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Toxicity of chlorpyrifos adsorbed on clay and humic acid to larval walleye

by

Todd Alan Phillips

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Major Professors: Robert C. Summerfelt and Gary J. Atchison

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# GENERAL INTRODUCTION

Agricultural practices pose one of the most serious threats to the continued ecological integrity of environmental systems (Thurman et al., 1991) and has been charged as the activity most responsible for loss of fish species in streams (Karr et al., 1985). Therefore, understanding the effects of agricultural runoff (non-point source pollution) on aquatic ecosystems is especially important in the former tallgrass prairie region of the midwestern United States that now forms the heart of the "corn belt," a 12-state area of intensive agriculture. Fish communities of this region have declined since the land was developed for agricultural use. Although all anthropogenic stressors affecting aquatic ecosystems have not been isolated, suspended solids, sediment, and pesticides are believed to be major factors.

Use of organophosphorus insecticides (OPs) has increased rapidly since the 1970s following the ban or restricted use of organochlorine insecticides because of their environmental persistence and biomagnification problems (Pait et al., 1992; Richmonds and Dutta, 1992; Friend and Franson, 1999). Organophosphorus insecticides are extensively used to control insect pests on agricultural crops, turf, and household pests (Giesy et al., 1999). In 1989, OPs represented nearly 40% of the insecticide market (Racke, 1993) and in 1993, the top three OPs used in the United States were chlorpyrifos (3 x $10^6$ kg), terbufos (2.5 x $10^6$ kg), and methyl parathion (2.3 x $10^6$ kg; ERS, 1994).

Chlorpyrifos is used extensively for both agricultural and nonagricultural control of pests because it is a broad-spectrum pesticide with insecticidal activity against a wide range of insect and arthropod pests (Racke, 1993). However, control of agricultural pests represents the major use of chlorpyrifos (Barron and Woodburn, 1995). For example, from 1990 to 1992, about 30% of the agricultural land (3 x $10^6$ ha) treated with insecticides was treated with chlorpyrifos (USEPA, 1996) and the majority was applied in the corn belt.

The extensive use of OPs represents both potential point and non-point sources of pollution. For example, agricultural use of OPs may lead to non-point source pollution from

runoff and spray drift, and turf and household use may lead to contamination of urban and rural storm water runoff (Giesy et al., 1999). Each of these uses provides a pathway for the transportation of OPs to aquatic ecosystems, and although they are less persistent (e.g., the half-life of chlorpyrifos ranges from 5.3 h to 28 d; Racke, 1993) in the environment than organochlorine insecticides, OPs often have a higher acute toxicity to nontarget species, especially aquatic organisms. For example, bluegill (Lepomis macrochirus) (weight = 0.5 to 0.8 g) exposed to chlorpyrifos were found to have a 96-h LC50 of 2.4 µg $L^{-1}$ (Mayer and Ellersieck, 1986).

Even though OPs are a suspected non-point source pollutant, because they generally have short half-lives, they are rarely reported in random surveys of water samples (Barron and Woodburn, 1995). In addition, the low water solubility of OPs (e.g., chlorpyrifos has a solubility of 1.39 mg $L^{-1}$ at 25°C) and high soil adsorption coefficients (Kd) (e.g., chlorpyrifos has a Kd value that ranges from 13.4 mL $g^{-1}$ to 1862 mL $g^{-1}$ depending of the soil type, climatic conditions, and application practices) (Harris, 1972; Racke, 1993) make detecting OPs in water samples from aquatic ecosystems even more difficult because OPs are often adsorbed on suspended solids.

Because OPs are rarely detected in water samples, measurement of cholinesterase (ChE) inhibition has been used as a bio-indicator of environmental exposure to OPs (Habig and Di Giulio, 1991). Cholinesterases are important enzymes of the nervous system found in all animals, and the attachment of OPs to the ChE enzyme prevents the enzyme from clearing acetylcholine (ACh), a neurotransmitter, from the synapse. Organophosphorus insecticide binding to ChE is largely irreversible, because a covalent bond is formed (Matsumara, 1985), which can cause inhibition of ChE for 2 to 6 weeks (van der Wel and Welling, 1989; Morgan et al., 1990; Carr et al., 1995). Therefore, OP exposure may be determined long after OP residues are no longer detected in water (Brock et al., 1992). Also, because ChE inhibition provides evidence that fish have been exposed to OPs, a survey of ChE inhibition is an

effective method to measure the contamination of OPs in the environment (Williams and Sova, 1966; Holland et al., 1967; Coppage and Matthews, 1974; Haines, 1981).

Although ChE inhibition is effective in measuring OP exposure in aquatic organisms, factors affecting ChE, other than from OPs, must be evaluated. For example, Rattner and Fairbrother (1991) suggested that differences in age, sex, and reproductive status and water quality parameters such as temperature and dissolved oxygen may be potential sources of variation in ChE activity. Zinkl et al. (1987) and Rath and Misra (1981) determined that larger fish had lower ChE activities than smaller fish, but no relationship between weight and ChE activity was found in bluegill (Cole, 1995). Hogan (1970) showed a significant correlation in adult bluegill and water temperature, but Beauvais (1997) found no correlation.

The effects of Finquel® (tricaine methane sulfonate), an anesthetic commonly used to anesthetize and euthanize fish, on ChE activity is unclear. If Finquel® effects ChE activity, comparisons of studies that euthanize fish by different methods will be invalid. The effects of stress on ChE activity is another factor that needs to be examined because handling during toxicity tests and collection from the field will certainly produce a physiological stress response (Molinero et al., 1997; Papoutsoglou et al., 1999). Finally, the effects of postmortem changes and long-term storage of tissue on ChE activity must be determined so that results among studies can be compared. Therefore, interpretations of findings may be ambiguous unless sources of variability in measuring ChE activity are evaluated for each species and environmental condition.

Understanding the most sensitive life stage of fish is critical because, although adult and juvenile fish are usually used to evaluate the toxicity of pesticides, the early life stages are commonly considered to be the most sensitive to waterborne toxicants (Macek and Sleight, 1977; McKim, 1977). In addition, although some studies have evaluated the toxicity of pesticides on early life stages of fish (Olson and Marking, 1973, 1975; Smith and Cole, 1973), little work has been done to evaluate the toxicity of OPs. However, it is critical that the effects

of OPs on different life stages be understood, because development of eggs and larvae of many fishes coincides with the spring application of many OPs (Heath et al., 1993a, 1993b; Havens et al., 1998).

In addition to determining the most sensitive life stage to OPs, the potential effects of OPs adsorbed on suspended colloids needs to be determined. It is possible that OPs adsorbed on suspended colloids present the greatest likelihood of adverse environmental consequences to aquatic life, because OPs are transported off site in surface runoff adsorbed to colloidal materials (organic and inorganic soil constituents).

Although research has not evaluated the biological availability of OPs transported by colloids to aquatic organisms, Misitano et al. (1994) demonstrated that polynuclear aromatic hydrocarbons and polychlorinated biphenyls sorbed to sediment were transferred to larval fish. The bioavailability of colloid adsorbed OPs depends on the strength of the colloid-OP bonding, the strength of interaction between OPs and gill tissue, and the extent of exposure of gills to colloids containing OPs. Thus, it seems that in aquatic environments, OPs adsorbed on suspended colloids may be absorbed by fish when the suspended colloids come in contact with gill tissue, a metabolically active epithelium.

Lastly, suspended solids may represent a threat to aquatic organisms. A substantial body of research on the effects of suspended solids on salmonid fishes in the western United States has demonstrated negative effects of suspended solids on survival, growth, feeding, reproduction, and behavior of fish (Swenson and Matson, 1976; Auld and Schubel, 1978; Sigler et al., 1984; Vandenbyllaardt et al., 1991). However, these studies have not attempted to characterize the nature of the inorganic components; no study has evaluated the influence of clay mineralogy. Ironically, in spite of water problems of non-point source pollution in Midwest streams, little work has been done to evaluate the effects of suspended solids on warmwater fishes of the Midwest, even though the most extensive damage to streams has been

in the agricultural Midwest where warmwater streams have been severely degraded (Waters, 1995).

## Objectives

The objectives of this dissertation were to: 1) evaluate sources of variability in measuring ChE activity in larval to juvenile walleye; 2) determine the most sensitive life stage of walleye (Stizostedion vitreum) exposed to chlorpyrifos by quantifying the concentration of chlorpyrifos that caused 50% mortality (LC50) in three larval stages and three ages of juvenile walleye; 3) evaluate the bioavailability (sublethal effect as measured by cholinesterase activity and acute toxicity) of chlorpyrifos adsorbed on humic acid (HA) and Panther Creek (PC) clay to larval walleye; 4) to determine the toxicity of Cedar River sediments and water to larval walleye.

## Dissertation organization

This dissertation consists of a general introduction, four journal papers, and a general conclusion. The first journal paper evaluates sources of variability in measuring ChE activity in larval to juvenile walleye. This paper will be submitted for publication to Hydrobiologia under the authorship of Todd A. Phillips, Robert C. Summerfelt, and Gary J. Atchison. The second journal paper describes the sensitivity of several life stages of walleye to chlorpyrifos by determining the LC50s for three larval stages and three ages of juvenile walleye. This paper will be submitted for publication to Aquatic Toxicology under the authorship of Todd A. Phillips, Robert C. Summerfelt, and Gary J. Atchison. The third journal paper evaluates the bioavailability (sublethal effect as measured by cholinesterase activity and acute toxicity) of chlorpyrifos adsorbed on HA and PC clay to larval walleye. This paper will be submitted for publication to Aquatic Toxicology under the authorship of Todd A. Phillips, Robert C. Summerfelt, Jigang Wu, and David A. Laird. The fourth journal paper evaluates the toxicity of Cedar River sediments and water to larval walleye. This paper will be submitted for

publication to the Journal of the Iowa Academy of Science under the authorship of Todd A. Phillips and Robert C. Summerfelt. Each chapter is in the style desired by each journal. The General Introduction and General Conclusion sections are presented using Aquatic Toxicology style. References cited in the General Introduction and General Conclusion are listed in the General References section.

# CHAPTER 1. FACTORS AFFECTING CHOLINESTERASE ACTIVITY IN WALLEYE (STIZOSTEDION VITREUM)

Todd A. Phillips, Robert C. Summerfelt, and Gary J. Atchison

## Abstract

Organophosphorus insecticide (OP) use has increased rapidly since the 1970s as a direct consequence of the ban or restricted use of organochlorine insecticides. The short half-lives of OPs makes direct monitoring of OPs in water samples from aquatic ecosystems difficult. Consequently, measurement of cholinesterase (ChE) inhibition has been used as a bio-indicator of environmental exposure to OPs. Factors affecting ChE activity must be considered and understood before ChE activity can be used as a dependable indicator of fish exposure to ChE inhibitors. We examined the effects of water temperature, size of larval and juvenile walleye (Stizostedion vitreum), stress, long-term storage, postmortem changes, and methods of euthanasia on ChE activity. Water temperature (17.2, 20.9, and 24.6°C), stress, long-term storage, postmortem changes, and the method of euthanasia had no effect on ChE activity of walleye. A positive relationship was observed between whole body ChE activity and total length (7.2 to 17.9 mm) of larval walleye. However, no relationship was found between brain ChE activity and total length (59 to 164 mm) of juvenile walleye. These results indicate that ChE is a reliable and effective bio-indicator of OP exposure in walleye.

## Introduction

Organophosphorus insecticide (OP) use has increased rapidly since the 1970s as a direct consequence of the ban or the restricted use of organochlorine insecticides because of their environmental persistence and biomagnification problems (Pait et al., 1992; Richmonds & Dutta, 1992; Friend & Franson, 1999). Although OPs are less persistent in the environment

than organochlorine insecticides, OPs often have a higher acute toxicity and may result in toxicity to nontarget species; the 96-h LC50s for bluegill (*Lepomis macrochirus*) are 1.8 µg L$^{-1}$ for terbufos and 2.4 µg L$^{-1}$ for chlorpyrifos (Mayer & Ellersieck, 1986).

Organophosphorus insecticides have short half-lives (e.g., chlorpyrifos has a half-life of < 6 h in pasture water under field conditions; Racke, 1993) which makes detection in water samples from aquatic ecosystems difficult. In addition, because of their high soil adsorption coefficients (Kd), OPs are strongly adsorbed to colloidal materials (e.g., chlorpyrifos has a Kd value that ranges from 13.4 mL g$^{-1}$ to 1,862 mL g$^{-1}$, depending of the soil type, climatic conditions, and application practices; Harris, 1972; Racke, 1993). Because of this, measurement of cholinesterase (ChE) inhibition has been used as a bio-indicator of environmental exposure to OPs (Habig & Di Giulio, 1991) because OPs inhibit ChE. Organophosphorus insecticides attach to the ChE enzyme, which prevents the enzyme from clearing acetylcholine (ACh). Because ChE is unable to clear ACh from the synapse, ACh accumulates, and when sufficient ACh is present, a rapid twitching of voluntary muscles, paralysis, and death may follow.

Ellman et al. (1961) developed a quantitative colorimetric assay to measure ChE activity in the tissues of organisms. This method has been used to monitor OP exposure in fish, birds, and rodents (Finlayson & Rudnicki, 1985; Johnson & Wallace, 1987; van der Wel & Welling, 1989). At this time, however, relatively little attention has been given to describing environmental and biological factors that affect ChE activity. ChE has been found to vary among and within fish species (Habig & Di Giulio, 1991). Hogan (1970) showed a significant correlation between ChE activity in adult bluegill and water temperature, but Zinkl et al. (1987) showed no relationship between water temperature and ChE activity in rainbow trout (*Oncorhynchus mykiss*). In addition, Zinkl et al. (1987) and Rath and Misra (1981) determined that large rainbow trout and tilapia had lower ChE activities than small rainbow

trout and tilapia, but no relationship between weight and ChE activity was found in bluegill (Cole, 1995).

The effects of Finquel® (tricaine methane sulfonate), an anesthetic commonly used to anesthetize and euthanize fish, on ChE activity is unclear. If Finquel® affects ChE activity, comparisons of studies that euthanize fish by different methods will be invalid. The effect of stress on ChE activity is another factor that needs to be quantified because fish are handled during toxicity tests and when collected from the field. The effects of stress on ChE activity are also unknown. Therefore, we sequentially sampled fish every 45 min for 135 min. This type of fish sampling often produces a rise in cortisol, a physiological indicator of stress (Molinero et al., 1997; Papoutsoglou et al., 1999). Finally, the effects of postmortem changes and long-term storage of tissue on ChE activity must be determined so that results among studies can be compared.

The objectives of this study were to: 1) optimize the ChE assay for use with larval and juvenile walleye (Stizostedion vitreum); 2) quantify the relationship between ChE activity and size or age of fish; 3) quantify the effects of anesthesia; 4) quantify the effects of water temperature; 5) quantify the effects of stress on ChE activity, 6) quantify the effects of postmortem changes of walleye euthanized and placed in 19°C water for 24 h; 7) quantify the effects of long-term storage of tissue at −80°C on ChE activity.

## Materials and Methods

### Fish

Eyed walleye eggs were obtained from the Rathbun Fish Hatchery, Moravia, Iowa on March 4, 1999 and from Spirit Lake Fish Hatchery, Spirit Lake, Iowa on April 21, 1999. Both groups of eggs were incubated at 13°C in standard hatching jars for 5 d before hatching began, but to maintain uniformity of age, only larvae that hatched within a 24-h interval were used in

this study. Mean length ± SE of 20 larvae at hatching was 7.2 ± 0.04 mm and 7.4 ± 0.05 mm for Rathbun and Spirit Lake fish, respectively.

## Culture conditions

Both groups of walleye were raised as described by Summerfelt (1996). For both groups of fish, at 3 d posthatch, larvae were stocked at a density of 20 larvae $L^{-1}$ (3,000 larvae) in a 150-L tank. Walleye were raised in turbid water at 16.6 ± 0.2°C (Rathbun) and 17.2 ± 0.2°C (Spirit Lake) for the first 30 d posthatch. Walleyes were fed different kinds and sizes of food over the course of the 250 d they were cultured. The different types of food are described by Summerfelt (1996).

## Water quality of culture tanks

Every day, temperature was measured to the nearest 0.1°C using a glass thermometer, and dissolved oxygen (DO) was measured to the nearest 0.1 mg $L^{-1}$ using an oxygen-sensitive membrane electrode (polarographic). Total ammonia-nitrogen ($NH_3$-N; TAN) was measured to the nearest 0.01 mg $L^{-1}$ twice a month using the Nesslerization method (APHA, 1998) and a spectrophotometer. Quality control samples (HACH Company, Loveland, Colorado) were analyzed along with water samples to verify the accuracy of the procedures used to measure TAN. Measured concentrations for the externally supplied TAN quality assurance samples were always within the certified 95% confidence interval. The pH was measured to the nearest 0.1 weekly with a standard combination electrode and meter standardized with pH 4.0, 7.0, and 10.0 buffers. Hardness was measured to the nearest 1 mg $L^{-1}$ twice a month using the Man Ver 2 burette titration method (HACH Company, Loveland, Colorado). Total alkalinity was measured twice a month to the nearest 1 mg $L^{-1}$ by titration with 0.02N $H_2SO_4$ (APHA, 1998).

Fish sampling

For both groups of fish, a sample of 5 fish was netted every day to 30 d posthatch. Walleye

were euthanized with 300 mg L$^{-1}$ Finquel®, measured to the nearest millimeter, and observed

microscopically for gas bladder inflation (GBI), presence of food in the gut, and deformities.

Observations of the day when the yolk sac and oil globule disappeared were used to describe

their larval stage as well as when gas bladder inflation began and first feeding occurred. The

three larval stages of walleye are: prolarval (yolk sac present: 1 to 5 d posthatch); postlarval I

(yolk sac absent and oil globule present: 6 to 14 d posthatch); and postlarval II (oil globule

absent: 15 to 21 d posthatch).


Analysis of cholinesterase

Cholinesterase activity (ChE) was analyzed in homogenates of whole individual larval fish,

heads of individual early juvenile fish, and the excised brain of individual juvenile fish. A

colorimetric method for analyzing whole body and brain ChE activity, modified for use on a

THERMOmax microplate reader and SOFTmax software (Molecular Devices Corporation,

Sunnyvale, California), was used to monitor the rate of formation of 5-thio-2-nitrobenzoate, a

yellow-colored anion. Hydrolysis of acetylthiocholine by ChE results in an acetate ion and a

negatively charged thiocholine complex that reacts with 5,5-dithiobis-2-nitrobenzoic acid

(DTNB) to form 5-thio-2-nitrobenzoate (Ellman et al., 1961; Hill and Fleming, 1982; Gard and

Hopper, 1993). The microplate reader was set in the kinetic mode to monitor increases in

absorbance at 405 nm for 2 min, read at 8 sec intervals with a 0 sec lag time, and a final

volume of 250 μl well$^{-1}$ at 25°C (Gard and Hopper, 1993; Beauvais, 1997). The optimal

substrate concentrations, ATI, for larval and juvenile walleye were determined prior to analysis

with non-test samples. The optimal concentration was determined to be 0.001 M ATI. The

Vmax and dilution factors were used to calculate ChE activities, expressed as μmoles AThCh

hydrolyzed $min^{-1}$ $g^{-1}$ of tissue. Hereafter, mention of ChE activity will be abbreviated as $\mu M$ AThCh.

## Quality assurance

All tissue samples analyzed for ChE activity were run in triplicate. If the CV among the triplicates was greater than 10%, samples were rerun (less than 5% of the samples had to be rerun). Because a commercial cholinesterase standard for walleye tissue was not available, a check standard was used. The check standard was made by pooling larval walleye or brains of juvenile walleye diluted 100-fold in pH 7.4 Tris buffer. The pooled tissue was homogenized and divided into 1-mL aliquots in 2 mL cryovials and placed in liquid nitrogen. These aliquots were run as check standards in triplicate along with each plate of treated samples throughout the study. If the CV was greater than 10%, all samples were rerun (less than 5% of the samples had to be rerun).

## Optimization

Larval walleye (whole body). To increase the precision and sensitivity of the ChE method, the concentration of substrate, ATI, that gave the greatest response in the enzyme sample from larval walleye was determined. Fifteen prolarvae (1 d posthatch; mean length ± SE = 7.2 ± 0.04 mm) and five postlarvae I (12 d posthatch; mean length ± SE = 11.5 ± 0.14 mm) walleye (Rathbun fish) were collected from the culture tank, euthanized, and analyzed immediately for ChE activity using a series of concentrations of ATI ranging from 0.01 to 0.0001 M. Because of their small size, three prolarvae were pooled for each sample. The concentration of ATI that was used in all analyses of larval walleye was 0.001 M, because this concentration gave the maximum ChE response.

Juvenile walleye (brain tissue). Five juvenile walleye (160 d posthatch) with a mean length ± SE of 125 ± 3.12 mm and a mean weight ± SE of 19.5 ± 1.82 g were analyzed for ChE using a

series of ATI concentrations ranging from 0.01 to 0.00001 M and set concentrations of the remaining agents. The concentration of ATI that gave the greatest response in the enzyme sample from excised brain tissue of juvenile walleye was determined. The concentration of ATI that was used in all analyses of juvenile walleye was 0.001 M, because this concentration gave the maximum ChE response.

### Effects of size and age on ChE activity

The effect of life stage on ChE activity was determined by measuring whole body ChE activity in three larval stages of walleye: prolarval; postlarval I; and postlarval II. For both groups of fish (Rathbun and Spirit Lake), ten larvae were removed from the culture tank on 3, 8, and 17 d posthatch, and five larvae were placed in each of two 2-L glass beakers filled with 1.5 L of system water. Fish were allowed to acclimate for 48-h. At the end of the 48 h, fish were euthanized with 300 mg L$^{-1}$ Finquel®, placed in freezer bags, and stored at −80°C until ChE analysis.

For both groups of fish, ten juvenile walleye were removed from the culture tank 28 and 88 d posthatch and five fish were placed in each of two 40-L aquaria filled with 20 L of system water. For Rathbun fish, ten juvenile walleye were also removed from the culture tank 78 and 200 d posthatch and five fish were placed in each of two 40-L aquaria filled with 20 L of system water. For the Spirit Lake fish, ten juvenile walleye were removed from the culture tank 21, 135, and 150 d posthatch and five fish were placed in each of two 40-L aquaria filled with 20 L of system water. All fish were allowed to acclimate for 48 h. At the end of the 48 h, fish were euthanized with 300 mg L$^{-1}$ Finquel®, placed in freezer bags, and stored at −80°C until ChE analysis. Cholinesterase activity was measured using whole bodies of larval and 23 d posthatch juvenile walleye. Whole heads of 30- and 37-d-old juvenile walleye, and brain tissue for all other juvenile walleye, were used to measure ChE activity.

