of chlorpyrifos is shown in the IC_{50} of 2.22 $\mu g \cdot L^{-1}$. These values can help in interpreting possible field exposures of bluegill by the determination of cholinesterase activities in fish captured in the field. ChE

Figure 1. Recovery of cholinesterase inhibition of bluegill. Fish were exposured to a nominal concentration of 3.0 $\mu g \cdot L^{-1}$ chlorpyrifos for 96-h, then placed in clean water for two weeks (n=2). The verticle lines represent ± SEM.


35
30
25
20
15
10
5
0
% ChE Inhibition
0
0.5
1
1.5
2
Weeks In Recovery

inhibition can be an important method to detect possible detrimental effects on fish.

Jarvinen et al. (1983) used fathead minnows (*Pimephales promelas*) to evaluate effects of long-term exposure (200-d) to chlorpyrifos (0.2-3.0 $\mu g \cdot L^{-1}$). They found that at >10-40% ChE inhibition, fish maturation and second-generation growth were reduced; at >50% inhibition, reproductive effects occurred; and at >80% inhibition, survival, growth, deformities, and hatchability were significantly affected. Such results indicate how damaging long exposures, even at low concentrations with little ChE inhibition, can be to fish.

The LC_{50} value of 7.74 $\mu g \cdot L^{-1}$ (static renewal) is higher than the LC_{50} of 2.4 $\mu g \cdot L^{-1}$ for chlorpyrifos and bluegill in a static test reported by Mayer and Ellersieck (1986). The magnitude of inhibition which is associated with death (80-90%) is similar to previous research (Coppage and Mathews 1974; Richmonds and Dutta 1992).

During the 96-h exposures, behavior changes were noted. After 24 h, 20 $\mu g \cdot L^{-1}$ treated bluegill expressed loss of equilibrium and a tendency to have extended pectoral fins. Fish exposed to 8 $\mu g \cdot L^{-1}$ showed some of the same signs after 90 h. Overall, treated fish were agitated and when disturbed often lost all muscle control. All treatment mortalities had their pectoral fins extended or pointed forward. These symptoms are consistent with other observations of OP

poisoning (Zinkle et al. 1991).

Habig and Di Giulio (1991) showed that recovery to non-inhibited levels of ChE in fish brain took approximately 4 wk. Other researchers found similar results and noted that recovery varied depending on duration and level of exposure (Post and Leasure 1974; Van Der Wel and Welling 1989). The recovery in this study was not shown to have statistical significance due to large differences between replicates and the variation in the cholinesterase activity.

Bluegill are very sensitive to chlorpyrifos exposure with low $\mu g \cdot L^{-1}$ concentration causing mortality and depressed ChE levels. Test fish exposed to low levels of chlorpyrifos (4 $\mu g \cdot L^{-1}$) exhibited symptoms of poisoning which could affect survival (U.S. EPA 1986). ChE activity depression was easily detected for such sublethal exposures and shows promise for field monitoring.

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GENERAL SUMMARY

The use of a cholinesterase inhibition assay in biomonitoring has its limitations. Cholinesterase activity is typically highly variable and standardized methods for analysis are only recently being developed. Optimizing the assay includes adjusting the procedures to specific types of tissue and adjusting instrumentation.

The effects of fish size on ChE activity was not determined to have a linear relationship. The effects of water temperature also proved important, again not showing a significant linear relationship. The primary concern based on results from this study is the increased variability in ChE activity introduced by highly variable water temperatures and fish size. Therefore, sample collection needs to be carefully done to keep fish size and water temperature similar.

The storage of enzyme samples for a long period was accomplished by storing them in a liquid nitrogen freezer which kept activities of the samples similar for at least 90 d. For shorter storage times, from sample collection in the field to the laboratory, the storage of whole fish for up to 8 h on ice did not result in significantly different ChE activity from the time 0 analyses.

Bluegill were very susceptible to ChE inhibition and produced an IC_{50}=2.22 $\mu g \bullet L^{-1}$ and an LC_{50}=7.74 $\mu g \bullet L^{-1}$. The

percentage of ChE inhibition associated with mortality was 80-90%. The recovery experiment showed that when inhibited fish were placed in clean water their ChE activities came back to near normal after 2 wk. However large variability in data precluded showing statistically significant recovery.

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APPENDIX A

Standard Operating Procedure for the Determination of Cholinesterase Activity in Bluegill (*Lepomis macrochirus*) Brain Tissue

I. Introduction - Justification

Cholinesterase activity is a measure of the amount/effectiveness of cholinesterase in tissues. Cholinesterase is an essential enzyme in the central and peripheral nervous systems, which hydrolyzes acetylcholine, a primary neuro-transmitter. The determination of cholinesterase activity can be used as a biomarker to determine if organisms have been exposed to organophosphorus, or carbamate insecticides, since both types of insecticides primary mode of action is the inhibition of cholinesterase activiy.