## Effects of anesthesia on ChE activity

Two methods used to sacrifice fish were compared to determine the effects of anesthesia on ChE activity in walleye: 1) immersion in 300 mg L$^{-1}$ Finquel® for 15 min and 2) severing the spinal cord. Five fish (Rathbun fish) from each of three culture tanks were removed at 80 d posthatch and euthanized in 300 mg L$^{-1}$ Finquel® for 15 min. After 15 min, each fish was weighed, measured, and stored individually in freezer bags at –80°C for later ChE analysis. Five fish from each of three culture tanks were removed at 80 d posthatch and sacrificed by severing the spinal cord. Each fish was weighed, measured, and stored individually in freezer bags at –80°C for later ChE analysis. Fish had a mean length ± SE of 63.1 ± 0.83 mm and a mean weight ± SE of 2.78 ± 0.11 g.

## Effects of temperature on cholinesterase activity

The effect of temperature on ChE activity was determined by measuring ChE activity of 149-d-old juvenile walleye (Spirit Lake fish) exposed to three different temperatures (17.2 ± 0.02°C; 20.9 ± 0.03°C; and 24.6 ± 0.04°C). Twenty-five juvenile walleye with a mean length ± SE of 129 ± 4.2 mm and a mean weight ± SE of 21.3 ± 2.33 g were stocked into each of nine 100-L tanks (three tanks per treatment) with black sides and a black bottom. To obtain three different temperatures, two systems of water were used. Water was 24.6°C in one system and 17.2°C in the other system. The 20.9°C water was obtained by mixing 17.2°C and 24.6°C water. To determine the effects of different temperatures on ChE activity, five walleye were removed from each tank after acclimation periods of 1 h, 4 d, and 7 d. Fish removed from each tank were euthanized with 300 mg L$^{-1}$ Finquel®, weighed, measured, placed in individual freezer bags, and stored at –80°C for later ChE analysis. Temperature, DO, pH, hardness, and alkalinity were measured at the beginning and at the end of the experiment. Differences in temperature, DO, TAN, pH, alkalinity, and hardness were not significantly different among treatments.

Effects of sequential sampling on ChE activity

To determine the effect of stress on ChE activity in 200-d-old walleye, five walleye were removed from each of three culture tanks every 45 min for 135 min. Fish were sampled at time 0, before they were disturbed, so baseline brain ChE activity could be determined. After the 90 min sampling, additional stress was induced by turning lights on and off and hitting the sides of the tanks. Fish sampled at each interval were euthanized in 300 mg L$^{-1}$ Finquel® for 15 min. After fish were euthanized, each fish was individually weighed, measured, placed in individual freezer bags, and frozen at $-80°C$ for later ChE analysis.

Effects of postmortem changes on ChE of walleye

Cholinesterase activity was evaluated in 136-d-old walleye (Spirit Lake) euthanized in 300 mg L$^{-1}$ Finquel® for 15 min and placed in 19°C water for up to 24 h. Thirty euthanized fish were placed in each of three, 100-L tanks and five fish were removed from each tank immediately after being euthanized and at 1, 4, 8, 12, and 24 h. After fish were removed from each tank, they were weighed, measured, placed in individual freezer bags, and frozen at $-80°C$ for later ChE analysis.

Effects of long-term storage on ChE activity

Cholinesterase activity was evaluated in 22-d-old walleye (Spirit Lake) euthanized in 300 mg L$^{-1}$ Finquel® for 15 min and stored at $-80°C$ for up to 180 d. Forty juvenile walleye with a mean length ± SE of 19.4 ± 2.33 mm were removed from each of three culture tanks and euthanized with 300 mg L$^{-1}$ Finquel®. After fish were euthanized, five fish were placed into each of 24 cryovials and stored at $-80°C$ for later ChE analysis. Juvenile walleye were analyzed for whole body ChE immediately and after 1, 7, 14, 30, 60, 90, and 180 d of storage at $-80°C$.

Statistical analysis

Differences among treatment effects (Finquel®, temperature, life stage, short-term, and long-term storage) on ChE activity were assessed by analysis of variance using Statview® (SAS, 1998). Regression analyses were done to determine relationships between size and ChE activity, temperature and ChE activity, sequential sampling and ChE activity, postmortem changes and ChE activity, and long-term storage and ChE activity.

## Results

Water quality of culture tanks

Mean temperature and DO values in the culture tank (from hatch to 250 d posthatch) containing Rathbun walleye were $19.3 \pm 0.10°C$ and $6.9 \pm 0.07$ mg $L^{-1}$, respectively. Mean pH, TAN, hardness, and alkalinity values were $7.3 \pm 0.02$, $0.411 \pm 0.033$ mg $L^{-1}$, $160 \pm 2.0$ mg $L^{-1}$, and $41 \pm 0.6$ mg $L^{-1}$, respectively. Mean temperature and DO values in the culture tank (from hatch to 250 d posthatch) containing Spirit Lake walleye were $19.5 \pm 0.12°C$ and $6.8 \pm 0.07$ mg $L^{-1}$, respectively. Mean pH, TAN, hardness, and alkalinity values were $7.3 \pm 0.02$, $0.520 \pm 0.043$ mg $L^{-1}$, $163 \pm 2.5$ mg $L^{-1}$, and $41 \pm 0.7$ mg $L^{-1}$, respectively.

Optimization

The concentration of acetylthiocholine iodide that gave the greatest response of ChE activity was 0.001 M for prolarvae, postlarvae I, and juvenile walleye (Table 1).

Effects of size and age on ChE activity

Larval walleye. Cholinesterase activity ranged from 3.77 μM AThCh in 1-d-old prolarvae to 10.41 μM AThCh in 19-d-old postlarvae II walleye (Rathbun; Table 2). Also, ChE activity ranged from 4.88 μM AThCh in 5-d-old prolarvae to 11.86 μM AThCh in 19-d-old postlarvae II walleye (Spirit Lake; Table 3). Because differences in ChE activity did not differ between

Rathbun and Spirit Lake larval walleye, all data were pooled. A positive relationship was found between whole body ChE activity and total length of larval walleye ($r^2 = 0.87$; $P < 0.01$; Figure 1).

Juvenile walleye. Brain ChE activity for juvenile fish ranged from 11.20 $\mu$M AThCh in 202 d posthatch walleye to 13.54 ± 0.15 $\mu$M in 80 d posthatch walleye (Rathbun; Table 2). Also, brain ChE activity ranged from 12.44 $\mu$M AThCh in 60 d posthatch walleye to 14.32 $\mu$M AThCh in 90 d posthatch walleye (Spirit Lake; Table 3). Because differences in ChE activity did not differ between Rathbun and Spirit Lake juvenile walleye, all data were pooled. No relationship was found between brain ChE activity and total length of juvenile walleye ($r^2 = 0.459$; $P = 0.10$; Figure 2).

## Effects of anesthesia on ChE activity

Mean ChE activity for juvenile walleye (80 d posthatch) sacrificed by severing the spinal cord was 14.30 $\mu$M AThCh and mean ChE activity for fish euthanized by immersion in 300 mg L$^{-1}$ Finquel® for 15 min was 13.80 $\mu$M AThCh. The differences in ChE activity were not significant ($P = 0.353$).

## Effects of temperature on ChE activity

Cholinesterase activity of juvenile walleye (149-d-old) acclimated to three different temperatures for 1 h ranged from 10.95 $\mu$M AThCh at 24.6°C to 11.78 $\mu$M AThCh at 17.2°C (Table 4). The differences in ChE activity were not significant ($P = 0.453$).

Juvenile walleye (149-d-old) acclimated to the three different for 4 d had ChE activity ranging from 11.08 $\mu$M AThCh in the 17.2°C treatment to 11.89 $\mu$M AThCh in the 20.9°C treatment (Table 4). The differences in ChE activity were not significant ($P = 0.486$).

Cholinesterase activity of juvenile walleye (149-d-old) acclimated to three temperatures for 7 d ranged from 11.61 µM AThCh at 20.9°C to 12.11 µM AThCh at 24.6°C (Table 4). The differences in ChE activity were not significant ($P = 0.780$).

## Effects of sequential sampling on ChE activity

There was no relationship ($r^2 = 0.327$; $P = 0.428$) between ChE activity and sequential sampling, as measured by brain ChE activity, in 200-d-old juvenile walleye (Figure 3). Cholinesterase activity ranged from a high of 11.69 µM AThCh 45 min after initial sampling to 11.16 ± 0.81 µM AThCh 90 min after initial sampling (Table 5).

## Effects of postmortem changes on ChE of walleye

Cholinesterase activity in juvenile walleye (136-d-old) that remained in 19°C water for up to 24 h after they were euthanized by a 15-min exposure to 300 mg $L^{-1}$ Finquel® ranged from 14.03 µM AThCh after 12-h to 11.80 µM AThCh after 24 h (Table 6). There was no relationship ($P = 0.382$) between ChE activity and time in 19°C water following death for a 24 h period ($r^2 = 0.194$; Figure 4).

## Effects of long-term storage on ChE activity

There was no pattern in ChE of 22-d-old walleye (Spirit Lake) that were stored at –80°C for 180 d. Cholinesterase activity ranged from 9.31 ± 0.12 µM AThCh after 7 d to 11.20 ± 0.18 µM AThCh after 90 d (Table 7). There was no significant relationship ($P = 0.153$) between days stored at –80°C and mean ChE activity ($r^2 = 0.308$; Figure 5).

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## Discussion

Effects of size and age on ChE activity

Because size, age, and development may affect ChE activity, fish of similar size should be used in experiments evaluating the effects of ChE inhibitors. If fish of similar size are not available, it is recommended that relationships between size, age, and development be understood so that estimates of variation in ChE activity can be made.

We found that whole body ChE activity increased throughout the larval stage of development for walleye (length = 7.2 to 17.9 mm). Similar results have been observed in birds and mice. Cholinesterase activity in eastern bluebirds (Sialia sialis) and European starlings (Sturnus vulgaris) increased from hatching to fledging (Gard & Hooper, 1993), and Fishwick et al. (1996) found that ChE activity increased two to three fold during the first few weeks of life in wood mice (Apodemus sylvaticus).

No relationship was found between brain ChE activity and length of juvenile walleye (59 to 163 mm). Cole (1995) also found no relationship between brain ChE activity and size of bluegill ranging from 40 to 180 mm. However, many studies have found that ChE activity does change with fish size. For example, Weiss (1961) found that brain ChE activity was inversely proportional to brain weight in largemouth bass (Micropterus salmoides), bluegill, golden shiner (Notemigonus crysoleucas), and goldfish (Carassius auratus). Similar results were found in bluegill ranging in size from 30 to 189 mm (Beauvais, 1997). Tilapia (Tilapia mossambica) ranging in size from 40 to 110 mm were also found to have a significant negative correlation between body weight and brain ChE activity (Rath & Misra, 1981). Rath and Misra (1981) suggested that the negative correlation was related to a decrease in enzyme activity during aging and that toxicity was directly related to active metabolism and inversely related to aging. Rath and Misra (1981) also found that increased age decreased inhibition of ChE in tilapia when exposed to dichlorvos, an OP insecticide. Zinkl et al. (1987) found that small (5 to 54 g) rainbow trout had significantly higher ChE activity than large (1,300 to 2,300 g)

rainbow trout. However, there was not a linear relationship between brain ChE activities and size of rainbow trout.

## Effects of anesthesia on ChE activity

Although anesthetics are frequently used to euthanize fish in experiments evaluating the effects of ChE inhibitors on fish (Eaton, 1970; Finlayson & Rudnicki, 1985), the effects of the type, concentration, and duration of exposure to the anesthetic have not been described. We found no significant differences in brain ChE activity in walleye euthanized by either an anesthetic (Finquel®) or by severing of the spinal cord. Beauvais (1997) also found no significant differences in ChE activity of bluegill euthanized by either Finquel® or severing of the spinal cord. Therefore, if appropriate concentrations of Finquel® are used to euthanize fish, it seems that effects of Finquel® anesthesia on ChE activity will be negligible.

## Effects of temperature on ChE activity

Cholinesterase activity did not differ significantly in walleye exposed to 17.2, 20.9, and 24.6°C for 7 d. Similarly, brain ChE activity was not significantly different for rainbow trout exposed to 9, 13, and 20°C for 7 d (Zinkl et al., 1987). Cole (1995) and Beauvais (1997) also found no significant difference in ChE activity in bluegill exposed to five temperatures ranging from 20–30°C for two weeks and 13.0–25.9°C, respectively.

However, other studies have found a positive relationship between brain ChE activity and fish acclimated to different water temperatures. Brain ChE activity was twice as high in goldfish exposed to 35°C as it was at 5°C (Hazel, 1969), and Hogan (1970) observed a positive relationship between water temperature and brain ChE activity of bluegill over a one year period when water temperatures ranged from 0 to 27°C. Pavlov (1994) also found that brain ChE activity of tilapia was greater at 26–27°C than at 23–24°C. However, the increase in

brain ChE activity observed by Hogan (1970) and Pavlov (1994) could have also been related to seasonal changes and increased reproductive activity.

Although we found no changes in brain ChE activity in walleye exposed to different temperatures in the present study, it is possible that a larger range of temperatures would have resulted in changes in brain ChE activity. However, the temperature range used in the present study ($17.2-24.6°C$) is representative of spring and summer water temperatures, when monitoring for OPs would most likely occur in lakes and rivers containing walleye.

Effects of stress on ChE activity

There was no relationship between brain ChE activity and sequential sampling in juvenile walleye. Our findings suggest that stress induced by fish collection and sampling have no effect on ChE activity. However, ChE activity has been reported to be affected by fish collecting gear (Beauvais, 1997). In that study, mean brain ChE activity in bluegill collected by trap nets was not significantly different from mean ChE activities collected by electrofishing; however, mean ChE activity of bluegill collected by angling was significantly lower than the mean ChE activity of bluegill collected by electrofishing.

Effects of postmortem changes on ChE of walleye

Because fish may not be collected for 24 h or more after a fish kill, understanding the effects of postmortem changes of fish on ChE activity is critical. If postmortem changes significantly decrease ChE activity, accurate conclusions may not be made concerning pesticide poisonings. We found no significant differences in mean ChE activity for euthanized walleye that were held in $19°C$ water for 24 h after death had occurred. Similarly, Stansley (1992) found that ChE activity in fathead minnow remaining in $19-22°C$ for up to 24 h after being euthanized did not differ significantly from initial ChE measurements.

Zinkl et al. (1987) found that brain ChE activity remained stable in rainbow trout stored at 4°C for 2 d and at ambient temperatures for 1 d, but ChE activity decreased significantly after 7 d at 4°C. Brain ChE activity of whole channel catfish (Ictalurus punctatus) stored at 7°C for 1 to 5 d decreased significantly with increased storage time (Finlayson and Rudnicki, 1985). ChE activity was also significantly inhibited by storing whole channel catfish at –5°C for 2 to 14 d.

These results suggest that collecting fish samples up to 24 h postmortem should not adversely affect ChE activity, thus removing the possibility of an erroneous diagnosis of pesticide poisoning.

## Effects of long-term storage on ChE activity

Because analysis of ChE activity may not be possible immediately after fish are collected, the ability to store fish for later ChE analysis is critical. Thus, it is critical to determine the length of time ChE remains stable.

There was no relationship in brain ChE activity of walleye tissue stored at –80°C for 180 d. Similar results were found in several other species. Brain ChE activity remained stable in rainbow trout stored at –80°C and –20°C (Zinkl, 1987) and in fathead minnows after 2 weeks at –18°C (Stansley, 1992). Weiss (1961) found that brain ChE activity is stable for up to 3 months at –25°C and Cole (1995) found that ChE activity in bluegill did not differ significantly over 90 d stored in liquid $N_2$. These results suggest that ChE remains stable for at least 180 d when stored at –80°C and at least 90 d when stored at –25°C.

## Acknowledgements

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Table 1. Optimization of 1 d posthatch (7.2 ± 0.04 mm) prolarvae and 12 d posthatch postlarvae I (11.5 ± 0.14 mm) Rathbun walleye.

| AThCh (M) | Prolarvae[1] (ChE Activity ± SE) | Postlarvae I[1] (ChE Activity ± SE) | Juvenile[2] (ChE Activity ± SE) |
|---|---|---|---|
| 0.01 | 3.37 ± 0.07 | 8.21 ± 0.40 | 12.8 ± 0.43 |
| 0.0032 | 3.47 ± 0.04 | 8.92 ± 0.30 | 13.9 ± 0.83 |
| 0.001 | 3.77 ± 0.29 | 9.07 ± 0.28 | 14.8 ± 0.88 |
| 0.00032 | 2.72 ± 0.12 | 7.44 ± 0.27 | 12.3 ± 0.87 |
| 0.0001 | 1.63 ± 0.10 | 5.26 ± 0.22 | 7.8 ± 0.62 |
| 0.000032 | ----- | ----- | 3.4 ± 0.31 |
| 0.00001 | ----- | ----- | 1.2 ± 0.05 |

[1]Whole body ChE

[2]Brain ChE

Table 2. Effects of size (mm) and age (d) on ChE activity in Rathbun walleye.

| Age (d) | Life stage | Tissue analyzed | Length (mm) ± SE | ChE Activity ± SE |
|---|---|---|---|---|
| 1 | prolarval | whole body | 7.2 ± 0.04 | 3.77 ± 0.29 |
| 5 | prolarval | whole body | 8.7 ± 0.04 | 5.11 ± 0.10 |
| 6 | prolarval | whole body | 9.0 ± 0.03 | 4.98 ± 0.42 |
| 10 | postlarval I | whole body | 10.3 ± 0.09 | 7.36 ± 0.26 |
| 11 | postlarval I | whole body | 10.8 ± 0.09 | 8.31 ± 0.38 |
| 18 | postlarval II | whole body | 15.6 ± 0.23 | 9.59 ± 0.37 |
| 19 | postlarval II | whole body | 16.0 ± 0.24 | 10.41 ± 0.93 |
| 37 | juvenile | head | 25.9 ± 0.19 | 8.25 ± 0.96 |
| 80 | juvenile | brain | 64.2 ± 1.05 | 13.54 ± 0.15 |
| 90 | juvenile | brain | 73.5 ± 1.93 | 13.40 ± 0.19 |
| 202 | juvenile | brain | 163.9 ± 4.32 | 11.20 ± 0.64 |

Table 3. Effects of size (mm) and age (d) on ChE activity in Spirit Lake walleye.

| Age (d) | Life stage | Tissue analyzed | Length (mm) ± SE | ChE Activity ± SE |
|---------|-----------|-----------------|------------------|-------------------|
| 5 | prolarval | whole body | 9.1 ± 0.02 | 4.88 ± 0.21 |
| 10 | postlarval I | whole body | 10.3 ± 0.10 | 8.56 ± 0.94 |
| 19 | postlarval II | whole body | 17.9 ± 0.13 | 11.86 ± 0.08 |
| 23 | juvenile | whole body | 20.4 ± 0.28 | 10.27 ± 0.30 |
| 30 | juvenile | head | 25.4 ± 1.74 | 11.41 ± 0.35 |
| 62 | juvenile | brain | 59.0 ± 1.50 | 12.44 ± 1.69 |
| 87 | juvenile | brain | 87.6 ± 1.83 | 14.32 ± 0.05 |
| 137 | juvenile | brain | 117.0 ± 2.77 | 12.54 ± 0.97 |
| 150 | juvenile | brain | 125.0 ± 3.36 | 12.70 ± 0.22 |

Table 4. ChE activity of juvenile (129 ± 4.2 mm) Spirit Lake walleye acclimated for three time intervals (1 h, 4 d, 7 d) to three different temperatures.

| Temp. (°C) | ChE Activity ± SE | | |
|------------|-------------------|-----------|-----------|
| ± SE | 1 hour | 4 day | 7 day |
| 17.2 ± 0.02 | 11.78 ± 0.38 | 11.08 ± 0.27 | 11.71 ± 0.31 |
| 20.9 ± 0.03 | 11.09 ± 0.66 | 11.89 ± 0.73 | 11.61 ± 0.57 |
| 24.6 ± 0.04 | 10.95 ± 0.28 | 11.78 ± 0.33 | 12.11 ± 0.63 |
| P-Value of ANOVA | 0.453 | 0.486 | 0.780 |

Table 5. Cholinesterase activity in 200-d-old juvenile Rathbun walleye 45, 90, and 135 min after stress was induced.

| Time after stress initiated (min) | Length (mm) ± SE | Weight (g) ± SE | ChE Activity ± SE |
|---|---|---|---|
| 0 | 163.7 ± 6.7 | 41.33 ± 4.22 | 11.20 ± 0.64 |
| 45 | 160.3 ± 1.3 | 41.70 ± 2.23 | 11.69 ± 0.30 |
| 90 | 162.4 ± 8.6 | 41.37 ± 6.45 | 11.16 ± 0.81 |
| 135 | 157.7 ± 4.2 | 38.37 ± 3.23 | 11.38 ± 0.49 |
| P-Value of ANOVA | 0.895 | 0.940 | 0.915 |

Table 6. Cholinesterase activity of 136-d-old juvenile walleye that remained in 19°C water for up to 24-h after being euthanized in 300 mg $L^{-1}$ Finquel® for 15-min.

| Time (hrs) | Length (mm) ± SE | Weight (g) ± SE | ChE activity ± SE |
|---|---|---|---|
| 0 | 117 ± 3.8 | 14.18 ± 0.71 | 12.54 ± 0.97 |
| 1 | 116 ± 1.2 | 14.82 ± 1.02 | 12.93 ± 0.82 |
| 4 | 117 ± 1.6 | 16.93 ± 0.44 | 13.77 ± 0.29 |
| 8 | 118 ± 9.2 | 15.46 ± 2.70 | 13.60 ± 0.52 |
| 12 | 124 ± 3.1 | 18.41 ± 2.05 | 14.03 ± 1.29 |
| 24 | 122 ± 0.9 | 17.30 ± 1.36 | 11.80 ± 0.16 |
| P-Value of ANOVA | 0.551 | 0.468 | 0.415 |

Table 7. Mean cholinesterase activity of 22-d-old Spirit Lake walleye stored at –80°C for 0 to

180 d after being euthanized in 300 mg L⁻¹ Finquel® for 15-min.

| Days in storage | ChE Activity ± SE |
|---|---|
| 0 | 10.28 ± 0.30[a,b,c] |
| 1 | 10.13 ± 0.20[a,b,c] |
| 7 | 9.31 ± 0.12[b] |
| 14 | 11.16 ± 0.73[c,e] |
| 30 | 9.64 ± 0.34[b,f] |
| 60 | 10.81 ± 0.22[a,c,f,g] |
| 90 | 11.20 ± 0.18[c,g] |
| 180 | 11.57 ± 0.60[d,e,g] |
| P-Value of ANOVA | <0.001 |

Figure Captions

Figure 1. Relationship between larval walleye (Rathbun and Spirit Lake) length and ChE activity. Cholinesterase activities are reported as μM AThCh.

Figure 2. Relationship between juvenile walleye (Rathbun and Spirit Lake) length and brain ChE activity. Cholinesterase activities are reported as μM AThCh.

Figure 3. Relationship between sequential sampling and ChE activity of juvenile walleye (Rathbun). Cholinesterase activities are reported as μM AThCh.

Figure 4. Relationship between juvenile walleye (Spirit Lake) ChE activity and hours in 19°C water following death. Cholinesterase activities are reported as μM AThCh.

Figure 5. Relationship between juvenile walleye (Spirit Lake) ChE activity and days stored at –80°C. Cholinesterase activities are reported as μM AThCh.

Figure 1.
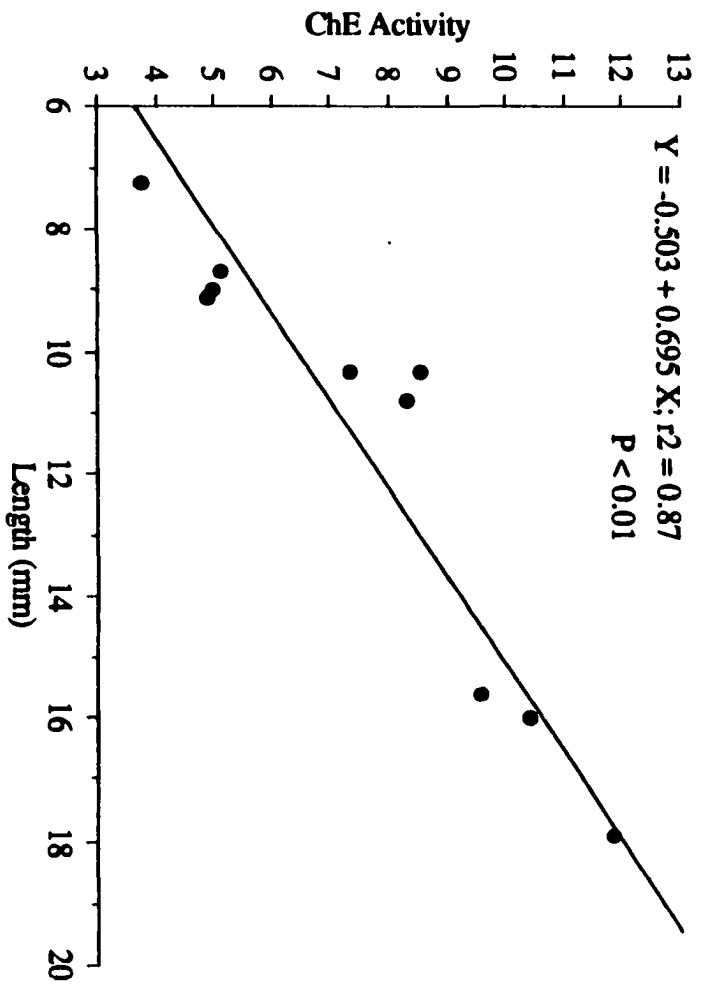
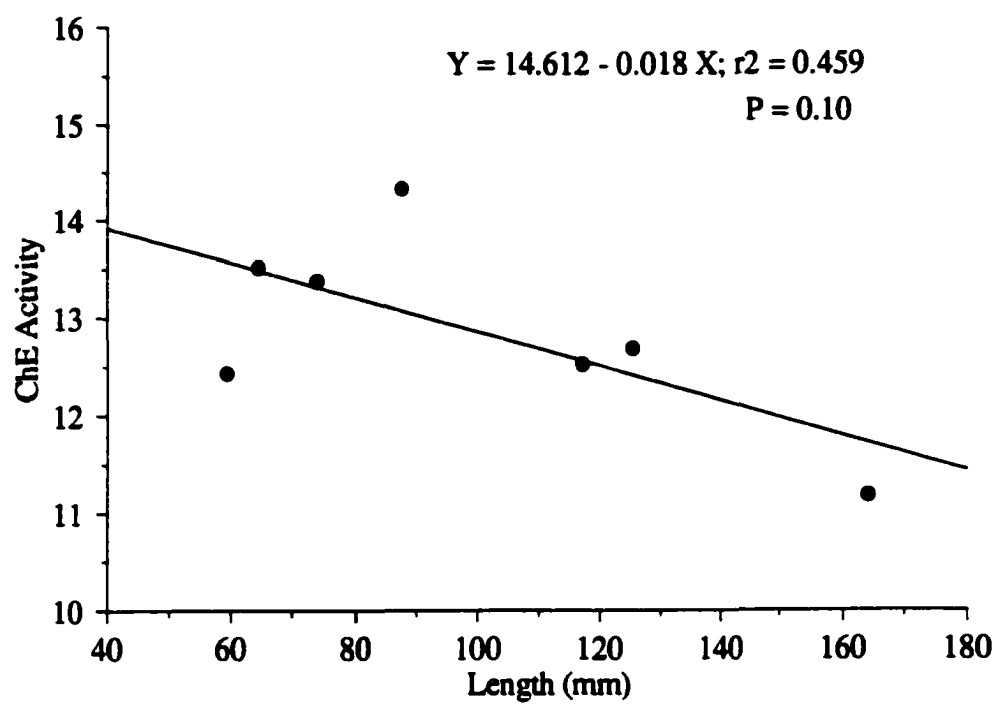Y = -0.503 + 0.695 X; r2 = 0.87

P < 0.01

ChE Activity

Length (mm)

32

Figure 2.

Figure 3.

$Y = 13.452 - 0.042\ X;\ r2 = 0.194$

$P = 0.382$

**Figure 4.**

$Y = 10.197 + 0.006\,X;\ r2 = 0.308$

$P = 0.153$
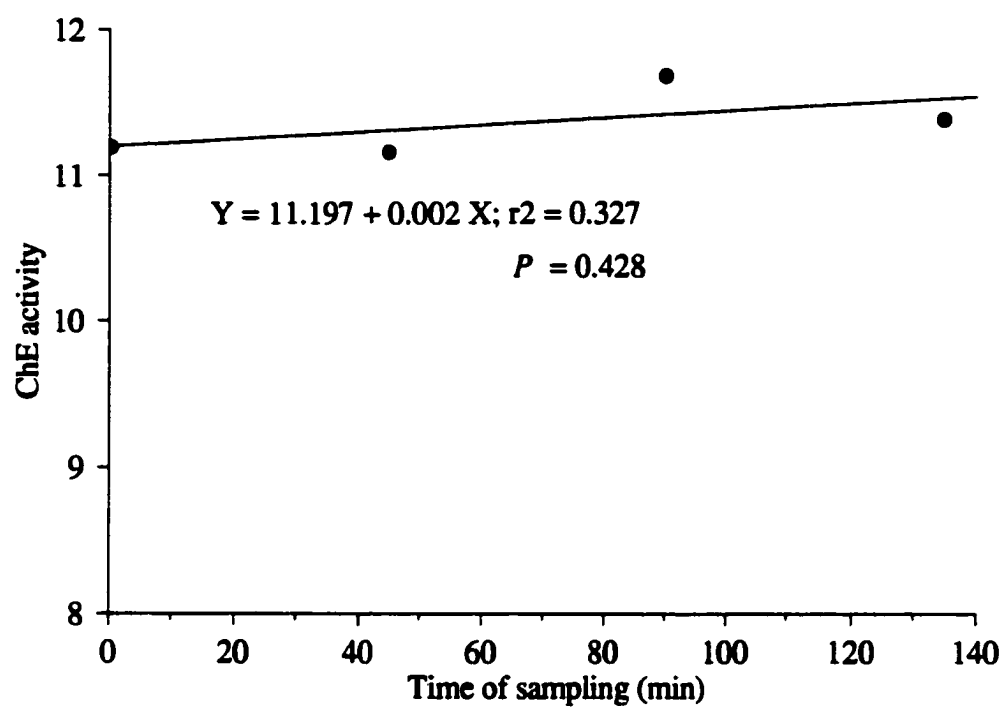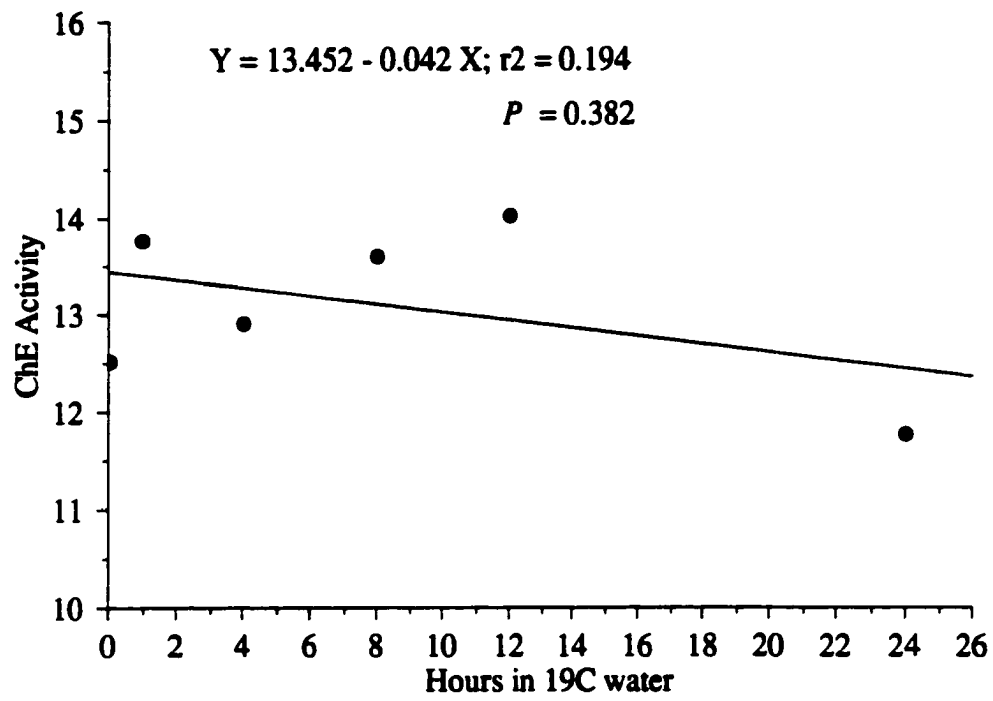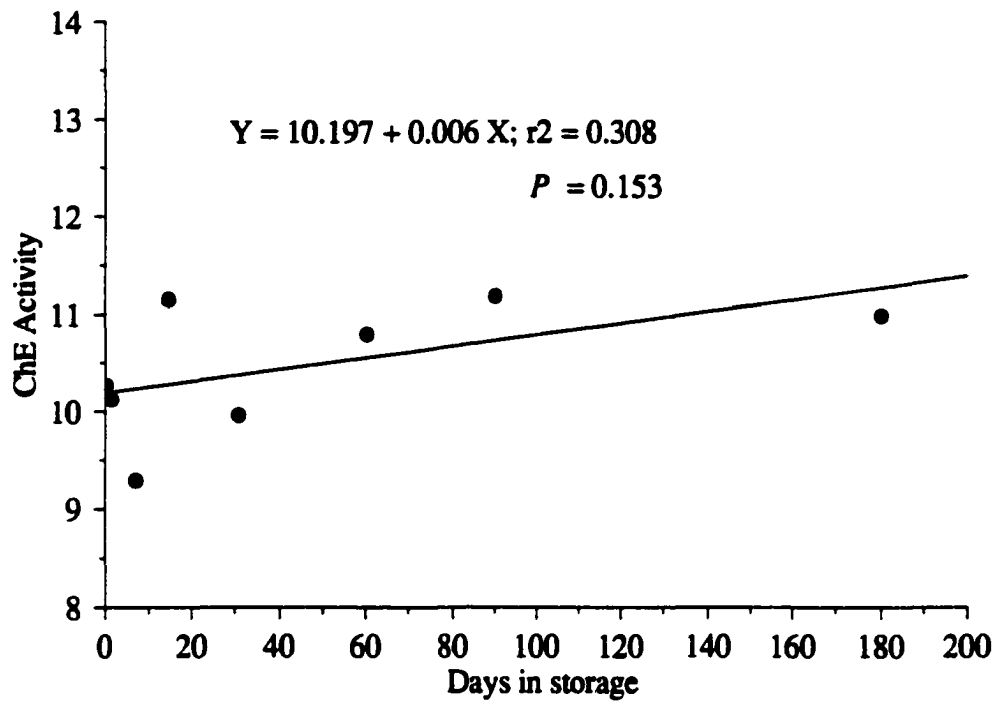
Figure 5.

# CHAPTER 2. EVALUATION OF ACUTE TOXICITY AND CHOLINESTERASE INHIBITION IN WALLEYE (STIZOSTEDION VITREUM) EXPOSED TO CHLORPYRIFOS

A paper to be submitted to Aquatic Toxicology

Todd A. Phillips, Robert C. Summerfelt, and Gary J. Atchison

## Abstract

It is critical that the effects of organophosphorus insecticides (OPs) on different life stages of fishes be understood because development of their eggs and larvae often coincides with the spring application of these chemicals. Our objective was to identify the most sensitive life stage of walleye (Stizostedion vitreum) to chlorpyrifos, a widely used OP in both agriculture and urban situations. The concentrations of chlorpyrifos that caused 50% mortality (LC50) and the amount of inhibition of cholinesterase (ChE) activity in three larval stages and three ages of juvenile walleye was determined. Prolarvae (yolk-sac larvae) were the least sensitive larval stage (LC50 = 225 to 316 $\mu$g $L^{-1}$), but there was a significant increase in toxicity of chlorpyrifos from the postlarvae I (LC50 = 24 to 29 $\mu$g $L^{-1}$) to postlarvae II stage (LC50 = 12 to 13 $\mu$g $L^{-1}$). However, the LC50 stabilized in 30- to 90-d-old juvenile walleye. The increased sensitivity of larval walleye may occur because gill filaments are not present until 3 d posthatch, and development of secondary lamellae does not occur until the end of the postlarvae I stage. Larvae walleye were able to survive when ChE activity was inhibited by as much as 90%, and 37 d posthatch walleye were able to survive when ChE was inhibited by 85%. However, 62 and 90 d posthatch juvenile walleye did not survive when ChE was inhibited more than 71%.

# 1. Introduction

Because organochlorine insecticides (OCs) have long environmental persistence and high biomagnification factors, their use has been banned or severely restricted in many countries. Consequently, organophosphorus insecticides (OPs) have replaced OCs for many applications (Pait et al., 1992; Richmonds and Dutta, 1992). In 1989, OPs were the most widely used group of insecticides in the world, encompassing nearly 40% of the insecticide market (Racke, 1993). Organophosphorus insecticide use is preferred over the use of OCs because OPs are less persistent and do not present biomagnification problems. However, OPs have a high acute toxicity to nontarget species, especially aquatic organisms. For example, bluegill (Lepomis macrochirus) exposed to chlorpyrifos were found to have a 96-h LC50 of 2.4 µg L$^{-1}$ (Mayer and Ellersieck, 1986). Freshwater fish in agricultural areas are especially susceptible to OP exposure caused by OPs moving off target sites in runoff water and sediment (Sancho et al., 1993; Havens et al., 1998).

Chlorpyrifos, one of the most widely used OP insecticides, is widely used in both agriculture and urban situations because of its versatility and wide spectrum insecticidal activity against a wide range of insects and other arthropod pests (Giesy et al., 1999). From 1990–1992, about 30% of the agricultural land treated with insecticides was treated with chlorpyrifos, which represented a total of 3 million ha of agricultural land treated with chlorpyrifos each year (USEPA, 1996). In addition, the majority of use in the United States occurred in the Midwestern corn belt which includes Iowa, Illinois, Indiana, Ohio, and Missouri. The wide spread use of chlorpyrifos makes it a probable non-point source contaminant.

Adult and juvenile fish are usually used to evaluate the toxicity of pesticides. Early life stages of fish are generally considered to be the most sensitive to waterborne toxicants (Macek and Sleight, 1977; McKim, 1977). However, little work has been done to evaluate the toxicity of OPs on early life stages of fish. It is critical that the effects of OPs on different life stages be

understood, because development of eggs and larvae of many fishes coincides with the spring application of many pesticides (Thurman et al., 1991; Heath et al., 1993a, 1993b; Havens et al., 1998).

Because of the short half-life of OPs, it is difficult to detect OPs in aquatic ecosystems from random water sample surveys. To overcome this problem, cholinesterase (ChE) activity has been used as a bio-indicator to diagnose environmental exposure to OPs because it is a ChE inhibiting chemical (Williams and Sova, 1966; Holland et al., 1967; Coppage and Matthews, 1974; Haines, 1981; Habig and Di Giulio, 1991). The OPs attach to the ChE enzyme, which prevents the enzyme from clearing acetylcholine (ACh). An accumulation of ACh may result in rapid twitching of voluntary muscles, paralysis, and death.

Ellman et al. (1961) developed a colorimetric assay to quantify cholinesterase (ChE) activity in the tissues of organisms. Because ChE inhibition is a specific response to OP exposure, measurement of ChE activity by this method has been used as a bio-indicator of OP contamination (Finlayson and Rudnicki, 1985; Johnson and Wallace, 1987; van der Wel and Welling, 1989).

Our first objective was to determine the most sensitive life stage of walleye (Stizostedion vitreum) exposed to chlorpyrifos by comparing the concentration of chlorpyrifos that caused 50% mortality (LC50) in three larval stages and three ages of juvenile walleye. Although several studies have examined the effects of sublethal exposures of juvenile and adult fishes to OPs, little work has been done on larvae because their small size makes biochemical and physiological measurements difficult (Heath et al., 1993a, 1993b). Therefore, our second objective was to compare the LC50s of three larval and three early juvenile stages of walleye to ChE activity to determine sublethal exposures and the amount of ChE inhibition necessary to cause mortality.

## 2. Materials and Methods

### 2.1. Fish

Eyed walleye eggs were obtained from the Rathbun Fish Hatchery, Moravia, Iowa on March 4, 1999 and from the Spirit Lake Fish Hatchery, Spirit Lake, Iowa on April 21, 1999. Both groups of eggs were incubated at 13°C in standard hatching jars for 5 d before hatching began, but to maintain uniformity of age, only larvae that hatched within a 24-h interval were used in this study. Mean length ± SE of 20 larvae at hatching was 7.2 ± 0.04 mm and 7.4 ± 0.05 mm for the Rathbun and Spirit Lake fish, respectively.

### 2.2. Culture conditions

Both groups of walleye were raised in a similar manner as described by Summerfelt (1996). Walleyes were raised in turbid water at 16.6 ± 0.2°C (Rathbun) and at 17.2 ± 0.2°C (Spirit Lake) for the first 30 d posthatch. At 3 d posthatch, both groups of larvae were each stocked at a density of 20 larvae L⁻¹ (3,000 larvae) in a 150-L tank. Walleye were fed different kinds and sizes of food over the course of 180 d they were cultured. The different types of food are described by Summerfelt (1996).

### 2.3. Fish sampling

For both groups of fish, a sample of 5 fish was netted every day to 30 d posthatch. Walleye were euthanized with 300 mg L⁻¹ Finquel®, measured to the nearest millimeter, and observed microscopically for gas bladder inflation (GBI), presence of food in the gut, and deformities. Observations of the day when the yolk sac and oil globule disappeared were used to describe their larval stage (prolarvae, postlarvae I and II) as well as when gas bladder inflation began and first feeding occurred. The three larval stages of walleye are: prolarval (yolk sac present: 1 to 5 d posthatch); postlarval I (yolk sac absent and oil globule present: 6 to 14 d posthatch); and postlarval II (oil globule absent: 15 to 21 d posthatch).

## 2.4. Acute toxicity assays

## 2.4.1. Larval walleye

Forty-eight hour static toxicity tests were conducted on three stages of larval walleye for both groups of fish using standard bioassay methods (APHA, 1998). Food was withheld 24 h preceding and during the 48-h exposure. Each static 48-h toxicity test consisted of at least five concentrations of chlorpyrifos, controls, and blanks. Two replicates were performed for each treatment. The control and treatment solutions contained equal amounts of the carrier solvent (ethanol), but the carrier solvent was not added to the blank exposure treatment. Tests were conducted in 2-L Pyrex beakers containing 1.5 L water. Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) (99% pure) stock solutions were prepared in ethanol (>99% pure) before each toxicity test. After chlorpyrifos was added to the beaker, 20 prolarvae and postlarvae I and five postlarvae II walleye were removed from the culture tank and placed in each beaker. Test chambers were kept in water baths throughout the 48-h exposure to maintain a constant temperature.

During the 48-h exposure, the test chambers were examined at 24 and 48 h to enumerate mortality. At the end of the 48-h exposure, all surviving fish were euthanized with Finquel® (tricaine methanesulfonate), placed in cryovials, and frozen in liquid nitrogen for later ChE analysis. Phillips (2000) found that exposure to this anesthetic had no effect on ChE activity.

Concentrations of chlorpyrifos were measured from each test chamber 5 min after chlorpyrifos was added and at the end of the 48-h exposure. Ten mL water samples were collected and placed in acetone-cleaned amber glass jars. After collection, jars were stored at 4°C until analysis. Concentrations of chlorpyrifos were measured using solid phase microextraction (SPME) and gas chromatography (GC).

An SPME fiber coated with 85 µm polyacrylate was completely immersed into the 10 mL water sample and stirred at a constant rate for 30 min. The SPME fiber was then inserted directly into the GC injector port of a Hewlett Packard 5890 series II gas chromatograph

equipped with a flame ionization detector for desorption and analysis of chlorpyrifos. A split/splitless GC injection port maintained at 220°C, and a 30 m x 0.25 mm ID DB-1701 fused silica capillary column with a 0.25 μm stationary film (J & W Scientific, Folsom, California) was used. The GC oven programming was: initial temperature of 50°C ramped to a final temperature of 260°C at a rate of 5°C/min. The final temperature was held for 10 min. Analyte desorption from the fiber and purge off time was 5.00 min. The carrier gas was helium with a head pressure set to 10 psi, and the detector temperature was maintained at 260°C. Spike and recovery analysis, procedural blanks, and calibration standards were measured for QA/QC.

## 2.4.2. Juvenile walleye

Forty-eight hour static toxicity tests were conducted at 37 and 90 d posthatch for Rathbun fish and at 30, 62, and 87 d posthatch for Spirit Lake fish using the methods described earlier, except the test chambers were 40-L glass aquaria containing 20 L of water. We placed ten 30- and 37-d-old juvenile walleye and five 62-, 87-, and 90-d-old juvenile walleye in each aquarium; fish were added to the test chambers after addition of chlorpyrifos. All juvenile toxicity tests were conducted at room temperature (19.5 to 22.0°C).

Throughout the 48-h exposure, walleye were examined every hour for the first 4 h, then at 8, 12, 24, 36, and 48 h for mortality. Fish that died during the 48-h exposure were removed, placed in individual freezer bags and frozen at –80°C until later ChE analysis. At the end of the 48-h exposure, all surviving fish were euthanized with 300 mg L$^{-1}$ Finquel® for 15 min, placed in individual freezer bags, and frozen at –80°C until later ChE analysis. Concentrations of chlorpyrifos were measured from each aquarium 5 min after chlorpyrifos was added and at the end of the 48-h exposure as previously described.