A spectrophotometric assay using a plate reader will be used to determine cholinesterase (ChE) activity in fish brain tissue (Ellman et al. 1961; Hill and Fleming 1982; Corvallis Environmental Research Laboratory 1987; The Institute for Wildlife and Environmental Toxicology 1991). ChE activity is determined from the result of two reactions occurring in the assay solution: acetylthiocholine hydrolysis and the nucleophilic attack by the reagent. The assay solution consists of a portion of the brain sample (ChE enzyme), acetylthiocholine (ATCH substrate), and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB reagent).

ATCH is an analogue of the natural ChE substrate, acetylcholine and the analogue has a sulfur atom which replaces the esteric oxygen of acetylcholine. The first reaction is the hydrolysis of ATCH by ChE which proceeds at the same rate as ChE hydrolysis of acetylcholine. Hydrolysis of ATCH results in the formation of a negatively charged thiocholine complex and an acetate ion.

The second reaction is the nucleophilic attack of the thiocholine complex on DTNB, generating a stable, yellow-colored anion (5-thio-2-nitrobenzoate) which absorbs light strongly at 412 nm. For every molecule of ATCH hydrolyzed, approximately one molecule of the anion is generated. The rate of formation of the yellow-colored anion can be measured and subsequent calculations can determine the ChE activity for the sample.

II. Materials

A. Chemicals

1. Acetylthiocholine iodide (ATCH)
2. 5,5-dithiobis-2-nitrobenzoic acid (DTNB)
3. Sodium bicarbonate
4. Trizma 7.4 pH pre-set crystals
5. Trizma 8.0 pH pre-set crystals
6. 1.0 N HCl
7. 1.0 N NaOH

B. Equipment

1. Spectrophotometer: e.g., automated kinetic microplate reader, Molecular Devices Corporation, Thermo max interfaced with a desk top computer (e.g. Zenith z-386/20) loaded with appropriate software package to run spectrophotometer (e.g. Softmax).

2. Constant temperature water bath set a 25°C.

3. Ice bucket and/or ice chest.

4. Crushed ice.

5. Disposable test tubes (13x100 mm).

6. Multi-aliquot, variable volume pipette (e.g., Eppendorf Combitip Pipette) with disposable tips 10 µl, 50 µl, 100 µl and 1000 µl (e.g., Eppendorf Combitips).

7. Single aliquot, variable volume pipette, 10-1000 µl range, with disposable tips.

8. Vortex mixer.

9. Magnetic stirrer and stir bars.

10. pH meter and standards.

11. 96 multi-well microplates, e.g., Dynatech Microtiter.

12. Analytical balance.

13. Volumetric flasks, 5-50 ml and 1000 ml.

14. Weigh boats, glass and plastic.

III. Preparation of buffers, reagents and substrate.

Nanopure or distilled water is used to mix solutions. Bottles containing solutions are labeled with chemical name, date, and preparer's name. Solutions are prepared according to the following procedures:

Trizma 7.4 pH buffer solution

1. Weigh 7.58g Trizma 7.4 pre-set crystals in a glass weigh boat and transfer to a 1-liter volumetric flask.
2. Make a complete transfer of chemical by rinsing the weigh boat with water.
3. Add water until the volumetric flask is approximately 1/3 full and shake until buffer is dissolved.
4. Bring the volumetric flask to volume.
5. Check pH and adjust to pH 7.4 with HCl or NaOH.
6. Pour buffer solution into a labeled bottle and store in the refrigerator (4°C). Buffer solution will be good for one week.

Trizma 8.0 pH buffer solution

1. Weigh 7.09g Trizma 8.0 pre-set crystals in a glass weigh boat and transfer to a 1-liter volumetric flask.
2. Make a complete transfer of chemical by rinsing the weigh boat with water.
3. Add water until the volumetric flask is approximately 1/3 full and shake until buffer is dissolved.
4. Bring the volumentric flask to volume.
5. Check pH and adjust to pH 8.0 with HCl or NaOH.
6. Pour buffer soution into a labeled bottle and store in the refrigerator (4°C). Buffer solution will be good for one week.

ATCH substrate

1. Weigh 0.4512g ATCH in a glass weigh boat and transfer to a 10 ml volumetric flask (make a complete transfer).

2. Fill the flask approximately 1/2 full and mix until ATCH is dissolved.

3. Bring the flask to volume.

4. Transfer to a labeled amber bottle and store in the refrigerator (4°C). Substrate solution will be good for 3 days.

DTNB reagent

1. Measure 50 mL of 7.4 trizma buffer solution in a graduated cylinder.

2. Weigh 0.198g of DTNB in a glass weigh boat and transfer to a labeled amber bottle.

3. Make a complete transfer using part of the measured buffer solution.

4. Weigh 0.075g sodium bicarbonate in a glass weigh boat and transfer to the same amber bottle. Again, make a complete transfer using part of the measured buffer solution.

5. Add the remaining buffer solution to the bottle and mix until dissolved. Store in the regrigerator (4°C). Solution will be good for 3 days.

IV. Analysis procedure:

1. Turn on ice machine and water bath $\geq$ 1 h prior to analysis.

2. Place appropriate volume of Trizma 8 pH buffer in water bath. If Trizma is cold (4°C) allow appropriate time in water bath for it to come to temperature (25°C).