## 2.5. Water quality of culture tanks

Water quality was measured in each test chamber at the beginning and end of each experiment. Temperature was measured to the nearest $0.1^{\circ}C$ using a glass thermometer and dissolved oxygen (DO) was measured to the nearest $0.1$ mg $L^{-1}$ using an oxygen-sensitive membrane electrode (polarographic). The pH was measured to the nearest 0.1 with a standard combination electrode and meter standardized with pH 4.0, 7.0, and 10.0 buffers. Hardness was measured to the nearest 1 mg $L^{-1}$ using the Man Ver 2 burette titration method (HACH Company, Loveland, Colorado) and total alkalinity was measured to the nearest 1 mg $L^{-1}$ by titration with 0.02N $H_2SO_4$ (APHA, 1998). Quality control samples (HACH Company, Loveland, Colorado) were analyzed along with water samples to verify the accuracy of the procedures.

## 2.6. Cholinesterase inhibition and analysis

Inhibition of ChE in larval and juvenile fish that survived the 48-h static acute toxicity tests was determined by measuring total ChE activity; differentiation between acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was not made in this study. Although all juvenile fish exposed to 48-h static toxicity tests were analyzed for ChE activity, larval fish that died during the 48-h exposure were not analyzed for ChE activity because they undergo rapid decomposition.

A colorimetric method for analyzing whole body and brain ChE activity, modified for use on a THERMOmax microplate reader and SOFTmax software (Molecular Devices Corporation, Sunnyvale, California), was used to monitor the rate of formation of 5-thio-2-nitrobenzoate, a yellow-colored anion. Hydrolysis of acetylthiocholine by ChE results in an acetate ion and a negatively charged thiocholine complex that reacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoate (Ellman et al., 1961; Hill and Fleming, 1982; Gard and Hopper, 1993). The microplate reader was set in the kinetic mode to monitor increases in

absorbance at 405 nm for 2 min, read at 8 sec intervals with a 0 sec lag time, and a final volume of 250 $\mu$l well$^{-1}$ at 25°C (Gard and Hooper, 1993; Beauvais, 1997). The optimal substrate concentrations, ATI, for larval and juvenile walleye were determined prior to analysis with non-test samples. The optimal concentration was determined to be 0.001 M ATI. The Vmax and dilution factors were used to calculate ChE activities, expressed as $\mu$moles AThCh hydrolyzed min$^{-1}$ g$^{-1}$ of tissue. Hereafter, mention of ChE activity will be abbreviated as $\mu$M AThCh.

## 2.7. Quality assurance

All tissue samples analyzed for ChE activity were run in triplicate on the microtiter plate. If the coefficient of variance (CV) among the triplicates was greater than 10%, samples were rerun (less than 5% of the samples had to be rerun). Because a commercial cholinesterase standard for walleye tissue was not available, a check standard was made by pooling 3-d-old larval walleye or brains of 60-d-old juvenile walleye diluted 100-fold in pH 7.4 trizma buffer. The pooled tissue was homogenized and divided into 1-mL aliquots in 2 mL cryovials and placed in liquid nitrogen. These aliquots were run as check standards in triplicate along with each plate of treated samples. If the CV of the check standard was greater than 10%, all samples were rerun (less than 5% of the samples had to be rerun).

## 2.8. Statistical methods

LC50 values and 95% confidence limits (CL) were calculated using the trimmed Spearman-Karber method (TOXSTAT 3.5) for each larval and juvenile stage of walleye exposed to chlorpyrifos. The survival count from each test chamber was counted as one replicate, and two replicates were conducted in each treatment. Mean measured concentrations of the test solutions and fish survival data were used to determine LC50s. The LC50s with

nonoverlapping 95% CLs were considered significantly different as described in other studies (Swartz et al., 1990; Green et al., 1993; Key and Fulton, 1993; Rice et al., 1997).

Analysis of variance (ANOVA) was done using Statview® (SAS, 1998) to determine any treatment effects on inhibition of ChE activity. When the F-value for the overall test was significant (P < 0.05), Fisher's least significant difference test was used to determine significance among treatments. Regression analyses were done to determine if linear relationships existed between ChE activity and concentration of chlorpyrifos.

## 3. Results

### 3.1. Prolarval walleye

The 48-h LC50 of Rathbun prolarval walleye was 225 μg L$^{-1}$, with 95% CLs of 187 to 263 μg L$^{-1}$ (Table 1). Whole body ChE activities of Rathbun prolarvae were 4.98 μM AThCh in the control group and ranged from 0.47 to 1.31 μM AThCh in walleye exposed to 302 and 46 μg L$^{-1}$ chlorpyrifos, respectively (Table 2). Cholinesterase activity was higher in the control walleye than in all groups treated with chlorpyrifos, but did not differ among the groups treated with chlorpyrifos. Water temperatures ranged from 16.0 to 16.3°C, DO ranged from 8.82 to 9.15 mg L$^{-1}$, pH ranged from 7.40 to 7.61, hardness ranged from 174 to 182 mg L$^{-1}$, and alkalinity ranged from 36 to 38 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

Spirit Lake prolarval walleye had a 48-h LC50 of 316 μg L$^{-1}$ (CLs 283 to 348 μg L$^{-1}$; Table 1). Whole body ChE activities of Spirit Lake prolarvae were 4.88 μM AThCh for the control group and 0.68 to 1.28 μM AThCh in walleye exposed to 238 and 60 μg L$^{-1}$ chlorpyrifos, respectively (Table 2). Cholinesterase activity was higher in the control group than in all groups treated with chlorpyrifos. Water temperatures ranged from 15.5 to 15.6°C, DO ranged from 6.85 to 8.84 mg L$^{-1}$, pH ranged from 7.09 to 7.32, hardness ranged from 162 to 168 mg

L$^{-1}$, and alkalinity ranged from 39 to 42 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

## 3.2. Postlarval I walleye

The 48-h LC50 for Rathbun postlarval I walleye was 29 μg L$^{-1}$ (CL 22 to 35 μg L$^{-1}$; Table 1). Whole body ChE activities were 5.72 μM AThCh in the control group and ranged from 0.73 to 1.34 μM AThCh in postlarvae I exposed to 17 to 340 μg L$^{-1}$ chlorpyrifos, respectively (Table 3). Cholinesterase activity was significantly higher in walleye exposed to the control group compared to fish exposed to chlorpyrifos at all concentrations. Water temperature ranged from 17.0 to 17.3°C, DO ranged from 6.03 to 6.73 mg L$^{-1}$, pH ranged from 7.06 to 7.36, hardness ranged from 170 to 174 mg L$^{-1}$, and alkalinity ranged from 36 to 39 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

Spirit Lake postlarvae I walleye had a 48-h LC50 of 24 μg L$^{-1}$ (CLs 18 to 29 μg L$^{-1}$; Table 1). Whole body ChE activities were 8.56 μM AThCh in the control group and ranged from 1.01 to 1.58 μM AThCh in postlarvae I exposed to 10 to 96 μg L$^{-1}$ chlorpyrifos, respectively (Table 3). Although ChE activity was significantly different between control walleye and walleye exposed to chlorpyrifos at all concentrations, no significant differences were determined among treatment groups exposed to chlorpyrifos. Water temperature ranged from 17.2 to 17.4°C, DO ranged from 6.99 to 8.27 mg L$^{-1}$, pH ranged from 7.11 to 7.41, hardness ranged from 148 to 156 mg L$^{-1}$, and alkalinity ranged from 38 to 41 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

## 3.3. Postlarval II walleye

Rathbun postlarvae II had a 48-h LC50 of 12 μg L$^{-1}$ (CLs 9 to 14 μg L$^{-1}$; Table 1). Whole body ChE activities were 10.41 μM AThCh in the control group and ranged from 0.94 to 3.08

μM AThCh in walleye exposed to 21 and 10 μg L$^{-1}$ chlorpyrifos, respectively (Table 4). Cholinesterase activity was higher in the control fish than any other group of postlarvae II fish treated with chlorpyrifos. Water temperature ranged from 17.5 to 17.8°C, DO ranged from 9.05 to 9.16 mg L$^{-1}$, pH ranged from 7.23 to 7.33, hardness ranged from 154 to 158 mg L$^{-1}$, and alkalinity ranged from 39 to 41 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

Spirit Lake postlarvae II had a 48-h LC50 of 13 μg L$^{-1}$ (CLs of 10 to 16 μg L$^{-1}$; Table 1). Whole body ChE activities were 11.86 μM AThCh in the control group and ranged from 1.66 to 2.31 μM AThCh in postlarvae II exposed to 5 to 26 μg L$^{-1}$ chlorpyrifos, respectively (Table 4). Water temperature ranged from 17.5 to 17.7°C, DO ranged from 6.4 to 7.6 mg L$^{-1}$, pH ranged from 7.1 to 7.6, hardness ranged from 148 to 158 mg L$^{-1}$, and alkalinity ranged from 39 to 43 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

## 3.4. Juvenile walleye

Rathbun juvenile walleye (37-d-old) had a 48-h LC50 of 26 μg/L (CLs 24 to 28 μg L$^{-1}$; Table 1). Cholinesterase activity was 8.25 μM AThCh in control fish and ranged from 1.23 to 1.69 μM AThCh in 37-d-old walleye exposed to 14 to 32 μg L$^{-1}$ chlorpyrifos, respectively (Table 5). Cholinesterase activity in control fish was higher than for the groups treated with chlorpyrifos, but did not differ among groups treated with chlorpyrifos. Water temperature ranged from 20.1 to 21.3°C, DO ranged from 6.4 to 8.5 mg L$^{-1}$, pH ranged from 7.03 to 7.22, hardness ranged from 154 to 164 mg L$^{-1}$, and alkalinity ranged from 39 to 41 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

Spirit Lake juvenile walleye (30-d-old) had a 48-h LC50 of 37 μg L$^{-1}$ (CLs 28 to 45 μg L$^{-1}$; Table 1). Cholinesterase activity was 11.41 μM AThCh in control fish and ranged from 2.46

to 3.11 μM AThCh for 30-d-old walleye exposed to 17 to 40 μg L$^{-1}$ chlorpyrifos, respectively (Table 5). Cholinesterase activity was higher in the control group than for the groups exposed to chlorpyrifos, but did not differ among groups treated with chlorpyrifos. Water temperature ranged from 19.5 to 20.3°C, DO ranged from 7.3 to 8.7 mg L$^{-1}$, pH ranged from 7.0 to 7.3, hardness ranged from 158 to 164 mg L$^{-1}$, and alkalinity ranged from 37 to 42 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments ($P >$ 0.05).

The 48-h LC50 for 62-d-old Spirit Lake juvenile walleye was 41 μg L$^{-1}$ (CLs 32 to 50 μg L$^{-1}$; Table 1). Brain ChE activity for the control group was 12.44 μM AThCh and ranged from 4.27 to 7.68 μM AThCh in walleye exposed to 5 and 64 μg L$^{-1}$ chlorpyrifos, respectively (Table 6). Water temperature ranged from 20.2 to 20.5°C, DO ranged from 7.4 to 8.8 mg L$^{-1}$, pH ranged from 7.0 to 7.2, hardness ranged from 156 to 164 mg L$^{-1}$, and alkalinity ranged from 40 to 43 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments ($P > 0.05$).

The 48-h LC50 for 87-d-old Spirit Lake walleye was 45 μg L$^{-1}$ (CLs 35 to 55 μg L$^{-1}$; Table 1). Brain ChE activity for the control fish was 14.32 μM AThCh and ranged from 4.15 to 10.73 μM AThCh in walleye exposed to 2 to 74 μg L$^{-1}$ chlorpyrifos, respectively (Table 7). Water temperature ranged from 20.6 to 22.0°C, DO ranged from 6.5 to 7.7 mg L$^{-1}$, pH ranged from 6.8 to 7.1, hardness ranged from 158 to 166 mg L$^{-1}$, and alkalinity ranged from 40 to 42 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments ($P > 0.05$).

The 48-h LC50 for 90-d-old Rathbun juvenile walleye was 43 μg L$^{-1}$ (CLs 36 to 50 μg L$^{-1}$; Table 1). Cholinesterase activity in the control group was 13.40 μM AThCh and ranged from 4.59 to 5.71 μM AThCh in walleye exposed to 14 to 70 μg L$^{-1}$ chlorpyrifos, respectively (Table 7). Water temperature ranged from 20.7 to 21.4°C, DO ranged from 6.1 to 7.9 mg L$^{-1}$, pH ranged from 7.0 to 7.2, hardness ranged from 154 to 162 mg L$^{-1}$, and alkalinity ranged

from 37 to 42 mg L$^{-1}$. Differences in water quality parameters were not significantly different among treatments (P > 0.05).

## 4. Discussion

Although the toxicity of chlorpyrifos has been determined on juvenile and adult fish, little work has been done on the toxicity to early life stages. We found that postlarvae II walleye (LC50 = 12–13 µg/L) were the most sensitive life stage, but prolarvae walleye (LC50 = 225–316 µg/L) were five times more tolerant to chlorpyrifos than any other life stage tested. Our findings are similar to those of Klaverkamp et al. (1977), who evaluated the toxicity of fenitrothion on different life stages of rainbow trout. They found that embryos were the least sensitive, sac fry were intermediate, and juvenile and adults were the most sensitive. Embryos and sac fry up to 5 d posthatch survived static 24-h exposures in 34 mg L$^{-1}$ fenitrothion, but survival decreased from 70% to 10% in 6 d and 9 d posthatch sac fry, respectively, and no survival was observed in 10 and 11 d posthatch sac fry. Klaverkamp et al. (1977) suggested that embryo and sac fry may be less sensitive because of an inability to take up or activate fenitrothion to a more toxic metabolite. The toxicity of OPs results from initial metabolic activation to form the oxon metabolite, which inactivates ChE at the neural junctions by phosphorylating the enzyme active site (Giesy et al., 1999).

The increased sensitivity of larval walleye from the prolarval to postlarval II stage may be related to gill development. Phillips and Summerfelt (1999) found that gill filaments were not present in walleye until 3 d posthatch, and the development of gill filaments coincides with the end of the prolarval stage when larvae make the transition from yolk sac respiration to branchial respiration. This may explain the significant increase, as determined by nonoverlapping 95% CLs, in toxicity between prolarvae and postlarvae I stage. Perhaps the significant increase in toxicity of chlorpyrifos from the postlarvae I to postlarvae II is related to development of secondary lamellae that occurs at the end of the postlarvae I stage. These results suggest that

the gills are probably the main site of uptake of OPs. Also, growth and increased number of gill filaments and secondary lamellae during the first 21 d of walleye development may provide an explanation for increased sensitivity of larval fish as they shift from yolk sac respiration to branchial respiration. Similar results have been found in larval walleye exposed to hydrogen peroxide and elevated pH. Bergerhouse (1992) found less than 10% of prolarval walleye died when exposed to pH 10.0, but over 50% of postlarvae II died when exposed to the same pH. Clayton and Summerfelt (1996) found that only 2% of postlarvae I walleye, but 80% of prolarvae, survived a 1-h exposure to 100 mL L$^{-1}$ hydrogen peroxide.

The acute toxicity of chlorpyrifos has been evaluated on many species of juvenile and adult fish. In our study, the 48-h static LC50s ranged from 26 to 45 µg L$^{-1}$ for 37 and 87 d posthatch juvenile walleye, respectively. Rainbow trout (Oncorhynchus mykiss), cutthroat trout (Oncorhynchus clarki), and bluegill are more sensitive to chlorpyrifos than walleye. The 96-h static LC50 was 15 µg L$^{-1}$ for rainbow trout (Mayer and Ellersieck, 1986) and 18 µg L$^{-1}$ for cutthroat trout (Johnson and Finley, 1980). The 96-h LC50 for bluegill ranged from 1.7 to 4.2 µg L$^{-1}$ (Mayer and Ellersieck, 1986). Fathead minnow (Pimephales promelas), channel catfish (Ictalurus punctatus), and Japanese medaka (Oryzias latipes) were less sensitive to chlorpyrifos than walleye. The 96-h LC50 for channel catfish and fathead minnows was 280 and 140 µg L$^{-1}$, respectively (Johnson and Finley, 1980; Jarvinen and Tanner, 1982). The 48-h LC50 for 30-d-old Japanese medaka was 250 µg L$^{-1}$ (Rice et al., 1997).

In the present study, inhibition of ChE activity was a sensitive indicator of chlorpyrifos exposure. Cholinesterase activity of larval and 30-d-old walleye was inhibited at least 70% even at the lowest concentrations of chlorpyrifos exposure in the 48-h toxicity tests. However, 62- and 90-d-old walleye had ChE activity inhibited 60% to 71% even at the highest concentrations of chlorpyrifos exposure. The magnitude of ChE inhibition that was associated with death in larval and 30-d-old fish ranged from 70–91%, but only 57–71% in 62- and 90-d-old walleye. Other investigators have also found that death commonly occurred when ChE

activity was inhibited 80–90% (Weiss, 1961; Coppage, 1972; Cole, 1995). However, Weiss (1961) found that mortality of fathead minnows and largemouth bass (Micropterus salmoides) occurred over a wide range (5 to 92%) of ChE activity when exposed to several OPs.

Although fish are able to survive when ChE is inhibited by as much as 90%, sublethal effects from exposure to OPs, as measured by ChE activity, can result in adverse effects (Dutta et al., 1992; Pavlov et al., 1992; Dutta et al., 1995). Dutta et al. (1992) found that bluegill with inhibited ChE activity after being exposed to an OP had impaired optomotor response and Pavlov et al. (1992) found decreased food consumption by bream (Abramis brama) after exposure to dichlorvos. In addition, chronic exposure and brief exposure can have detrimental effects on fish (Jarvinen et al., 1983, 1988). Survival and growth effects were observed during a 200-d partial toxicity test on fathead minnows exposed to 1.21 $\mu$g L$^{-1}$ of chlorpyrifos and reproductive effects were observed at a concentration of 0.63 $\mu$g L$^{-1}$ chlorpyrifos (Jarvinen et al., 1983). Reductions in growth and increases in deformities were observed in fathead minnows exposed to a continuous exposure of 155 $\mu$g L$^{-1}$ chlorpyrifos for 5 h (Jarvinen et al., 1988). Therefore, future research should evaluate the effects of sublethal exposures of fish to OPs, and attempt to relate the levels of ChE inhibition to specific biological responses.

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Table 1. Acute toxicity of chlorpyrifos to larval and juvenile walleye at different stages of development.

| Life Stage | Rathbun 48-h LC50 (95% CL) ($\mu$g L$^{-1}$) | Spirit Lake 48-h LC50 (95% CL) ($\mu$g L$^{-1}$) |
|---|---|---|
| Prolarvae | 225 (187 – 263) | 316 (283 – 348) |
| Postlarvae I | 29 (22 – 35) | 24 (18 – 29) |
| Postlarvae II | 12 (9 – 14) | 13 (10 – 16) |
| 30 d posthatch | ----- | 37 (28 – 45) |
| 37 d posthatch | 26 (24 – 28) | ----- |
| 62 d posthatch | ----- | 41 (32 – 50) |
| 87 d posthatch | ----- | 45 (35 – 55) |
| 90 d posthatch | 43 (36 – 50) | ----- |

Table 2. ChE inhibition of surviving prolarval (6-d-old) walleye exposed to chlorpyrifos for 48 h. ChE values followed by the same letter are not significantly different (P $\geq$ 0.05, Fisher's test of least significant difference).

| Rathbun | | | Spirit Lake | | |
|---|---|---|---|---|---|
| Concentration ($\mu$g L$^{-1}$ $\pm$ SE) | ChE Activity $\pm$ SE | % Inhibition | Concentration ($\mu$g L$^{-1}$ $\pm$ SE) | ChE Activity $\pm$ SE | % Inhibition |
| 0 | 4.98 $\pm$ 0.42[a] | 0.0 | 0 | 4.88 $\pm$ 0.21[a] | 0.0 |
| 46 $\pm$ 6.5 | 1.31 $\pm$ 0.19[b] | 73.7 | 60 $\pm$ 8.0 | 1.28 $\pm$ 0.20[b] | 73.8 |
| 91 $\pm$ 8.0 | 0.80 $\pm$ 0.24[b] | 83.9 | 96 $\pm$ 6.0 | 1.16 $\pm$ 0.01[b,c] | 76.2 |
| 136 $\pm$ 9.5 | 0.96 $\pm$ 0.24[b] | 80.7 | 124 $\pm$ 3.0 | 0.86 $\pm$ 0.09[c,d] | 82.4 |
| 178 $\pm$ 3.0 | 0.89 $\pm$ 0.35[b] | 82.1 | 202 $\pm$ 17.5 | 0.83 $\pm$ 0.04[c,d] | 83.0 |
| 224 $\pm$ 25.5 | 0.93 $\pm$ 0.05[b] | 81.3 | 238 $\pm$ 7.5 | 0.68 $\pm$ 0.03[d] | 86.1 |
| 302 $\pm$ 51.0 | 0.47 $\pm$ 0.04[b] | 90.6 | 260 $\pm$ 10.0 | 0.77 $\pm$ 0.07[d] | 84.2 |
| 365 $\pm$ 19.1 | ----- | ----- | 333 $\pm$ 30.5 | 0.70 $\pm$ 0.02[d] | 85.7 |
| P-Value of ANOVA | <0.001 | | | <0.001 | |

Table 3. ChE inhibition of postlarval I (10-d-old) walleye exposed to chlorpyrifos for 48 h. ChE values followed by the same letter are not significantly different (P ≥ 0.05, Fisher's test of least significant difference).

| | Rathbun | | | Spirit Lake | | |
|---|---|---|---|---|---|---|
| Concentration (μg $L^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition | Concentration (μg $L^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition |
| 0 | 5.72 ± 1.38[a] | 0.0 | 0 | 8.56 ± 0.94[a] | 0.0 |
| 17 ± 1.5 | 1.34 ± 0.17[b] | 76.6 | 10 ± 2.5 | 1.49 ± 0.13[b] | 82.6 |
| 57 ± 7.5 | 0.83 ± 0.10[b] | 85.5 | 20 ± 1.5 | 1.58 ± 0.41[b] | 81.5 |
| 113 ± 4.5 | ----- | NA | 23 ± 2.0 | 1.39 ± 0.19[b] | 83.8 |
| 142 ± 8.0 | ----- | NA | 41 ± 3.5 | 1.28 ± 0.18[b] | 85.1 |
| 174 ± 4.5 | 0.73[b] | 87.2 | 47 ± 0.5 | 1.01[b] | 88.2 |
| 252 ± 39.5 | ----- | NA | 96 ± 16.0 | ----- | ----- |
| 340 ± 24.5 | ----- | NA | | | |
| P-Value of ANOVA | 0.012 | | | 0.003 | |

Table 4. ChE inhibition of postlarval II (19-d-old) walleye exposed to chlorpyrifos for 48 h.
ChE values followed by the same letter are not significantly different (P ≥ 0.05, Fisher's test of
least significant difference).

| Rathbun | | | Spirit Lake | | |
|---|---|---|---|---|---|
| Concentration (μg L$^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition | Concentration (μg L$^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition |
| 0 | 10.41 ± 0.93[a] | 0.0 | 0 | 11.86 ± 0.08[a] | 0.0 |
| 5 ± 1.5 | 2.34 ± 0.0.2[b,c] | 77.5 | 5 ± 0.5 | 2.31 ± 0.01[b] | 80.5 |
| 10 ± 1.5 | 3.08 ± 0.32[b] | 70.4 | 8 ± 1.0 | 2.19 ± 0.10[b] | 81.5 |
| 12 ± 0.0 | 2.03 ± 0.01[b,c] | 80.5 | 11 ± 0.0 | 1.71 ± 0.09[c] | 85.6 |
| 16 ± 2.0 | 2.00 ± 0.13[b,c] | 80.8 | 15 ± 0.5 | 2.16 ± 0.03[b] | 81.8 |
| 21 ± 1.0 | 0.94[c] | 91.0 | 20 ± 2.0 | 1.66 ± 0.12[c] | 86.0 |
| | | | 26 ± 0.5 | ----- | ----- |
| P-Value of ANOVA | <0.001 | | | <0.001 | |

Table 5. ChE inhibition in whole head tissue of 30 and 37 d posthatch juvenile walleye exposed to chlorpyrifos for 48 h. ChE values followed by the same letter are not significantly different (P ≥ 0.05, Fisher's test of least significant difference).

| 37-d-old (Rathbun) | | | 30-d-old (Spirit Lake) | | |
|---|---|---|---|---|---|
| Concentration ($\mu$g L$^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition | Concentration ($\mu$g L$^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition |
| 0 | 8.25 ± 0.96[a] | 0.0 | 0 | 11.41 ± 0.35[a] | 0.0 |
| 14 ± 0.0 | 1.69 ± 0.16[b] | 79.5 | 17 ± 1.0 | 3.11 ± 0.15[b] | 72.7 |
| 18 ± 1.0 | 1.67 ± 0.04[b] | 79.8 | 20 ± 0.5 | 2.46 ± 0.12[b] | 78.4 |
| 24 ± 0.0 | 1.55 ± 0.10[b] | 81.2 | 26 ± 0.0 | 2.99 ± 0.24[b] | 73.8 |
| 26 ± 0.0 | 1.44 ± 0.04[b] | 82.6 | 29 ± 0.0 | 2.46 ± 0.26[b] | 78.4 |
| 32 ± 1.0 | 1.23 ± 0.05[b] | 85.1 | 34 ± 1.0 | 2.77 ± 0.04[b] | 75.7 |
| | | | 40 ± 0.5 | 2.63 ± 0.10[b] | 77.0 |
| P-Value of ANOVA | <0.001 | | | <0.001 | |

Table 6. ChE inhibition in brain tissue of 62 d posthatch juvenile walleye (Spirit Lake) exposed to chlorpyrifos for 48 h. ChE values followed by the same letter are not significantly different (P ≥ 0.05, Fisher's test of least significant difference).

| Concentration (μg L$^{-1}$ ± SE) | ChE Activity ± SE | % Inhibition |
|---|---|---|
| 0 | 12.44 ± 1.69[a] | 0.0 |
| 5 ± 0.5 | 7.68 ± 1.05[b] | 38.3 |
| 9 ± 0.5 | 4.92 ± 0.21[c] | 60.5 |
| 15 ± 0.5 | 5.13 ± 0.03[c] | 58.8 |
| 19 ± 0.0 | 4.32 ± 0.05[c] | 65.3 |
| 25 ± 2.0 | 4.27 ± 0.08[c] | 65.7 |
| 43 ± 5.0 | 4.36 ± 0.29[c] | 65.0 |
| 64 ± 3.0 | 4.87 ± 0.70[c] | 60.9 |
| P-Value of ANOVA | <0.001 | |

Table 7. ChE inhibition in brain tissue of 87 and 90 d posthatch juvenile walleye exposed to chlorpyrifos for 48 h. ChE values followed by the same letter are not significantly different (P $\geq$ 0.05, Fisher's test of least significant difference).

| 90-d-old (Rathbun) | | | 87-d-old (Spirit Lake) | | |
|---|---|---|---|---|---|
| Concentration ($\mu$g L$^{-1}$ $\pm$ SE) | ChE Activity $\pm$ SE | % Inhibition | Concentration ($\mu$g L$^{-1}$ $\pm$ SE) | ChE Activity $\pm$ SE | % Inhibition |
| 0 | 13.40 $\pm$ 0.19[a] | 0.0 | 0 | 14.32 $\pm$ 0.05[a] | 0.0 |
| 14 $\pm$ 0.0 | 5.30 $\pm$ 0.74[b,c] | 60.5 | 2 $\pm$ 1.0 | 10.73 $\pm$ 0.65[b] | 25.1 |
| 21 $\pm$ 3.0 | 5.20 $\pm$ 0.16[b,c] | 61.2 | 6 $\pm$ 0.5 | 7.83 $\pm$ 0.16[c] | 45.3 |
| 31 $\pm$ 2.0 | 4.59 $\pm$ 0.12[b] | 65.8 | 21 $\pm$ 4.5 | 4.69 $\pm$ 1.62[d] | 67.3 |
| 42 $\pm$ 2.5 | 5.05 $\pm$ 0.10[b,c] | 62.3 | 26 $\pm$ 1.0 | 4.43 $\pm$ 0.70[d] | 69.1 |
| 57 $\pm$ 2.5 | 5.33 $\pm$ 0.11[b,c] | 60.2 | 57 $\pm$ 1.5 | 4.15 $\pm$ 0.37[d] | 71.0 |
| 70 $\pm$ 1.5 | 5.71 $\pm$ 0.15[c] | 57.4 | 74 $\pm$ 6.5 | 4.37 $\pm$ 0.50[d] | 69.5 |
| P-Value of ANOVA | <0.001 | | | <0.001 | |

# CHAPTER 3. TOXICITY OF CHLORPYRIFOS ADSORBED ON HUMIC ACID AND A SMECTITIC CLAY TO LARVAL WALLEYE

Todd A. Phillips, Robert C. Summerfelt, Jigang Wu, and David A. Laird

## Abstract

Organophosphorus insecticides (OPs) are often strongly adsorbed to colloidal materials, but little research has been done to evaluate the bioavailability of OPs transported by colloids to aquatic organisms. Therefore, 48-h static toxicity tests were conducted to determine the toxicity of chlorpyrifos adsorbed on humic acid (HA) and Panther Creek (PC) clay to three larval stages of walleye (Stizostedion vitreum). We found that chlorpyrifos adsorbed on HA reduced survival of larval walleye, which indicates that chlorpyrifos desorbed from HA. Panther Creek clay without chlorpyrifos and PC–chlorpyrifos (10 µg chlorpyrifos $g^{-1}$ PC clay) were highly toxic to postlarvae I and II walleye, but not prolarvae. This indicates that PC clay, itself, is highly toxic to postlarvae I and II walleye. The toxicity of PC clay coincides with the development of the gills (during postlarvae I and postlarvae II) which may explain the increased toxicity. Extrapolating the sensitivity of larval walleye to chlorpyrifos–HA complexes and PC clay in these laboratory experiments to field conditions indicates potential toxicity of chlorpyrifos–soil complexes to larval fish.

## 1. Introduction

Since the 1970s, use of organochlorine insecticides in the United States has been banned or greatly curtailed due to problems from environmental persistence and biomagnification (Pait et al., 1992; Richmonds and Dutta, 1992; Friend and Franson, 1999). By 1989, organophosphorus insecticides (OPs) were the most widely used group of insecticides in the world, representing nearly 40% of the insecticide market (Racke, 1993). In 1993, in the

United States, the most widely used insecticide was chlorpyrifos, more than 3 million kg were applied to field crops (ERS, 1994).

Because OPs have low water solubilities (e.g., chlorpyrifos has a solubility of 1.39 mg $L^{-1}$ at $25^\circ C$) and high soil adsorption coefficients (Kd) (e.g., chlorpyrifos has a Kd value ranging from 13.4 to 1,862 mL $g^{-1}$ depending of the soil type, climatic conditions, and application practices) (Harris, 1972; Racke, 1993), they are most often transported adsorbed to suspended solids. Thus, the greatest likelihood of adverse environmental consequences to aquatic life may occur when OPs are transported off site adsorbed to colloidal materials (organic and inorganic soil constituents). Although research has not evaluated the biological availability of OPs transported by colloids to aquatic organisms, Misitano et al. (1994) demonstrated that polynuclear aromatic hydrocarbons and polychlorinated biphenyls sorbed to sediment were transferred to larval fish, providing evidence for toxicity of hydrophobic compounds adsorbed to sediment.

In aquatic environments, OPs adsorbed on suspended colloids may be absorbed by fish when the suspended colloids come in contact with gill tissue, a metabolically active epithelium (Moyle and Cech, 1996). Consequently, the bioavailability of colloid-adsorbed OPs depends on the strength of the colloid-OP insecticide bonding, the strength of interaction between OP insecticides and gill tissue, and the extent of exposure of gills to colloids containing OP insecticides.

Interactions between OPs and smectites (the dominant clay mineral in most suspended solids) have been extensively studied by several authors (Bowman et al., 1970; Saltzman and Yariv, 1976; Sanchez-Camazano and Sanchez-Martin, 1980, 1991; Sanchez-Martin and Sanchez-Camazano, 1980, 1984; Rodriguez et al., 1988). In general, these investigations revealed that OPs are strongly adsorbed on smectite surfaces, and sometimes smectites have been shown to catalyze hydrolysis of OPs.

The fate and effect of chlorpyrifos in the environment is related to its transformation

products (Figure 1) by abiotic hydrolysis and other mechanisms—e.g., photolysis and

microbial degradation (Racke, 1993). Barron et al. (1993) found that parent chlorpyrifos was

rapidly absorbed from exposure water (t1/2 < 1 h) by channel catfish (Ictaluras punctatus).

Trichloropyridinol (TCP) was the major metabolite in blood and glucuronide conjugate of TCP

was the major metabolite in urine and bile (Barron et al., 1993). The insecticidal property of

chlorpyrifos is caused by the metabolite chlorpyrifos oxon, which was not detected in the

channel catfish. Chlorpyrifos oxon is generally not detected in tissues of vertebrates because of

rapid hydrolysis to TCP (Sultatos and Murphy, 1983).

The question that has yet to be considered in risk assessment for aquatic life is whether OPs

can be desorbed at the epithelial surface directly from suspended colloids. The gill epithelium

is a site for active transfer of oxygen and for certain ions ($Na^+$, $Cl^-$, $HCO^{3+}$) from the water into

fish, and for transfer of carbon dioxide and ammonia from fish to the environment (Moyle and

Cech, 1996). Therefore, if the biochemical activity of the gill facilitates desorption of OPs

from colloids, then OPs bound to suspended colloids may be a greater threat to aquatic life than

might be assumed from their low water solubility. The objective of this study was to evaluate

the bioavailability (sublethal effect as measured by cholinesterase activity and acute toxicity) of

chlorpyrifos adsorbed on humic acid (HA) and Panther Creek (PC) clay to larval walleye

(Stizostedion vitreum). Panther Creek clay was chosen as the test clay because it has a high

partition coefficient (Kd = 1,187 mL $g^{-1}$) for chlorpyrifos and has physical and chemical

properties similar to those found in natural sediment in Iowa.


## 2. Materials and Methods

### 2.1. Test organisms and culture conditions

Eyed walleye eggs were obtained from Spirit Lake Fish Hatchery, Spirit Lake, Iowa, on

April 21, 1999, and were incubated at 13.0°C in standard hatching jars for 5 d before hatching

began. To maintain uniformity of age, only larvae that hatched within a 24-h interval were used in this study. Mean length ± SE of 20 larvae at hatching was 7.4 ± 0.05 mm. At 3 d posthatch, larvae were stocked at a density of 20 larvae $L^{-1}$ in a 150-L culture tank (3,000 larvae tank$^{-1}$). Walleye were raised at 17.2 ± 0.2°C as described by Summerfelt (1996) and fed Fry Feed Kyowa B-400 and C-700 diets (BioKyowa, Inc., Chesterfield, Missouri) every 5 min, 22 h d$^{-1}$ during the larval stages of development. Feeding was suspended for 2 h d$^{-1}$ to clean the tanks.

## 2.2. Water quality of experimental test chambers

At the beginning and end of each static 48-h toxicity test, temperature, DO, hardness, alkalinity, pH, and ammonia were measured in each test chamber. Temperature (± 0.1°C) was measured using a glass thermometer and dissolved oxygen (DO) was measured to the nearest 0.1 mg $L^{-1}$ using an oxygen-sensitive membrane electrode (polarographic). Total ammonia-nitrogen ($NH_3$-N; TAN) was measured to the nearest 0.01 mg $L^{-1}$ using the Nesslerization method (APHA, 1998) and a spectrophotometer, and pH was measured to the nearest 0.1 with a standard combination electrode and meter standardized with pH 4.0, 7.0, and 10.0 buffers. Hardness was measured to the nearest 1 mg $L^{-1}$ using the Man Ver 2 burette titration method (HACH Company, Loveland, CO) and total alkalinity was measured to the nearest 1 mg $L^{-1}$ by titration with 0.02N $H_2SO_4$ (APHA, 1998). Quality control samples (HACH Company, Loveland, Colorado) were analyzed along with water samples to verify the accuracy of the procedures used to measure TAN. Measured concentrations for the externally supplied TAN quality assurance samples were always within the certified 95% confidence interval.

## 2.3. Fish sampling

Observations of the day when the yolk sac and oil globule disappeared were used to describe their larval stage (prolarvae, postlarvae I and II) as well as when gas bladder inflation

began and first feeding occurred. A sample of 5 fish were netted every day for 21 d. Walleye

were euthanized with 300 mg L$^{-1}$ Finquel® (tricaine methanesulfonate), measured (mm), and

observed microscopically for gas bladder inflation (GBI), presence of food in the gut, and

deformities. The three larval stages of walleye are: prolarval (yolk sac present: 1 to 5 d

posthatch); postlarval I (yolk sac absent and oil globule present: 6 to 14 d posthatch); and

postlarval II (oil globule absent: 15 to 21 d posthatch).

## 2.4. Preparation of chlorpyrifos–HA and chlorpyrifos–PC clay complexes

To determine the bioavailability of chlorpyrifos adsorbed on suspended colloids to larval

walleye, chlorpyrifos was adsorbed to HA and PC clay, predominantly comprised of

aluminum silicate (Table 1). For each larval stage, three concentrations of chlorpyrifos (34 µg,

109 µg, and 340 µg) were added to 2 g of HA and added to test chambers containing 3.5 L of

water. After a 24-h equilibration period, the concentrations of chlorpyrifos adsorbed on HA

for the three treatment groups used in experiments for the three larval stages were: 11.8 to

13.5 µg g$^{-1}$ HA (low), 47.0 to 51.0 µg g$^{-1}$ HA (medium), and 160.7 to 163.0 µg g$^{-1}$ HA

(high), depending on the concentration of chlorpyrifos that desorbed from the HA. For PC

clay, 109 µg chlorpyrifos was added to 4 g of PC clay and added to test chambers containing

3.5 L of water. After a 24-h equilibration period, the concentrations of chlorpyrifos adsorbed

on PC clay were 21.1, 21.7, and 20.0 µg g$^{-1}$ PC clay for the prolarval, postlarval I and II

experiments, respectively.

## 2.5. Acute toxicity assays

Forty-eight hour static acute toxicity tests were conducted on three stages of larval walleye

using standard methods (APHA, 1998). Food was withheld 24 h preceding and during the 48-

h exposure. Each static 48-h toxicity test consisted of seven treatment groups: HA control, PC

control, chlorpyrifos with no HA or PC clay, low chlorpyrifos–HA, medium chlorpyrifos–

HA, high chlorpyrifos–HA, and chlorpyrifos–PC clay. Three replicates were performed for each treatment. Fifty prolarvae and postlarvae I and twenty-five postlarvae II walleye were removed from the culture tank and placed in each test chamber. The test chambers were similar to those described by Schmidt-Dallmier et al. (1992), except instead of glass baffles or stainless steel mesh screen, 1.0-mm mesh screen was used to cover the notches cut at the top and bottom edges of the glass funnel to prevent larval walleye from passing through the notches. Suspension of PC clay and HA was maintained with a propeller-tipped stirring rod driven by an electric motor with a rheostat. The revolution rates of all stir rods were synchronized at 1500 rpm with a stroboscope before additions of sediment to the chambers. Test chambers were incubated in 15.5 to 18.0°C water baths (depending on the larval stage) throughout the 48-h exposure to maintain a constant temperature. At the end of the 48-h exposure, all surviving fish were euthanized with Finquel® (tricaine methanesulfonate), placed in cryovials, and frozen in liquid nitrogen for later cholinesterase (ChE) analysis.

Concentrations of chlorpyrifos were measured from each test chamber midway through and at the end of each 48-h static toxicity test. Water samples were collected (10 mL) and placed in acetone-cleaned amber glass jars. After collection, jars were stored at 4°C until analysis. Concentrations of chlorpyrifos were measured using solid phase microextraction (SPME) and gas chromatography (GC).

An SPME fiber coated with 85 µm polyacrylate was completely immersed into the 10 mL water sample and stirred at a constant rate for 30 min. The SPME fiber was then inserted directly into the GC injector port of a Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionization detector for desorption and analysis of chlorpyrifos. A split/splitless GC injection port maintained at 220°C, and a 30 m x 0.25 mm ID DB-1701 fused silica capillary column with a 0.25 µm stationary film (J & W Scientific, Folsom, California) was used. The GC oven programming was: initial temperature of 50°C ramped to a final temperature of 260°C at a rate of 5°C/min. The final temperature was held for 10 min. Analyte

desorption from the fiber and purge off time was 5.00 min. The carrier gas was helium with a head pressure set to 10 psi, and the detector temperature was maintained at 260°C. Spike and recovery analysis, procedural blanks, and calibration standards were measured for QA/QC.

## 2.6. Cholinesterase inhibition and analysis

Fifteen individual prolarvae (three prolarvae were pooled for each analysis) and five postlarvae I and II walleye from each test chamber that survived the 48-h static acute toxicity tests were analyzed to determine total ChE activity. Differentiation between acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was not made in this study. Larvae fish that died during the 48-h exposure were not analyzed for ChE activity, because rapid decomposition made the analysis unreliable.

A colorimetric method for analyzing whole body ChE activity modified for use on a THERMOmax microplate reader and SOFTmax software (Molecular Devices Corporation, Sunnyvale, California) was used to monitor the rate of formation of 5-thio-2-nitrobenzoate, a yellow-colored anion. Hydrolysis of acetylthiocholine (AThCh) by ChE results in an acetate ion, and a negatively charged thiocholine complex reacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoate (Ellman et al., 1961; Hill and Fleming, 1982; Gard and Hopper, 1993). The microplate reader was set in the kinetic mode to monitor increases in absorbance at 405 nm for 2 min, read at 8 sec intervals with a 0 sec lag time, and a final volume of 250 $\mu$l well$^{-1}$ at 25°C (Gard and Hooper, 1993; Beauvais, 1997). The optimal substrate concentrations, acetylthiocholine iodide (ATI), for larval walleye was determined prior to analysis with non-test samples. The optimal concentration was determined to be 0.001 M ATI. The Vmax and dilution factors were used to calculate ChE activities, reported as micromoles AThCh hydrolyzed per min per g of tissue (from here on will be abbreviated as $\mu$M AThCh).

All tissue samples analyzed for ChE activity were run in triplicate on the microtiter plate. If the coefficient of variance (CV) among the triplicates was greater than 10%, samples were rerun (less than 5% of the samples had to be rerun). Because a commercial cholinesterase standard for walleye tissue was not available, a check standard was used. The check standard was made by pooling larval walleye diluted 100-fold in pH 7.4 trizma buffer. The pooled tissue was homogenized and divided into 1-mL aliquots that were stored in 2 mL cryovials and placed in liquid nitrogen. These aliquots were run as check standards in triplicate along with each plate of treated samples. If the CV of the check standard was greater than 10%, all samples were rerun (less than 5% of the samples had to be rerun).

## 2.7. Statistical methods

Differences in treatment effects on survival and/or ChE activity were assessed by analysis of variance using Statview® (SAS, 1998) to determine any treatment effects on survival and/or ChE activity. When the F-value for the overall test was significant ($P < 0.05$), Fisher's least significant difference test was used to determine significance among treatments. Survival data were transformed to a normal distribution before analysis using the following formula: transformed survival = arcsine (survival proportion)$^{1/2}$ (Zar, 1984).

## 3. Results

### 3.1. Prolarvae

Survival of 3-d-old prolarval walleye (mean length ± SE = 7.3 ± 0.04 mm) ranged from 45 to 96% after a 48-h exposure to the 162.4 μg chlorpyrifos g$^{-1}$ HA and 21.1 μg chlorpyrifos g$^{-1}$ PC clay treatments (from here on will be abbreviated μg g$^{-1}$ of HA or PC clay), respectively (Table 2). Although survival of prolarvae exposed to the 162.4 μg g$^{-1}$ HA treatment was significantly lower than those for all other treatments; survival in other treatments were similar.

In addition to survival, ChE was measured to determine sublethal exposures. Cholinesterase activity of prolarvae that survived the 48-h exposure ranged from 5.24 $\mu$M AThCh in the HA control treatment to 1.68 $\mu$M AThCh in the 21.1 $\mu$g g$^{-1}$ PC clay treatment (Table 2). Prolarvae in both control treatments (PC and HA) had significantly higher ChE activity than those in any treatment group. Cholinesterase activity in prolarvae exposed to 162.4 $\mu$g g$^{-1}$ HA, 21.1 $\mu$g g$^{-1}$ PC clay, and chlorpyrifos treatments were significantly lower than those for the 13.5 and 49.3 $\mu$g g$^{-1}$ HA.

Although chlorpyrifos was strongly adsorbed to HA and PC clay, low concentrations of chlorpyrifos were detected in the aqueous phase after a 24-h equilibration period (Table 2). However, the concentrations in solution were not high enough to be acutely toxic to prolarvae.

The overall means $\pm$ SE for DO and pH were 9.2 $\pm$ 0.03 mg L$^{-1}$ and 7.6 $\pm$ 0.06, respectively; hardness was 162 $\pm$ 0.5 mg L$^{-1}$; alkalinity was 40 $\pm$ 0.4 mg L$^{-1}$; and the temperature maintained by the water bath was 15.9 $\pm$ 0.01°C. Water quality variables among individual test chambers did not differ significantly.

## 3.2. Postlarvae I

Survival of 9-d-old postlarvae I walleye (mean length $\pm$ SE = 9.4 $\pm$ 0.09 mm) after a 48-h exposure ranged from 67% in the HA control treatment to 0% in the 163 $\mu$g g$^{-1}$ HA and 21.7 $\mu$g g$^{-1}$ PC clay treatments (Table 3). Survival was significantly higher in the HA control, 13.5 $\mu$g g$^{-1}$ HA, and chlorpyrifos treatments than all other treatments. Although survival was not significantly different among the HA control, 13.5 $\mu$g g$^{-1}$ HA, and chlorpyrifos treatments, ChE activity was significantly higher in the HA control than the other two treatments (Table 3). Although survival was significantly lower in the PC control than HA control fish, ChE activity did not differ.