3. Turn on the spectrophotometer (Thermo Max) and control computer. Run the controling software (double click the Softmax icon). Turn the incubator on and set the temperature to 25° C under the control heading. Open the appropriate file (bgche) with the analysis parameters as listed below.

    A. wavelength: 405 nm
    B. run time: 2:00 min
    C. read interval: 7 s
    D. OD limit: 0.500 OD
    E. lag time: 0.00 s
    F. auto mix ON

4. Remove check standards from liquid nitrogen freezer and place in ice to thaw.

5. Euthanize sample fish by severing the spinal column. Remove brain tissue by cutting away the top of the skull, severing the optic nerves and then lifting out the tissue. Keep the brain tissue in iced pH 7.4 Trizma buffer until analysis. Homogenize tissue in pH 7.4 Trizma buffer with a motorized teflon pestle and glass tube. Dilute tissue homogenate using Trizma 7.4 pH to an activity appropriate for the spectrophotometer (usually 200-fold). Record the fish size data on form #1 and the weights of the brain tissue and appropriate dilutions on form #2.

6. Prepare cholinesterase assay plate reader set-up form (#3) indicating the positions of the various samples and check standards and their respective dilution factors.

7. Mark microplate to indicate where particular samples will be placed.

8. Pipette appropriate amounts of reagent into each well for each determination to be performed. Place the DTNB and ATCH on ice next to the analysis station. All samples should be assayed in triplicate.

Volumes of reagents for the various wells are as follows (in µl):

	Blank	ChE
Trizma 8.0 pH	200	170
DTNB	20	20
Enzyme	0	30
ATCH	30	30

9. Add compounds to wells in the order shown in the table. Once the ATCH is added the reaction begins. Immediately select read under the control heading in the software. The drawer will then open for a few seconds to allow for locking of the plate into place.

10. After the analysis is complete, type in comments on the data screen and save the file under an appropriate name. Print off a hardcopy of the file.

11. Check the data for any signs of error. Samples with a coefficient of variance (CV) greater then 10 % should be rerun. Also check if the check standards are in control.

12. Convert mOD output units into international units of enzyme activity using the following equation:

(((enzyme mOD/min)-(blank mOD/min))/1000) x 0.817 x dilution factor = (µmoles ATCH hydrolyzed/min) / gram tissue.

The above equation is derived from Ellman et al. (1961).

Form No. 1

CHOLINESTERASE ASSAY FISH
SIZE DATA SHEET

Date:_____
Study:_____
Species:_____

Id. Number	Length (mm)	Mass (g to 10^{-1})	Id. Number	Length (mm)	Mass (g to 10^{-1})

initials:_____

Form No. 2

CHOLINESTERASE ASSAY TISSUE PREPARATION DATA SHEET

Date:_____
Study:_____
Species:_____
Tissue:_____

Id. Number	[Vessel + sample + buffer] wt. (g)	Vessel wt. (g)	Sample wt. (g)	Dilution factor [d.f.] (fold - 1)	Buffer added [d.f. * sample wt.] (ml to 10^{-3})	Comments (i.e.dilution used, sample condition , etc.)

initials:_____

Form No. 3

CHOLINESTERASE ASSAY PLATE
READER SET-UP

Date:_____
Study:_____
Species:_____
Tissue:_____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

		Blank	Sample
Buffer	200 µl	_____	
Buffer	150 µl		_____
Buffer	20 µl		_____
DTNB	20 µl	_____	_____
Enzyme	30 µl		_____
C. Standard	30 µl		_____
AThCh	30 µl	_____	_____

Initials:_____
Filename:_____
Comments:_____
_____
_____
_____
_____

REFERENCES

Corvallis Environmental Research Laboratory. 1987. Cholinesterase determination procedure. Wildlife Toxicology Team SOP No. 5.5.1. U.S. EPA, Corvallis, OR. 17 pp.

Ellman, G.L., K.D. Courtney, V. Andres, Jr., and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95.

Hill, E.F., and W.J. Fleming. 1982. Anticholinesterase poisoning of birds: field monitoring and diagnosis of acute poisoning. Environ. Toxicol. Chem. 1:27-38.

The Institute for Wildlife and Environmental Toxicology. 1991. Cholinesterase activity determination procedure. SOP No. 202-06-03. TIWET, Clemson, SC. 7pp.

APPENDIX B

ANOVA Tables for Statistical Analyses

The last line represents the error term used in the analysis for each experiment.

Size experiment

source	d.f.
weight class	3
fish (weight class)	36

Temperature experiment

source	d.f.
temp. class	4
tank (temp. class)	5

Long-term storage

source	d.f.
time	5
rep. (time)	6

Short-term storage

source	d.f.
treatment	4
time	1
condition	1
time * condition	1
rep. (treatment)	4

Cholinesterase inhibition test

source	d.f.
concentration	4
tank (concentration)	5

Acute lethality test

source	d.f.
concentration	4
tank (concentration)	5

Cholinesterase recovery test

source	d.f.
time	2
rep. (time)	3