Although chlorpyrifos was strongly adsorbed to HA and PC clay, low concentrations of chlorpyrifos were detected in the aqueous phase after a 24-h equilibration period (Table 3).

However, the concentrations in solution were not high enough to be acutely toxic to postlarvae I.

The overall means ± SE for DO and pH were 8.4 ± 0.05 mg L$^{-1}$ and 7.4 ± 0.03, respectively; hardness was 168 ± 1.0 mg L$^{-1}$; alkalinity was 48 ± 1.9 mg L$^{-1}$; and the temperature maintained by the water bath was 17.7 ± 0.02°C. Water quality variables among individual test chambers did not differ significantly.

### 3.3. Postlarvae II

Survival of 16-d-old postlarvae II walleye (mean length ± SE = 12.8 ± 0.21 mm) after a 48-h exposure ranged from 67% in the chlorpyrifos treatment to 0% in the 160.7 µg g$^{-1}$ HA, 20.0 µg g$^{-1}$ PC, and PC control treatment groups (Table 4). Survival of walleye exposed to the chlorpyrifos and HA control treatments was significantly higher than any other treatment. However, ChE activity in walleye exposed to the chlorpyrifos treatment was only 41% of the ChE activity of fish exposed to the HA control treatment.

Although chlorpyrifos was strongly adsorbed to HA and PC clay, low concentrations of chlorpyrifos were detected in the aqueous phase after a 24-h equilibration period (Table 4). However, the concentrations in solution were not high enough to be acutely toxic to postlarvae II.

The overall means ± SE for DO and pH were 7.7 ± 0.16 mg L$^{-1}$ and 7.4 ± 0.04, respectively; hardness was 177 ± 2.4 mg L$^{-1}$; alkalinity was 49 ± 2.1 mg L$^{-1}$; and the temperature maintained by the water bath was 17.8 ± 0.04°C. Water quality variables among individual test chambers did not differ significantly.

## 4. Discussion

Compared with the HA control, chlorpyrifos adsorbed on HA reduced survival of all stages of larval walleye. These data suggest that chlorpyrifos may be biologically active in the aquatic

environment when it is adsorbed on the organic fraction of suspended solids. Although little work has been done to evaluate the toxicity of OPs adsorbed on suspended solids to fish, Misitano et al. (1994) demonstrated that radiolabeled polynuclear aromatic hydrocarbons and polychlorinated biphenyls sorbed to sediment were accumulated by larval surf smelt (Hypomesus pretiosus). These results provide evidence for an exposure route of hydrophobic compounds directly from sediment to fish. In addition, sediment-associated chlorpyrifos was shown to be highly toxic to a marine, sediment-dwelling copepod (Amphiascus tenuiremis) (Green et al., 1996). Sediment and pore water were determined to be the most likely routes of exposure. However, because A. tenuiremis ingests sediment particles directly and indirectly when feeding, exposure from chlorpyrifos associated with sediment particles is possible.

Cholinesterase activity of prolarvae exposed to the 162.4 µg g$^{-1}$ HA treatment was not significantly different than chlorpyrifos and 21.1 µg g$^{-1}$ PC clay treatments even though survival was significantly lower for prolarvae exposed to the 162.4 µg g$^{-1}$ HA treatment. These results indicate that exposure to chlorpyrifos adsorbed on HA contributed to the mortality of larval walleye. Cholinesterase activity was only inhibited 66% in prolarvae exposed to 162.4 µg g$^{-1}$ HA whereas, 80–90% inhibition is usually required for death to occur (Weiss, 1961; Coppage, 1972; Cole, 1995). In addition, ChE activity was inhibited 68% in prolarvae exposed to 21.1 µg g$^{-1}$ PC clay, but survival was 96%. Cholinesterase activity was inhibited 72 and 71% in postlarvae I walleye exposed to the 51.0 µg g$^{-1}$ HA and chlorpyrifos treatments, respectively. However, survival was only 2% in the 51.0 µg g$^{-1}$ HA treatment compared to 58% in the chlorpyrifos treatment. Therefore, these results indicate that the chlorpyrifos-HA complexes had toxic effects to larval walleye.

Cholinesterase activity was significantly lower in postlarvae II walleye exposed to the 47.0 µg g$^{-1}$ HA treatment than the chlorpyrifos treatment. The decreased ChE activity in postlarvae II walleye exposed to the 47.0 µg g$^{-1}$ HA treatment may be explained by increased chlorpyrifos exposure from chlorpyrifos desorbing directly from the HA to the fish. This explanation is

possible because similar concentrations of chlorpyrifos were in the aqueous phase of both treatments.

Panther Creek clay alone, without chlorpyrifos, was highly toxic to postlarvae I and II walleye, but not prolarvae. Auld and Schubel (1978) found that striped bass (Morone saxatilis) and yellow perch (Perca flavescens) were able to tolerate high concentrations of suspended sediment containing illite, chlorite, and kaolinite (1–4 $\mu$m particles) collected from the Chesapeake Bay ($\geq$ 500 mg L$^{-1}$), but survival of American shad larvae (Alosa sapidissima) decreased when concentrations were $\geq$ 100 mg L$^{-1}$. Phillips (1996) found that survival of prolarvae and postlarvae I and II walleye was not affected by exposure to a commercial aluminum-silicate clay at concentrations that ranged from 2–360 mg L$^{-1}$.

Overall, the postlarvae II larval stage was found to be the most sensitive life stage to chlorpyrifos–HA complexes and PC clay followed by the postlarvae I and prolarvae life stages, respectively. Similar results have been found in larval walleye exposed to hydrogen peroxide and elevated pH. Bergerhouse (1992) found less than 10% of prolarval walleye died when exposed to pH 10.0, but over 50% of postlarvae II died when exposed to the same pH. Clayton and Summerfelt (1996) found that only 2% of postlarvae I walleye, but 80% of prolarvae survived a 1-h exposure to 100 mL L$^{-1}$ hydrogen peroxide.

The increased sensitivity of larval fish to toxicants may be related to development of the gills. Phillips and Summerfelt (1999) found that gill filaments were not present in walleye until 3 d posthatch, and the development of gill filaments coincides with the end of the prolarval stage when larvae make the transition from yolk sac respiration to branchial respiration. This may explain the significant increase in toxicity that occurs when walleye change from prolarvae to postlarvae I walleye. In addition, the significant increase in toxicity from postlarvae I to postlarvae II may be explained by the development of secondary lamellae that occurs at the end of the postlarvae I stage. Therefore, these findings suggest that growth and increased number of gill filaments and secondary lamellae during the first 21 d of walleye development may

provide an explanation for increased sensitivity of larval fish as they shift from yolk sac respiration to branchial respiration.

Extrapolating the sensitivity of larval walleye to chlorpyrifos–HA complexes and PC clay in these laboratory experiments to field conditions suggests a potential toxicity hazard from OPs adsorbed to eroded soils from fields which were treated with OPs. In agricultural watersheds, larval fishes may be exposed to multiple environmental and anthropogenic stressors which may lead to additive or synergistic effects. For example, the development of eggs and larvae of many fishes coincides with the spring application of many pesticides. In addition, walleye are fish with a high fecundity which results in high mortality rates especially through the larval stage of development. This is potentially a major problem because it has been determined that very small increases or decreases in survival rates of larval fish can have a significant impact on adult populations (Moyle and Cech, 1996).

This study suggests that chlorpyrifos may desorb from HA and be transferred directly to the fish. Therefore, there may be an exposure route for OPs directly from suspended solids to fish. However, further research is needed to evaluate the bioavailability of OPs and other toxicants adsorbed on suspended solids to aquatic organisms.

## Acknowledgements

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Table 1. The elemental composition of Panther Creek

bentonite clay used in the 48-h toxicity tests.

| Elemental Composition | (g/kg) |
|---|---|
| $SiO_2$ | 645.6 |
| $Al_2O_3$ | 214.9 |
| $Fe_2O_3$ | 75.5 |
| CaO | 25.7 |
| MgO | 22.2 |
| $TiO_2$ | 8.3 |
| $K_2O$ | 7.6 |

Table 2. Survival and ChE activity ($\mu$M acetylthiocholine hydrolyzed min$^{-1}$ g$^{-1}$ tissue) of prolarvae (3-d-old) walleye after a 48-h exposure to chlorpyrifos–HA and chlorpyrifos–PC clay complexes. Analysis was conducted on survival data the were arcsine transformed. Survival and ChE activity of treatment groups followed by the same letter are not statistically significant (P = 0.05).

| Treatment (Chlorpyrifos on solid phase; $\mu$g g$^{-1}$)[3] | Chlorpyrifos in solution ($\mu$g/L)[2] | | Survival (% ± SE) | ChE Activity |
|---|---|---|---|---|
| | Midway | End | | |
| HA-Control | 0 | 0 | 87 ± 2.9[a] | 5.24 ± 0.11[a] |
| 13.5 HA | 2 | 2 | 88 ± 8.3[a] | 2.78 ± 0.15[b] |
| 49.3 HA | 3 | 2 | 91 ± 2.7[a] | 2.41 ± 0.18[b] |
| 162.4 HA | 4 | 4 | 45 ± 19.0[b] | 1.77 ± 0.20[c] |
| PC Control | 0 | 0 | 81 ± 8.7[a] | 4.97 ± 0.12[a] |
| 21.1 PC | 7 | 5 | 96 ± 1.2[a] | 1.68 ± 0.27[c] |
| Chlorpyrifos[1] | 8 | 0 | 92 ± 6.0[a] | 1.93 ± 0.07[c] |
| P-Value of ANOVA | | | 0.031 | <0.001 |

[1]Water samples for analysis of chlorpyrifos were collected at the beginning and end of the 48 h toxicity test.

[2]Concentration of chlorpyrifos was measured by GC, limit of detection is 1.0 $\mu$g/L.

[3]Concentration of chlorpyrifos was calculated by the difference between chlorpyrifos in the aqueous phase and total chlorpyrifos added.

Table 3. Survival and ChE activity ($\mu$M acetylthiocholine hydrolyzed min$^{-1}$ g$^{-1}$ tissue) of postlarvae I (9-d-old) walleye after a 48-h exposure to chlorpyrifos–HA and chlorpyrifos–PC clay complexes.. Analysis was conducted on survival data the were arcsine transformed. Survival and ChE activity of treatment groups followed by the same letter are not statistically significant (P = 0.05).

| Treatment (Chlorpyrifos on solid phase; $\mu$g g$^{-1}$)[3] | Chlorpyrifos in solution ($\mu$g/L)[2] | | Survival (% ± SE) | ChE Activity |
|---|---|---|---|---|
| | Midway | End | | |
| HA-Control | 0 | 0 | 67 ± 8.4[a] | 8.22 ± 0.85[a] |
| 13.5 HA | 2 | 1 | 49 ± 9.8[a] | 2.73 ± 0.32[b] |
| 51.0 HA | 2 | 2 | 2 ± 2.0[b] | 2.25 |
| 163.0 $\mu$g g$^{-1}$ HA | 4 | 3 | 0 | NA |
| PC Control | 0 | 0 | 5 ± 1.8[b] | 8.05 ± 1.08[a] |
| 21.7 PC clay | 6 | 4 | 0 | NA |
| Chlorpyrifos[1] | 8 | 0 | 58 ± 13.0[a] | 2.40 ± 0.29[b] |
| P-Value of ANOVA | | | <0.001 | <0.001 |

[1]Water samples for analysis of chlorpyrifos were collected at the beginning and end of the 48 h toxicity test.

[2]Concentration of chlorpyrifos was measured by GC.

[3]Concentration of chlorpyrifos was calculated by the difference between chlorpyrifos in the aqueous phase and total chlorpyrifos added.

Table 4. Survival and ChE activity ($\mu$M acetylthiocholine hydrolyzed min$^{-1}$ g$^{-1}$ tissue) of postlarvae II (16-d-old) walleye after a 48-h exposure to chlorpyrifos–HA and chlorpyrifos–PC clay complexes. Analysis was conducted on survival data the were arcsine transformed. Survival and ChE activity of treatment groups followed by the same letter are not statistically significant (P = 0.05).

| Treatment (Chlorpyrifos on solid phase; $\mu$g g$^{-1}$)[3] | Chlorpyrifos in solution ($\mu$g L$^{-1}$)[2] | | Survival (% ± SE) | ChE Activity |
|---|---|---|---|---|
| | Midway | End | | |
| HA-Control | 0 | 0 | 56 ± 6.9[a] | 9.59 ± 0.37[a] |
| 11.8 HA | 3 | 1 | 21 ± 11.6[b] | 3.63 ± 0.31[b,c] |
| 47.0 HA | 4 | 2 | 17 ± 8.7[b] | 2.55 ± 0.28[b] |
| 160.7 HA | 5 | 4 | 0 | NA |
| PC Control | 0 | 0 | 0 | NA |
| 20.0 PC | 8 | 6 | 0 | NA |
| Chlorpyrifos[1] | 5 | 0 | 67 ± 13.5[a] | 3.97 ± 0.26[c] |
| P-Value of ANOVA | | | <0.001 | <0.001 |

[1]Water samples for analysis of chlorpyrifos were collected at the beginning and end of the 48 h toxicity test.

[2]Concentration of chlorpyrifos was measured by GC.

[3]Concentration of chlorpyrifos was calculated by the difference between chlorpyrifos in the aqueous phase and total chlorpyrifos added.

Figure Caption

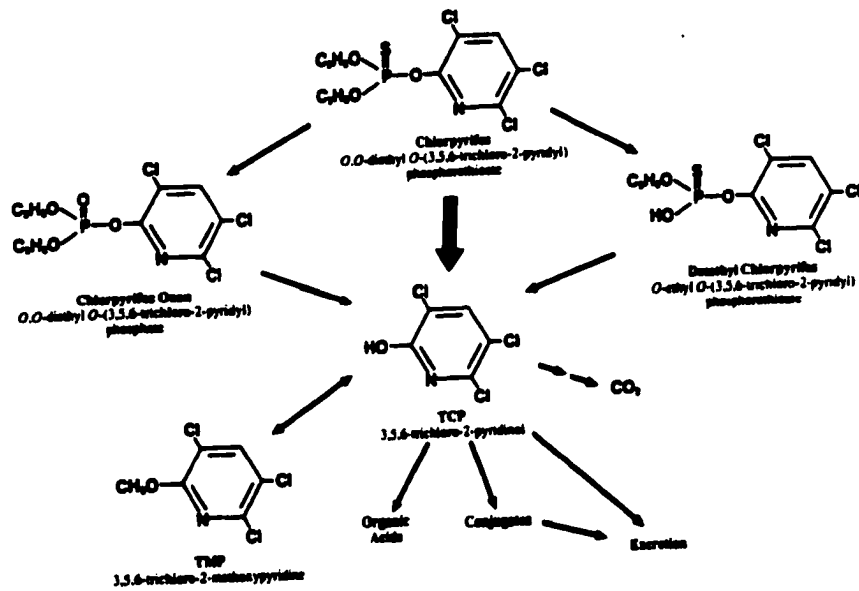Figure 1. Generalized pathways of chlorpyrifos transformation in the environment (from Racke, 1993).

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Figure 1.

# CHAPTER 4. TOXICITY OF CEDAR RIVER WATER AND SEDIMENT
# TO LARVAL WALLEYE

A paper to be submitted to the Journal of the Iowa Academy of Science

Todd A. Phillips and Robert C. Summerfelt

## ABSTRACT

Although all anthropogenic stressors affecting aquatic ecosystems have not been isolated, suspended solids, sediment, and pesticides are believed to be major factors in agroecosystems. In the spring of 1998 and 1999, static 48-h tests were conducted to determine the toxicity of water and sediment collected from the Cedar River to prolarval, postlarval I, and postlarval II walleye (Stizostedion vitreum). River water and sediment were not more toxic to any larval stage of walleye than reference water and sediment. Cedar River sediments, suspended solids, and water were examined for occurrence of the most common herbicides and insecticides in Iowa. No pesticides were found in sediments or suspended solids, but metolachlor, desethyl atrazine, acetochlor, and parathion were detected in water samples. However, no adverse effects were observed in larval walleye exposed to Cedar River water containing these pesticides. In addition, ChE activity in postlarvae I walleye exposed to Cedar River water containing parathion was not significantly different from postlarvae I exposed to control water. At the time of the study, the results indicate that pesticides do not seem to be a threat to larval walleye in the Cedar River.

## INTRODUCTION

In the U.S., agriculture contributes 65% of non-point source pollution to rivers, lakes, and streams, which is more than three times the amount contributed by the next leading source (USEPA, 1990). Therefore, agricultural practices pose one of the most serious threats to the continued ecological integrity of environmental systems (Thurman et al., 1991), and agriculture

has been charged as the activity most responsible for loss of fish species in streams (Karr et al., 1985). Fish communities in the former tallgrass prairie region of the midwestern United States, which now forms the heart of the "corn belt," a 12-state area of intensive agricultural land-use, have declined since the land was first developed for agriculture. Although all anthropogenic stressors affecting aquatic ecosystems in the corn belt have not been isolated, suspended solids, sediment, and pesticides are believed to be major factors.

A substantial body of research on the effects of suspended solids on salmonid fishes in the western United States has demonstrated negative effects of suspended solids on survival, growth, feeding, reproduction, and behavior (Swenson and Matson, 1976; Auld and Schubel, 1978; Sigler et al., 1984; Vandenbyllaardt et al., 1991). However, these studies have not attempted to characterize the nature of the inorganic components, and none evaluated the influence of clay mineralogy. Even though the most extensive damage to streams has been in the agricultural Midwest where warmwater streams have been severely degraded, little work has been done to evaluate the effects of suspended solids on warmwater fishes of the Midwest (Waters, 1995). Suspended solids, acting alone without pesticide adsorption, at concentrations observed in nature produce little or no direct mortality on juvenile and adult fish (the same cannot be said of impacts on larval fish). Wallen (1951) evaluated the toxicity of montmorrillonite clay (aluminum silicate) to 16 species of warmwater fish; lethal concentrations of clay ranged from 69,000 to 222,000 mg/L, but most species of fish survived exposure to 100,000 mg/L of clay for a week or longer. Thus, it seems that soil particles in of themselves are seldom toxic. Some studies, however, indicate that suspended solids may cause sublethal effects such as reduced feeding, depressed growth, and decreased tolerance to disease and toxicants (McLeay et al., 1984; Redding et al., 1987; Goldes et al., 1988).

In Iowa, suspended solids are of major concern because the landscape has changed substantially over the past 100-years. For example, in the late 1800's, the Cedar River basin was mainly covered by tallgrass prairie and the stream corridors were lined with timber (Meek,

1893); by the 1980's more than 90% of the Cedar River basin was used for the production of corn and soybeans (Menzel et al., 1984). As a result of intensive row-cropping, after a major storm, suspended solid concentrations as high as 3,230 mg/L can occur in Iowa rivers (USGS, 1994). However, streams in the Midwest rarely have suspended solid concentrations exceeding 300 mg/L for extended periods of time (USGS, 1994).

Along with increased quantities of suspended solids, the widespread use of herbicides and insecticides for corn production (Table 1) represents an additional threat from non-point source contaminants in Iowa's streams. During the 1990 growing season, 95% of corn acres and 97% of soybean acres in Iowa received herbicide treatment and 35% of corn acres received insecticide treatment (Hartzler and Wintersteen, 1991). Atrazine was the most widely used herbicide in Iowa; it was applied to more than 61% of the corn acres. Chlorpyrifos and terbufos (OPs) were the most widely used insecticides; they were applied to more than 20% of the corn acres in Iowa (Hartzler and Wintersteen, 1991).

Pesticides are most likely to represent a threat of acute mortality when spawning of fish coincides with application dates for pre-emergent pesticides. The timing of pre-emergent pesticide application is significant, because high concentrations of these pesticides can occur in rivers and streams after heavy spring rains (Thurman et al., 1991). In a literature review on atrazine, Solomon et al. (1996) reported that atrazine concentrations in streams and rivers in agricultural watersheds are episodic, with major peaks in spring and early summer following applications which typically occur in May and June. This would result in exposure of larval fish at their most sensitive life stage to peak concentrations of pesticides.

Although it has been assumed that suspended solids, herbicides, and insecticides have detrimental effects on the survival of fishes in Iowa, little work has been done to quantify these factors. Several ichthyofaunistic studies in Iowa have reported a reduction in fish species in Iowa streams. Menzel (1981) reported that eight environmentally sensitive species were absent or present in only small numbers in 10 headwater streams of the Cedar River basin, and all

streams examined were affected by moderate to intensive agricultural land use in their watersheds. Although the cause of the decline in the fish populations in these streams was not identified, Menzel (1981) implied that habitat degradation resulted from high levels of sediment and agricultural chemicals that were transported by the streams by runoff.

Larval walleye (Stizostedion vitreum) are the subjects of this study because they have been described as "extremely sensitive species" to suspended sediment (Alabaster and Lloyd, 1980), their status in Iowa's rivers has been evaluated from the late 1800's to the present, they are high on the food chain, and have economic value as a sport and food fish. Walleye is a favorite recreational species in Iowa as well as in the rest of the Midwest (Conover, 1986) and are native to large river systems in Iowa (Harlan et al., 1987). A statewide angler survey in 1994 indicated that walleye were third among fish most frequently eaten by Iowa anglers (Lutz et al., 1995).

From the late 1800's to the early 1950's walleye were commonly observed in the Iowan surface, a geologically distinct region of the state that encompasses the Cedar River basin (Meek, 1892; Cleary, 1953). However, riverine habitat degradation has affected many walleye fisheries in North America (Paragamian, 1989a), and an extensive statewide investigation of fish populations in the 1980's found walleye to be rare in Iowa's inland rivers (Paragamian, 1990).

The decline of walleye in inland streams of Iowa seems to be a function of poor recruitment (Paragamian, 1989b). As a result, a stocking program was initiated in 1951 to improve river walleye fisheries (Cleary and Mayhew, 1961). However, the success of larvae walleye survival was found to be very poor. In a four year study (1986 to 1990) conducted on rivers in the Iowan surface, natural reproduction and survival of stocked walleye larvae was poor (Kingery, 1991). Prior to the stocking of juvenile walleye, no young-of-the-year (YOY) walleye were observed (Paragamian, 1987, 1988, 1989a, 1990); however, survival of stocked juvenile walleye was as high as 22% (Kingery, 1991). Therefore, poor survival resulting from

stocking of larval fish, but good survival resulting from stocking juvenile, indicates that the larval stage (from hatching to juvenile) is the critical period for walleye survival. Although the eggs of walleye may settle to the bottom and be smothered by deposition of suspended solids, the reasons larval walleye are unable to survive is unknown. Therefore, the objectives of this study were to evaluate the toxicity of Cedar River sediments and water to larval walleye.

## MATERIALS AND METHODS

### Study area

The Cedar River, located in the Iowan Surface, a geologically distinct region of the state, was chosen for this study because it is one of the most intensively investigated inland rivers in Iowa, and walleye larvae stocking and research have been conducted in this stream for more than 30 years (Cleary and Mayhew, 1961; Mauldin, 1999).

Land-use data of the Cedar River watershed, according to the Anderson Land Cover Classification system, showed that over 93% of the land is in agricultural use, 3.5% is woodlands, 1.6% is urban, and 1.2% is wetlands (Table 2). Because so much of the land is used for agriculture, agricultural runoff represents the major non-point source of pollution. In addition, point source pollution may also be affecting the Cedar River. Three landfills (LaBounty Dump, Shaw Avenue Dump, and White Farm Equipment Landfill) were designated as Superfund hazardous waste sites in Charles City, Iowa, located 117 km upstream from the sample site at Janesville, Iowa (Iowa Department of Public Health, 1995). The LaBounty Site covers 3.4 ha on the Cedar River flood plain; volatile organic compounds and arsenic leached into groundwater and surface water from this disposal site (Iowa Department of Public Health, 1995). All cleanup activities have been completed at this site and the site was removed as a Superfund site in 1993. The Shaw Avenue Dump site is a 3.2-ha city dump located about 152-m east of the Cedar River in southeastern Charles City. The groundwater, soils, and Cedar River were contaminated with arsenic from this disposal site (Iowa Department of Public

Health, 1995). The White Farm Equipment Landfill occupies about 8.1 ha along the northern border of Charles City. The groundwater is contaminated by heavy metals (arsenic, chromium, copper, lead, nickel, and zinc) and volatile organic compounds from the waste site (Iowa Department of Public Health, 1995). Sediments, soils, and surface water may contain heavy metals that may also be affecting the Cedar River wetlands.

## Analysis of eggs of walleye collected from the Cedar River

One female walleye (length = 480 mm; weight = 1,056 g) was collected from the Cedar River by angling on April 16, 1998 and one female walleye (length = 523 mm; weight = 1,243 g) was collected by angling on April 9, 1999. Eggs were stripped from both females, placed in glass jars, and sent to the Iowa Hygienic Laboratory (Iowa City, Iowa) for analysis of chlorinated hydrocarbon insecticides. Eggs were evaluated for chlorinated hydrocarbons because these insecticides are extremely persistent and have high biomagnification factors (Pait et al., 1992; Richmonds and Dutta, 1992).

## Collection and analysis of sediment and water samples from the Cedar River

River sediment and water samples were collected once in the fall 1997 (September 18), five times in spring 1998 (March 31; April 16; May 3; May 6; May 13), and five times in spring 1999 (April 9; April 16; April 29; May 4; May 10) from Janesville, Iowa (USGS Station number 05458500). Sediment was collected with a petite ponar dredge from areas of the river where sediment had been deposited; the top 3 cm of sediment was collected and placed in glass jars. River water, including suspended solids, was collected in two 20-L carboys and transported to the Iowa State laboratory. Sediment and water collected from the Cedar River were sent to the Iowa Hygienic laboratory for pesticide analysis. The river water was filtered through a glass microfiber filter (1.5-$\mu$m pore size) and the suspended solids were collected. After filtering the water, the suspended solids, water, and sediment were analyzed for the most

common herbicides (atrazine, alachlor, cyanazine, metolachlor, and acetochlor) and insecticides (terbufos, fonofos, chlorpyrifos, and phorate) used in Iowa.

## Test organisms and culture conditions

Eyed walleye eggs were obtained from Rathbun Fish Hatchery, Moravia, Iowa, on April 28, 1998 and from Spirit Lake Fish Hatchery, Spirit Lake, Iowa, on April 21, 1999. Eggs were incubated at 14.0°C for 4 d and 13.0°C for 5 d in standard hatching jars before hatching began in Rathbun and Spirit Lake fish, respectively. To maintain uniformity of age, only larvae that hatched within a 24-h interval were used in the experiments. Mean length ± SE of 20 larvae at hatching was 7.8 ± 0.04 and 7.4 ± 0.05 mm for Rathbun and Spirit Lake fish, respectively. For both groups of fish, larvae were stocked at a density of 20 larvae/L (3,000 larvae) in a 150-L tank at 3 d posthatch. Fish stocked in this tank were used for later toxicity tests.

Rathbun and Spirit Lake larval walleye were raised at 16.9 ± 0.4°C and 16.5 ± 0.3°C for 30 d, respectively, following procedures described by Summerfelt (1996). Both groups of walleye were fed Fry Feed Kyowa B-400 and C-700 diets (BioKyowa, Inc., Chesterfield, Missouri) every 5 min, 22 h/d during the larval stages of development.

## Water quality of experimental test chambers

At the beginning and end of each static 48-h toxicity test, temperature, DO, hardness, alkalinity, pH, and ammonia were measured in each test chamber. Temperature (± 0.1°C) was measured using a glass thermometer and dissolved oxygen (DO) was measured to the nearest 0.1 mg $L^{-1}$ using an oxygen-sensitive membrane electrode (polarographic). Total ammonia-nitrogen ($NH_3$-N; TAN) was measured to the nearest 0.01 mg $L^{-1}$ using the Nesslerization method (APHA, 1998) and a spectrophotometer, and pH was measured to the nearest 0.1 with a standard combination electrode and meter standardized with pH 4.0, 7.0, and 10.0 buffers.

Hardness was measured to the nearest 1 mg L$^{-1}$ using the Man Ver 2 burette titration method

(HACH Company, Loveland, CO) and total alkalinity was measured to the nearest 1 mg L$^{-1}$ by

titration with 0.02N $H_2SO_4$ (APHA, 1998). Quality control samples (HACH Company,

Loveland, Colorado) were analyzed along with water samples to verify the accuracy of the

procedures used to measure TAN. Measured concentrations for the externally supplied TAN

quality assurance samples were always within the certified 95% confidence interval.


## Fish sampling

For both groups of fish, a sample of 5 fish were netted every day, and 20 larvae were

removed at 7 d posthatch. Walleye were euthanized with 300 mg/L tricaine methanesulfonate

(Finquel®), measured (mm), and observed microscopically for gas bladder inflation (GBI),

presence of food in the gut, and deformities. Observations of the day when the yolk sac and

oil globule disappeared were used to describe their larval stage (prolarvae, postlarvae I and II)

as well as when gas bladder inflation began and first feeding occurred. The three larval stages

of walleye are: prolarval (yolk sac present: 1 to 5 d posthatch); postlarval I (yolk sac absent

and oil globule present: 6 to 14 d posthatch); and postlarval II (oil globule absent: 15 to 21 d

posthatch).


## Acute toxicity assays

Static 48-h acute toxicity tests were conducted on three stages of larval walleye to determine

the toxicity of river sediment and river water collected from the Cedar River in 1998 and 1999

using standard methods (APHA, 1998). Food was withheld 24 h preceding and during the 48-

h exposure. Also, the sediment and water used in this toxicity tests was collected at the same

time and same site as the sediment analyzed by the Iowa Hygienic Laboratory. Therefore, the

concentration of pesticides that the larval walleye were exposed to was known.

Toxicity of Cedar River sediments. In both 1998 and 1999, the toxicity of sediments to three stages of larval walleye were determined by stocking 14 prolarvae and postlarvae I walleye/L (50 fish per test chamber) and 7 postlarvae II walleye/L into twelve 4-L test chambers. Test chambers described by Schmidt-Dallmier et al. (1992) were used; however, several modifications were made to the chambers. Glass baffles and a small-mesh stainless steel wire screen were removed, and a 1-mm mesh screen was used to cover the notches cut at the top and bottom of the funnel to prevent larval walleye from passing through the notches. Sediment suspension was maintained with a propeller-tipped stirring rod driven by an electric motor with a rheostat. The revolution rates of all stir rods were synchronized at 1,500 rpm with a stroboscope before additions of sediment to the chambers.

To obtain the desired concentrations of suspended solids, Cedar River sediment was added to 3.5 L of reference water (water used in the university aquaculture laboratories) until turbidity levels of 500, 250, and 100 NTUs, measured with a 90° light-scattering turbidimeter (HACH model 2100P, HACH Company, Loveland, Colorado), were obtained. However, in 1998, suspended solid concentrations were significantly lower than desired after the 48-h experiment because coarse materials settled to the bottom of the test chambers. Therefore, in 1999, Cedar River sediment was added to 10 L of reference water in a 20-L bucket, stirred, and allowed to settle for 5 min. This method was repeated until the desired turbidities were obtained. This allowed finer particles of suspended solids to be used, resulting in increased suspended solid concentrations in 1999. In addition, in 1999, test chambers were incubated in a water bath to maintain constant water temperatures.

A control (no riverine sediment), and three concentrations (high, medium, and low) of river sediment were used to determine the toxicity of the sediment (three replicates per treatment). Fish survival for each treatment was determined from counts at the end of the static 48-h test. Toxicity of Cedar River water. In 1998 and 1999, static 48-h tests were conducted to determine the toxicity of water collected from the Cedar River to prolarval, postlarval I, and

postlarval II walleye. The water used to accomplish this objective was collected from the Janesville site three times in the post-spawning interval as described. This experiment was run simultaneously with the sediment toxicity tests and fish from the same hatch were used in both experiments. Reference water (water used in the university aquaculture laboratories) was used as the control, and two concentrations of river water were used: 50:50 river water:reference water and 100% river water. Larvae were stocked into nine 4-L test chambers as described. Survival for each treatment was determined from counts at the end of the static 48-h test.

## Cholinesterase inhibition and analysis

Parathion, an organophosphorus insecticide (OP), was detected in the Cedar River water on May 4, 1999. Therefore, because OPs inhibit cholinesterase (ChE) activity, postlarvae I walleye exposed to this treatment were analyzed for ChE activity. Five postlarvae I walleye from each test chamber containing Cedar River water and from the control water that survived the 48-h static acute toxicity tests were analyzed to determine total ChE activity. Larval fish that died during the 48-h exposure were not analyzed for ChE activity because rapid decomposition made the analysis unreliable.

A colorimetric method for analyzing whole body ChE activity modified for use on a THERMOmax microplate reader and SOFTmax software (Molecular Devices Corporation, Sunnyvale, California) was used to monitor the rate of formation of 5-thio-2-nitrobenzoate, a yellow-colored anion. Hydrolysis of acetylthiocholine (AThCh) by ChE results in an acetate ion and a negatively charged thiocholine complex reacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoate (Ellman et al., 1961; Hill and Fleming, 1982; Gard and Hopper, 1993). The microplate reader was set in the kinetic mode to monitor increases in absorbance at 405 nm for 2 min, read at 8 sec intervals with a 0 sec lag time, and a final volume of 250 μl/well at 25°C (Gard and Hooper, 1993; Beauvais, 1997). The optimal substrate concentrations, acetylthiocholine iodide (ATI), for larval walleye was determined

prior to analysis with non-test samples. The optimal concentration was determined to be 0.001 M ATI. The Vmax and dilution factors were used to calculate ChE activities, reported as micromoles AThCh hydrolyzed per min per g of tissue (from here on will be abbreviated as µM AThCh).

All tissue samples analyzed for ChE activity were run in triplicate on the microtiter plate. If the coefficient of variance (CV) among the triplicates was greater than 10%, samples were rerun (less than 5% of the samples had to be rerun). Because a commercial cholinesterase standard for walleye tissue was not available, a check standard was used. The check standard was made by pooling larval walleye diluted 100-fold in pH 7.4 trizma buffer. The pooled tissue was homogenized and divided into 1-mL aliquots that were stored in 2 mL cryovials and placed in liquid nitrogen. These aliquots were run as check standards in triplicate along with each plate of treated samples. If the CV of the check standard was greater than 10%, all samples were rerun (less than 5% of the samples had to be rerun).

## Statistical analysis

Differences in survival and/or ChE activity due to treatment effects were assessed by analysis of variance (SAS, 1998). When the F-value for the overall test was significant ($P < 0.05$), Fisher's least significant difference test was used to determine significance among treatments. Survival data was transformed to a normal distribution before analysis using the following formula: transformed survival = arcsine (survival proportion)$^{1/2}$ (Zar, 1984).

## RESULTS

### Analysis of eggs of walleye collected from the Cedar River

In 1998, DDE (0.034 mg/kg) was the only chlorinated hydrocarbon present in the eggs of one female (Table 3). In 1999, DDD (0.018 mg/kg), DDE (0.16 mg/kg), and dieldrin (0.039 mg/kg) were present in the eggs collected from one female (Table 3).

## Pesticide analyses of water, suspended solids, and sediment

Suspended solids, water samples, and river sediment were analyzed for the most common herbicides (atrazine, alachlor, cyanazine, and metolachlor) and most common OPs (terbufos, fonofos, chlorpyrifos, and phorate) used in Iowa. No detectable concentrations of these pesticides were found in the September 18, 1997 sample when flow rates are normally low (Figure 1). In 1998, metolachlor was present in water samples collected on March 31 (0.31 µg/L) and May 3 (0.35 µg/L) when flow rates in the river were at their highest (Figure 2); desethyl atrazine was detected on April 16 (0.12 µg/L) and May 6 (0.10 µg/L; Figure 2). Pesticides were not found in the suspended solids or river sediment samples on any date.

Five spring 1999 samples (April 9, April 16, April 29, May 4, May 10) of suspended solids, bottom sediment, and water samples were analyzed for the most common herbicides (atrazine, alachlor, cyanazine, metolachlor, and acetochlor) and most common OPs (terbufos, fonofos, chlorpyrifos, and phorate) when flow rates in the river were at some of their highest levels (Figure 3). No detectable concentrations of these pesticides were found in suspended solids and sediment samples collected on any of the five dates. Metolachlor was present in water samples collected on four of the five collections: April 9 (0.27 µg/L), April 16 (0.33 µg/L), April 29 (0.14 µg/L), and May 4 (0.11 µg/L; Figure 3). Acetochlor was present in a water sample collected on May 10 (0.16 µg/L). Parathion, an organophosphorus insecticide, was detected in a water sample collected on May 4 (0.37 µg/L; Figure 3). No other pesticides were detected in water samples on any date.

## Toxicity of Cedar River water and sediments

Prolarvae. In 1998, survival of 3-d-old prolarvae (mean length ± SE = 9.0 ± 0.03 mm) exposed for 48-h to reference water, a 50:50 ratio of river water:reference water, and 100% river water did not differ from larval survival in the reference water. Likewise, differences in survival of prolarvae exposed to 25, 50, and 85 mg/L of Cedar River sediment did not differ

from survival of fish exposed to 132 mg/L of the reference (clay) treatment (Table 4). Also, differences in measures of water quality (temperature, dissolved oxygen, total ammonia-nitrogen, hardness, and alkalinity) among treatments were not significantly different at the beginning and end of the experiment.

In 1999, survival of 3-d-old prolarvae walleye (mean length ± SE = 8.8 ± 0.05 mm) exposed for 48-h to reference water, a 50:50 ratio of river water:reference water, and 100% river water did not differ from larval survival in reference water. Likewise, differences in survival of prolarvae exposed to 83, 280, and 392 mg/L of Cedar River sediment did not differ from survival of fish exposed to 340 mg/L of the reference (clay) treatment (Table 5). Differences in measures of water quality (temperature, dissolved oxygen, total ammonia-nitrogen, hardness, and alkalinity) among treatments were not significantly different at the beginning and end of the experiment.

Postlarvae I. In 1998, differences in survival of 6-d-old postlarvae I (mean length ± SE = 9.6 ± 0.06 mm) after a 48-h exposure to reference water, a 50:50 ratio of reference water:Cedar River water, and 100% Cedar River water were not different. Likewise, differences in survival of postlarvae I exposed to 12, 20, and 27 mg/L of Cedar River sediments did not differ from survival of fish exposed to 32 mg/L of the reference (clay) treatment (Table 4). Also, differences in measures of water quality (temperature, dissolved oxygen, total ammonia-nitrogen, hardness, and alkalinity) among treatments were not significantly different at the beginning and end of the experiment.

In 1999, differences in survival of 8-d-old postlarvae I (mean length ± SE = 10.2 ± 0.10 mm) after a 48-h exposure to reference water, a 50:50 ratio of reference water:Cedar River water, and 100% Cedar River water were not different (Table 5). Likewise, differences in survival of postlarvae I exposed to 56, 169, and 291 mg/L of Cedar River sediments did not differ significantly from survival of fish exposed to 250 mg/L of the reference (clay) treatment (Table 5). Also, differences in measures of water quality (temperature, dissolved oxygen, total

ammonia-nitrogen, hardness, and alkalinity) among treatments were not significantly different at the beginning and end of the experiment.

Because parathion, an OP insecticide, was found in river water, ChE activity was measured in postlarvae I walleye exposed to the reference water, 50:50 Cedar River:reference water, and 100% Cedar River water treatments. Mean ChE activity was $8.18 \pm 0.29$ μM AThCh for postlarvae I exposed to reference water and $8.34 \pm 0.44$ and $9.32 \pm 0.40$ μM AThCh for the Cedar River/reference water and 100% Cedar River water treatments, respectively. ChE activities did not differ among treatments.

Postlarvae II. In 1998, differences in survival of 13-d-old postlarvae II (mean length $\pm$ SE = $12.3 \pm 0.15$ mm) after a 48-h exposure to reference water, a 50:50 ratio of Cedar River water and reference water, and 100% Cedar River water were not different (Table 4). Likewise, differences in survival of postlarvae II exposed to 12, 34, and 42 mg/L of Cedar River sediments did not differ from survival of fish exposed to 74 mg/L of the reference (clay) treatment (Table 4). Also, differences in measures of water quality (temperature, dissolved oxygen, total ammonia-nitrogen, hardness, and alkalinity) among treatments were not significantly different at the beginning and end of the experiment.

In 1999, differences in survival after a 48-h exposure, starting with 14-d-old postlarvae II (mean length $\pm$ SE = $13.8 \pm 0.14$ mm) to reference water, a 50:50 ratio of Cedar River water and reference water, and 100% Cedar River water were not different (Table 5). Likewise, differences in survival of postlarvae II exposed to 81, 155, and 366 mg/L of Cedar River sediments did not differ from survival of fish exposed to 288 mg/L of the reference (clay) treatment (Table 5). Also, differences in measures of water quality (temperature, dissolved oxygen, total ammonia-nitrogen, hardness, and alkalinity) among treatments were not significantly different at the beginning and end of the experiment.

## DISCUSSION

The experiments conducted on three larval stages of walleye in 1998 and 1999 indicate that Cedar River water and sediment are not more toxic to larval walleye, at any larval stage than reference water and sediment. In both years, survival of postlarvae I walleye was lower than survival of prolarvae and postlarvae II walleye. Although this may suggest a toxic effect of the Cedar River sediment and water to postlarvae I, survival in these treatments did not differ significantly from the survival of postlarvae I walleye in the reference clay and water. Therefore, the decreased survival in postlarvae I walleye may be attributed to the high mortality that naturally occurs during this larval stage, when walleye switch from endogenous to exogenous feeding (Summerfelt, 1996).

Other investigators have also found that suspended solids have a low toxicity to fish (Wallen, 1951). Auld and Schubel (1978) determined that striped bass (Morone saxatilis) and yellow perch (Perca flavescens) were able to tolerate high concentrations of suspended sediment containing illite, chlorite, and kaolinite (1-4 $\mu$m particles) collected from the Chesapeake Bay ($\geq$ 500 mg/L). Similarly, Phillips (1996) found that a 28-d exposure of larval and early juvenile walleye to a ball clay (56% $SiO_2$ with 72% of the particles <1.0 $\mu$m) was not harmful at concentrations as high as 360 mg/L. However, Panther Creek clay (64.6% $SiO_2$), a bentonite clay, was found to be highly toxic to postlarval I and II walleye (Phillips, 2000). This indicates that suspended solids may be acutely toxic to fish depending on the physical and chemical characteristics of the suspended solid.

No pesticides were detected in Cedar River sediments or suspended solids, but metolachlor, desethyl atrazine, acetochlor and parathion were detected in Cedar River water. However, no adverse effects were observed in larval walleye exposed to Cedar River water containing these pesticides. In addition, ChE activity in postlarvae I walleye exposed to Cedar River water containing parathion was not significantly different from postlarvae I exposed to control water.

The lack of toxicity of Cedar River sediment and water in April and May 1998 and 1999, the time that walleye eggs and larvae were collected in the Cedar River (Mauldin, 1999), does not completely eliminate pesticides as a possible contributor to mortality of larval fish in this river, because concentrations of pesticides may vary with the nature of runoff events and time of year. However, the results of these experiments do not implicate pesticides as a problem to larval walleye in the Cedar River. Other factors limiting natural recruitment of walleye in the Cedar River may include a lack of nursery habitat and scarcity of zooplankton, as described by Mauldin (1999).

Although Cedar River water and sediment were not acutely toxic to the three stages of larval walleye, eggs collected from walleye of the Cedar River in 1998 and 1999 were found to contain DDE and DDD (metabolites of DDT) and dieldrin (a cyclodiene insecticide). The extreme persistence (years), biomagnification, and lipophilic characteristics of these three compounds is well established (Ware, 1994). As a result, a federal ban on the use of DDT was declared by the EPA in 1973 and most agricultural uses of the cyclodiene insecticides were canceled by the EPA between 1975 and 1980 (Ware, 1994). However, the presence of organochlorine insecticides in aquatic organisms is not uncommon. For example, although the concentrations were low (4.22 to 111.9 $\mu$g/kg), dieldrin, heptachlor epoxide, and chlordane were detected in whole three-year-old common carp (Cyprinus carpio) collected from the Des Moines River in Iowa (Lutz and Cavender, 1997). Newsome et al. (1993) found mean organochlorine contents (including hexachlorobenzenes, chlordane, DDTs, HCH, nonachlor, coctachlorostyrene, heptachlor, heptachlor epoxide, dieldrin, mirex, and toxaphene) in several species of commercial fish from the Great Lakes ranged from 44 to 138 mg/kg (lipid basis).

Although concentrations of DDD and DDE were very low (0.018 to 0.039 mg/kg) in walleye eggs collected from the Cedar River, DDD may represent a potential problem to walleye in the Cedar River because it has recently been determined to have estrogenic activity

(Klotz et al. 1996). Therefore, future research should examine the effects DDD may have on fish when it acts as an environmental estrogen.

## ACKNOWLEDGMENTS

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Table 1. Major herbicides and insecticides applied to Iowa farmland used for corn production: 1985 and 1990 data from Hartler and Wintersteen (1991); 1995 data from Hallberg (G. R. Hallberg, Hygienic Laboratory). Abbreviations: a.i., kg of active ingredient; NA, not available.

| | 1985 | | 1990 | | 1995 | |
|---|---|---|---|---|---|---|
| | % ha | kg. a.i. (1,000) | % ha | kg. a.i. (1,000) | % ha | kg. a.i. (1,000) |
| **Herbicide** | | | | | | |
| acetochlor | | | | | 26.0 | 2,815 |
| alachlor | 33.7 | 4,491 | 22.3 | 2,833 | 3.0 | 347 |
| atrazine | 49.0 | 4,407 | 61.0 | 3,424 | 62.0 | 2,944 |
| cyanazine | 33.9 | 4,702 | 19.6 | 2,322 | 18.0 | 2,402 |
| metolachlor | 37.2 | 4,692 | 34.4 | 4,265 | 33.0 | 3,798 |
| Percent acres treated[1] | 97.0 | | 95.0 | | 99.0 | |
| **Insecticide** | | | | | | |
| chlorpyrifos | 11.5 | 824 | 7.5 | 492 | N/A | N/A |
| fonofos | 7.0 | 439 | 4.0 | 195 | N/A | N/A |
| phorate | 4.7 | 351 | 2.9 | 197 | N/A | N/A |
| terbufos | 13.2 | 996 | 17.9 | 1,175 | N/A | N/A |
| Percent acres treated[2] | 43.0 | | 35.0 | | 28.0 | |

[1]Percent of Iowa corn ha treated with pesticides; it is not the sum of the column because some ha are treated with more than one pesticide.

Table 2. Cedar River watershed land use data during the early 1980's according to the Anderson Land Cover Classification.

| Basin | Subbasin | Urban | Agriculture | Woodland | Water | Wetland | Barren | MN[1] | Total Hectares |
|---|---|---|---|---|---|---|---|---|---|
| Cedar | Baskins Run | 108.1 | 6,268.0 | 624.9 | | 27.9 | 12.5 | | 7,041.4 |
| Little Cedar | Beaver | 11.3 | 4,366.7 | 242.4 | | | | | 4,620.5 |
| Little Cedar | Bur Oak | | 7,733.0 | 40.9 | | | | | 7,773.9 |
| Cedar | Cedar | 2,477.6 | 55,092.6 | 3,324.2 | 619.6 | 1,676.3 | 162.3 | | 63,351.7 |
| Cedar | Cedar | 6.1 | 3,376.8 | | | 2.4 | | | 3,384.9 |
| Cedar | Cedar | 575.5 | 17,632.8 | 1,189.0 | 151.8 | | 63.9 | 83,416.0 | 103,028.9 |
| Cedar | Cedar | | 2,520.9 | 44.1 | | | | 1,484.4 | 4,049.4 |
| Cedar | Deer | 193.0 | 20,267.8 | 400.7 | | | 2.4 | 5,737.4 | 16,601.3 |
| Cedar | Dry Run | 11.7 | 12,864.6 | 79.7 | | 32.0 | | | 12,987.6 |
| Little Cedar | Little Cedar | 250.1 | 46,515.4 | 1,496.6 | 5.7 | 1,284.9 | 62.3 | 17,395.2 | 67,010.2 |
| Cedar | Otter | 8.5 | 7,563.4 | 64.8 | | | | 8,588.5 | 16,225.2 |
| Cedar | Quarter Section Run | 265.1 | 14,470.9 | 481.6 | | | 18.6 | | 15,236.1 |
| Cedar | Rock | 4.5 | 17,444.6 | 427.8 | | | 19.4 | | 17,895.8 |
| Cedar | Rose | | | | | | | 17,958.2 | 17,958.2 |
| Little Cedar | Soap | | 3,282.1 | 39.3 | | | 12.5 | | 3,333.9 |
| Cedar | Spring | 38.4 | 10,147.9 | 234.3 | | | | | 10,420.6 |
| Cedar | Turtle (IA) | 133.6 | 5,745.9 | 6.1 | | | 29.5 | | 5,915.1 |
| Cedar | Turtle (MN) | | | | | | | 44,225.2 | 42,225.2 |
| Cedar | Woodbury | | | | | | | 10,924.5 | 10,924.5 |
| Total Hectares | | 4,083.8 | 235,292.6 | 8,695.4 | 776.6 | 3,023.1 | 383.7 | 187,729.0 | 439,984.6 |
| Percentage[2] | | 2.0 | 93.3 | 3.5 | 0.3 | 1.2 | 0.2 | | |

[1] Land use data for the Cedar River watershed in Minnesota were not available. Therefore, total hectares for the different land use data are for only the 252,255.6 hectares in Iowa of the total (439,984.6).

[2] Percentage based on 252,255.6 hectares in the Iowa portion of the watershed. There were no hectares in the "range" category of the Anderson land cover classification.

Table 3. Concentrations of chlorinated hydrocarbon insecticides in eggs of walleye collected from the Cedar River on April 16, 1998 and April 9, 1999.

| Organochlorine Insecticide | 1998 (mg/kg) | 1999 (mg/kg) | Quantitation Limit (mg/kg) |
| --- | --- | --- | --- |
| Aldrin | <0.01 | <0.01 | 0.01 |
| alpha-BHC | <0.01 | <0.01 | 0.01 |
| beta-BHC | <0.01 | <0.01 | 0.01 |
| delta-BHC | <0.01 | <0.01 | 0.01 |
| Lindane (gamma-BHC) | <0.01 | <0.01 | 0.01 |
| DDD | <0.01 | 0.02 | 0.01 |
| DDE | 0.034 | 0.16 | 0.01 |
| DDT | <0.01 | <0.01 | 0.01 |
| Dieldrin | <0.01 | 0.04 | 0.01 |
| Endosulfan I | <0.01 | <0.01 | 0.01 |
| Endosulfan II | <0.01 | <0.01 | 0.01 |
| Endosulfan sulfate | <0.01 | <0.01 | 0.01 |
| Endrin | <0.01 | <0.01 | 0.01 |
| Endrin aldehyde | <0.01 | <0.01 | 0.01 |
| Endrin ketone | <0.01 | <0.01 | 0.01 |
| Heptachlor | <0.01 | <0.01 | 0.01 |
| Heptachlor epoxide | <0.01 | <0.01 | 0.01 |
| Methoxychlor | <0.01 | <0.01 | 0.01 |
| Chlordane | <0.04 | <0.04 | 0.04 |
| Toxaphene | <2.00 | <2.00 | 2.00 |
| alpha-Chlordane | <0.01 | <0.01 | 0.01 |
| gamma-Chlordane | <0.01 | <0.01 | 0.01 |
| cis-Nonachlor | <0.01 | <0.01 | 0.01 |
| trans-Nonachlor | <0.01 | <0.01 | 0.01 |
| Oxychlordane | <0.01 | <0.01 | 0.01 |

Table 4. Survival of three larval stages (prolarval, postlarval I, and II) of walleye in a 48-h static toxicity tests in Cedar River water and in several concentrations of Cedar River sediment (Sed.) collected in 1998.

| Treatment | Prolarval | | Postlarval I | | Postlarval II | |
|---|---|---|---|---|---|---|
| | Water (%) | % Survival ± SE | Water (%) | % Survival ± SE | Water (%) | % Survival ± SE |
| Reference water | 100 | 96 ± 1.2 | 100 | 56 ± 13.1 | 100 | 48 ± 4.2 |
| Cedar R. | 50/50 | 92 ± 4.2 | 50/50 | 52 ± 14.0 | 50/50 | 40 ± 1.2 |
| Cedar R. | 100 | 87 ± 11.7 | 100 | 61 ± 5.8 | 1000 | 44 ± 5.5 |
| P-value of ANOVA | 0.45 | | 0.88 | | 0.29 | |
| | Conc. clay (mg/L) | % Survival ± SE | Clay (mg/L) | % Survival ± SE | Clay (mg/L) | % Survival ± SE |
| Reference clay | 132 ± 6.7 | 84 ± 3.1 | 32 ± 0.9 | 38 ± 10.1 | 74 ± 20.4 | 44 ± 2.4 |
| Cedar R. sed. | 25 ± 3.2 | 91 ± 2.9 | 12 ± 3.1 | 55 ± 12.2 | 12 ± 6.7 | 52 ± 2.4 |
| Cedar R. sed. | 50 ± 2.8 | 94 ± 2.0 | 20 ± 1.5 | 39 ± 11.4 | 34 ± 0.9 | 48 ± 3.5 |
| Cedar R. sed. | 85 ± 11.9 | 90 ± 6.1 | 27 ± 1.8 | 17 ± 5.2 | 42 ± 2.6 | 44 ± 5.3 |
| P-value of ANOVA | 0.37 | | 0.11 | | 0.31 | |

Table 5. Survival of three larval stages (prolarval, postlarval I, and II) of walleye in a 48-h static toxicity tests in Cedar River water and in several concentrations of Cedar River sediment (Sed.) collected in 1999.

| Treatment | Prolarvae | | Postlarvae I | | Postlarvae II | |
|---|---|---|---|---|---|---|
| | Water (%) | % Survival ± SE | Water (%) | % Survival ± SE | Water (%) | % Survival ± SE |
| Reference water | 100 | 91 ± 4.1 | 100 | 83 ± 1.8 | 100 | 88 ± 3.1 |
| Cedar R. | 50/50 | 91 ± 3.3 | 50/50 | 77 ± 5.5 | 50/50 | 81 ± 2.4 |
| Cedar R. | 100 | 85 ± 2.7 | 100 | 69 ± 10.1 | 1000 | 96 ± 1.2 |
| P-value of ANOVA | 0.39 | | 0.45 | | 0.12 | |
| | Conc. clay (mg/L) | % Survival ± SE | Clay (mg/L) | % Survival ± SE | Clay (mg/L) | % Survival ± SE |
| Reference clay | 340 ± 82 | 93 ± 4.4 | 250 ± 17 | 57 ± 2.4 | 288 ± 26 | 93 ± 1.8 |
| Cedar R. sed. | 83 ± 14 | 94 ± 3.5 | 56 ± 15 | 73 ± 5.9 | 81 ± 13 | 95 ± 1.8 |
| Cedar R. sed. | 280 ± 86 | 96 ± 1.2 | 169 ± 18 | 56 ± 12.9 | 155 ± 12 | 92 ± 1.2 |
| Cedar R. sed. | 392 ± 55 | 87 ± 3.7 | 291 ± 46 | 43 ± 16.3 | 366 ± 12 | 80 ± 5.0 |
| P-value of ANOVA | 0.40 | | 0.35 | | 0.33 | |

Figure Captions

Figure 1. Total flow ($m^3$/sec) in the Cedar River at Janesville, Iowa (Station 05458500) in 1997. No detectable concentrations of the eight were found in water, suspended solids, or sediment samples.

Figure 2. Total flow ($m^3$/sec) in the Cedar River at Janesville, Iowa (Station 05458500) in 1998. Metolachlor (M) and desethyl atrazine (DA) were detected in water samples. No detectable concentrations of pesticides were found in suspended solid or sediment samples.

Figure 3. Total flow ($m^3$/sec) in the Cedar River at Janesville, Iowa (Station 05458500) in 1999. Metolachlor (M) and parathion (P) were detected in water samples. No detectable concentrations of pesticides were found in suspended solid or sediment samples.
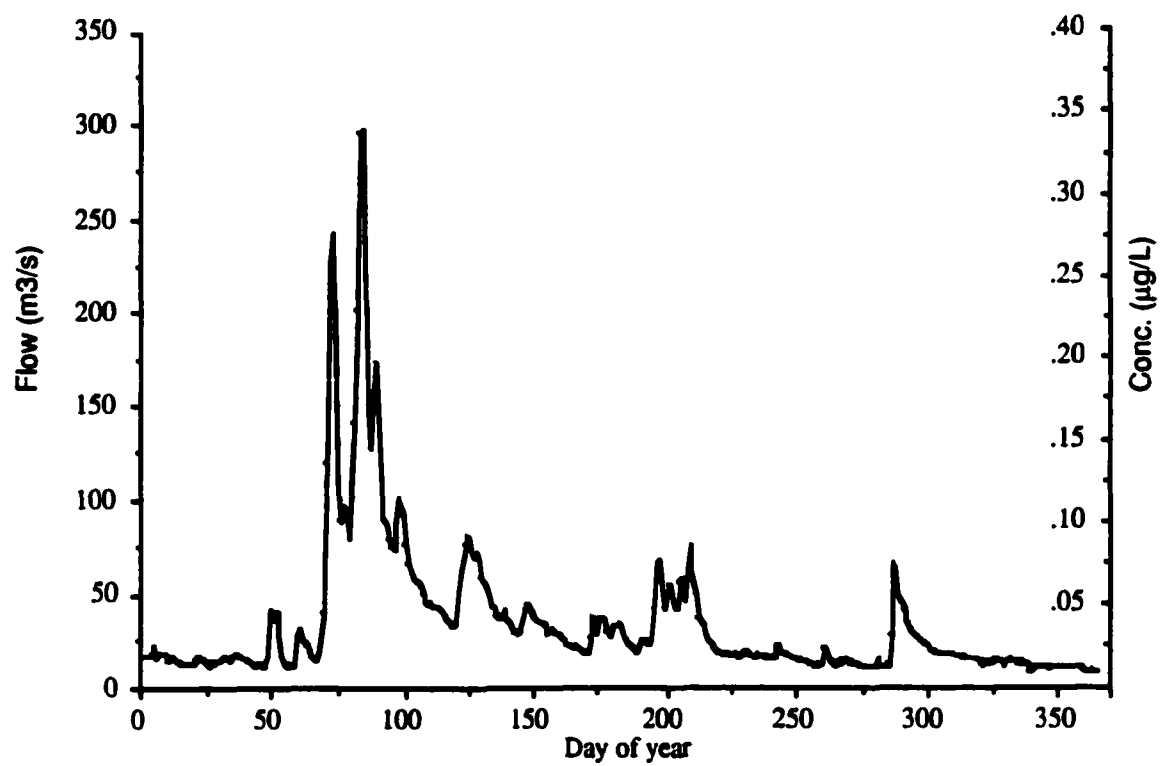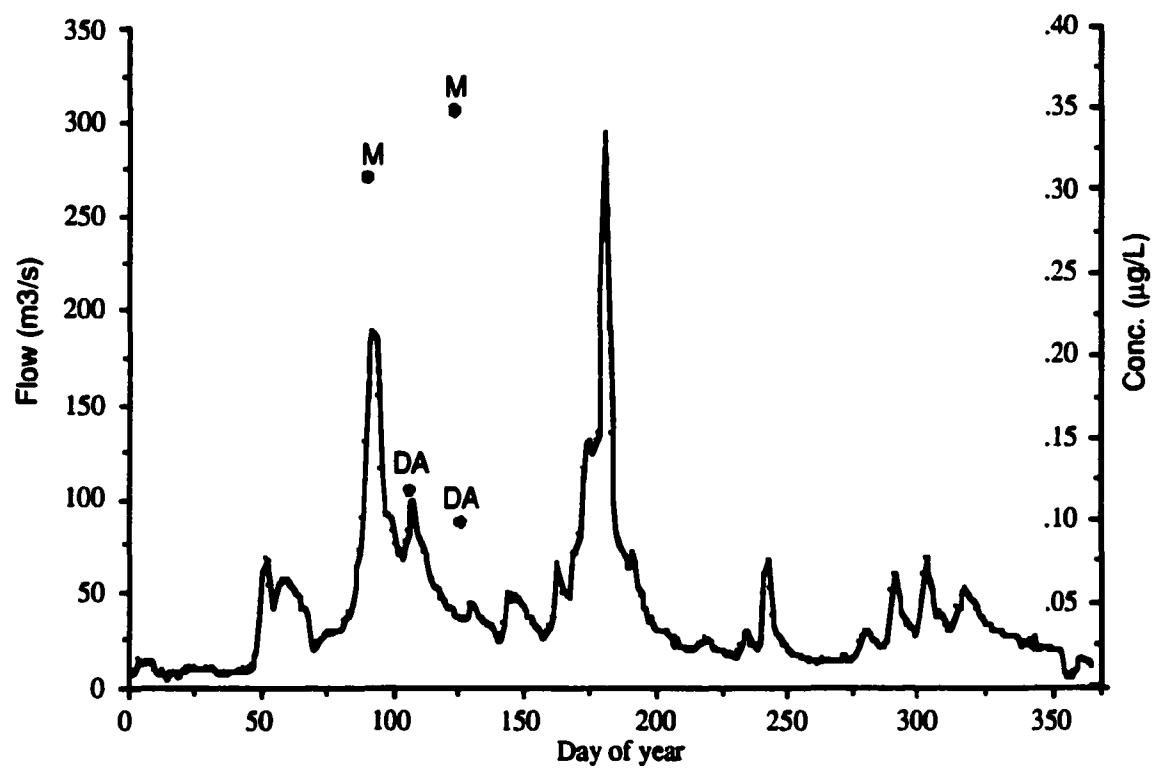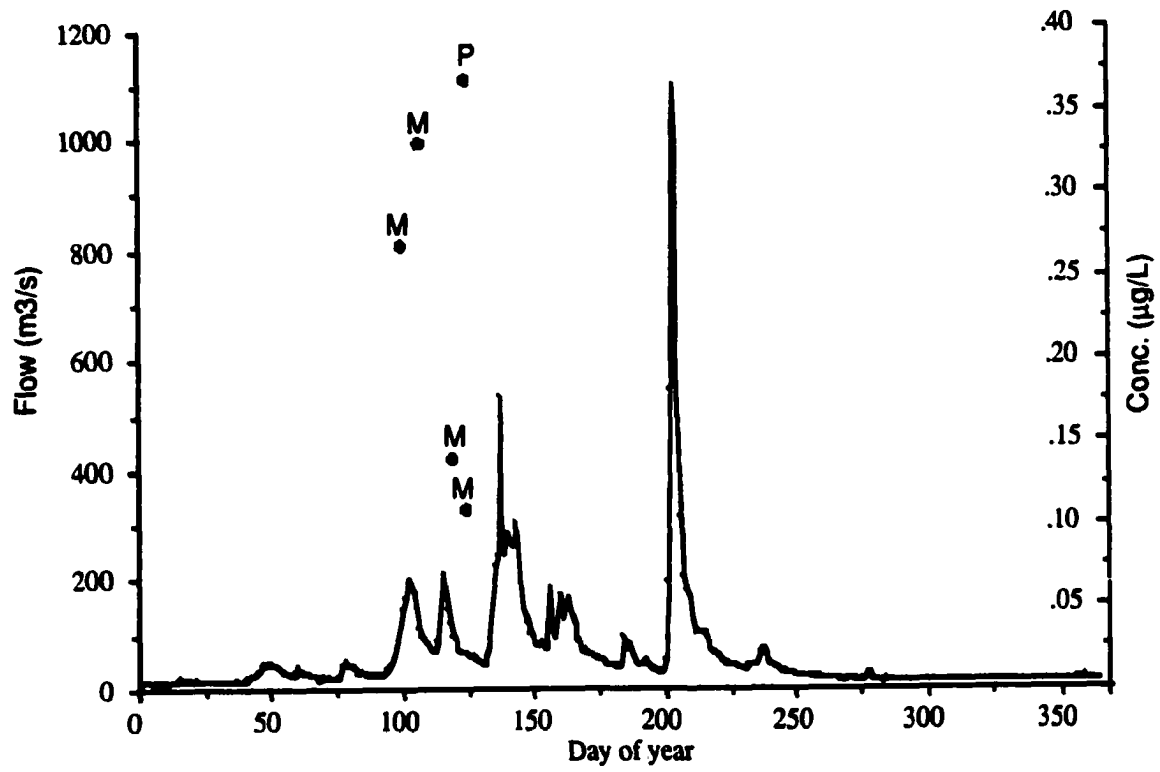
**Figure 1.**

Figure 2.

**Figure 3.**

## GENERAL CONCLUSIONS

In chapter one, the effects of water temperature, size of larval and juvenile walleye, stress, storage, and euthanasia techniques on ChE activity were examined. Water temperature (17.2, 20.9, and 24.6°C), stress, and the method of euthanasia had no effect on ChE activity of walleye. Cholinesterase activity of walleye stored at −80°C for 180 d did not differ from ChE activity of fresh specimens. However, a significant positive relationship was observed between whole body ChE activity and total length of larval walleye. No significant relationship was found between total length of juvenile walleye (59 to 163 mm) and brain ChE activity. The results from this study indicate that ChE inhibition is a reliable and sensitive indicator of OP exposure to walleye and is not affected by environmental factors. The only factor that affected ChE activity was larval development. Therefore, it is important to use larval walleye of similar size in experiments evaluating the effects of OPs; otherwise, ChE measurements may not be valid.

In chapter two, the toxicity to several life stages was examined. Prolarvae (yolk-sac larvae) were the least sensitive larval stage, but there was a significant increase in toxicity of chlorpyrifos from the prolarvae to postlarvae I stage and again from the postlarvae I to postlarvae II stage. The increased sensitivity of postlarval I walleye may occur because gill filaments are not well developed until the postlarvae I stage and are not even present until 3 d posthatch. The increased sensitivity of postlarval II walleye may be related to the development of secondary lamellae which occurs at the end of the postlarvae I stage. The LC50 concentration stabilized in 30- to 90-d-old juvenile walleye.

Larvae walleye were able to survive when ChE activity was inhibited by as much as 90%, and 37 d posthatch walleye were able to survive when ChE was inhibited by 85%. However, 62 and 90 d posthatch juvenile walleye did not survive when ChE was inhibited by more than 71%. Although fish were able to survive when ChE activity was inhibited by as much as 90%, the effects of ChE inhibition are unknown. Therefore, future research should evaluate the

effects of sublethal exposures of fish to OPs, and attempt to relate the level of ChE inhibition to specific biological responses.

In chapter three, 48-h static toxicity tests were conducted to determine the toxicity of chlorpyrifos adsorbed on humic acid (HA) and Panther Creek (PC) clay to three larval stages of walleye. Chlorpyrifos adsorbed on humic acid reduced survival of larval walleye indicating that chlorpyrifos desorbs from HA. Thus, chlorpyrifos-HA complexes represent a potential exposure route for OPs to fish. Also, PC clay alone, without chlorpyrifos, was highly toxic to postlarvae I and postlarvae II walleye, but not to prolarvae. The toxicity of PC clay coincides with the development of the gills (during postlarvae I and postlarvae II) which may explain the increased toxicity. Extrapolating the sensitivity of larval walleye to chlorpyrifos-HA complexes and PC clay in these laboratory experiments to field conditions suggests potential harm to walleye in streams because small increases or decreases in survival rates of larval fish can have a significantly impact on adult populations (Moyle and Cech, 1996). Results from this study should benefit agencies in evaluating the environmental risk to aquatic organisms from the use of OPs.

In chapter four, static 48-h bioassays were conducted to determine the toxicity of water and sediment collected from the Cedar River to prolarval, postlarval I, and postlarval II walleye. Neither river water nor sediment were toxic to larval walleye. Analyses were conducted for the most common pesticides used in Iowa, but none were found in Cedar River sediments or suspended solids. However, metolachlor, desethyl atrazine, acetochlor, and parathion were detected in river water. Bioassays with river water did not indicate adverse effects on larval walleye. In addition, ChE activity in postlarvae I walleye exposed to Cedar River water containing parathion was not significantly different from postlarvae I exposed to control water. The lack of toxicity of Cedar River sediment and water in these experiments does not completely eliminate pesticides as a possible contributor to decreased fish populations in this

region, but the results indicate that other factors such as lack of habitat or extremes in flow rates may be contributing to decreased fish populations (Mauldin, 1999).

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