2006

Biomonitoring organochlorine and cholinesterase inhibiting insecticide in eastern Iowa streams

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BIOMONITORING ORGANOCHLORINE AND CHOLINESTERASE INHIBITING INSECTICIDE IN EASTERN IOWA STREAMS

An Abstract of a Thesis

Submitted

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Matthew Thomas Fisher

University of Northern Iowa

May 2006

ABSTRACT

The four chapters in this thesis provide results from five separate investigations. Chapter One describes *Isonychia bicolor* acetylcholinesterase (AChE) activity in Northeast Iowa rivers. During 2002 and 2003 insects were collected from 10 sites during May, July and September, three sites on the Volga River were sampled weekly during May and June and one Cedar River site was sampled monthly. Also, in 2003, three sites on the Upper Iowa were sampled weekly in May and June. Sampling of ten sites yielded few discernable trends, however decreasing AChE activity from upstream to downstream sites was apparent on several occasions on the Volga and Upper Iowa Rivers. AChE activity decreased following a number of storm events on the Volga and Upper Iowa Rivers, possibly indicating exposure to insecticide runoff. No significant changes occurred during monthly Cedar River sampling.

Chapter Two encompasses two studies. One study investigated the effects of body size on *I. bicolor* AChE activity. Three size classes were sampled for AChE activity during June and August, 2002 from the Cedar River in Cedar Falls. No significant differences were found among sizes in either month. Another study maintained *I. bicolor* under three photoperiod treatments in stream microcosms. Weekly sampling over three weeks found no significant differences among treatments.

Chapter Three investigated the effects of the insecticide terbufos on *I. bicolor* AChE activity. Stream microcosms were dosed 0.0, 2.5, 5, 10 and 20 μg/L terbufos for 24 hours then purged with clean water. *I. bicolor* were sampled 24 h, 48 h and 9 d post

exposure. AChE activity in *I. bicolor* exposed to $\leq 10\mu g/L$ terbufos rebounded to control activity levels in 9 d. $20\mu g/L$ terbufos for 24 h was lethal to *I. bicolor* within 9 d.

Chapter Four investigated the benthic community composition of an urban trout stream. Periphyton samples were collected for determination of the Autotrophic Index and macroinvertebrates were analyzed for the pesticide chlordane. Macroinvertebrate communities consisted largely of Diptera and Oligochaeta, and Autotrophic Index values were high throughout the study indicating organic enrichment. No chlordane was found in macroinvertebrate samples.

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has been approved as meeting the thesis requirements for the Degree of Masters of Science.

2-28-06
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2-28-06
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Dedicated to the memory of Nate Bertram and Tommy Arjes.

ACKNOWLEDGEMENTS

This research was supported, in part, by three contracts (01-04HA-02 Ammendment 1, 01-04HA-02 Ammendment 2 and 03-04HA-03) amounting to $105,825 from the Iowa Department of Natural Resources.

This work would not be possible without the support and guidance of Kurt Pontasch. Thanks, Doc. Dr. Mercer and Dr. Clayton, have also been wonderful. I would like to thank all of the other students who helped in this project, especially Brian Andersen, Jon Eastman and Chris Jungling; without their assistance and friendship I could not have completed this project. Finally, I have to express great thanks to all of my friends and family. Their support, guidance, hot meals and good humor have been greatly appreciated.

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PROLOGUE

Several methods for examining the extent of pollution in streams exist, and each has advantages and disadvantages. These methods include chemical analysis, biomarkers, toxicity testing, and biomonitoring at the community level. Chemical analysis of stream water may seem to be the most accurate method of monitoring stream pollution, but, while chemical analysis may offer a precise measurement of contaminants, it has many drawbacks. Often the contaminant of concern is unknown, and protocols do not exist to screen for all substances. Also, when protocols do exist, chemical analysis only determines contamination at one point in time, while levels of contamination may vary dramatically over time. Comprehensive chemical analyses may be cost prohibitive and do not indicate the bioavailability of contaminants to organisms. For example, many chemicals of concern are nonpolar and tend to be sequestered in organic sediments. Therefore, to find these substances sediment samples must be analyzed and these analyses carry their own unique problems and added costs. In addition, quantifying a chemical's concentration will not give an accurate prediction of toxicity since species differ in tolerance to a given chemical, and the same species may respond differently to the same concentration of a compound under different environmental conditions.

Toxicity testing is the only method available to predict the hazards of various chemicals to ecosystems (Cairns, 1986). Extensive literature exists on toxicity testing methods, and a review of these procedures is far beyond the scope of this

thesis. However, biomarkers, a relatively new category of toxicity testing endpoints, are important in this discussion.

Biomarkers, or biological markers are "xenobiotically induced alterations in cellular or biochemical components or processes, structures, or functions that are measurable in a biological system or sample," (ATSDR, 1994, as cited by Kendall *et al.*, 1996). Typically biomarkers are measured in two ways, by measuring induction of detoxifying enzymes or by reduction of the activity of enzymes sensitive to inhibition by a specific xenobiotic (Callaghan *et al.*, 2002). Biomarker use is well established in clinical diagnoses of human health issues. However, the use of biomarkers for ecological risk assessment in aquatic ecosystems is more recent, and, in most cases, poorly understood.

Use of biomarkers may determine sublethal effects that could adversely affect sensitive species. Biomarkers are more effective at illustrating bioavailability of contaminants than chemical analysis. However, a detailed understanding of the biological system in question, as well as the system's responses to natural and unnatural environmental fluctuations is necessary. Henderson *et al.* (1989) describe an ideal biomarker as "...one that is chemical specific, detectable in trace quantities,...and quantitatively relatable to a prior exposure regimen. An ideal biomarker of an effect or of a disease state is unique to the disease state in question and quantitatively relatable to the degree or stage of the disease." This description refers to human biomarkers of disease, however environmental biomarkers should meet similar criteria.

When effective protocols exist for a biomarker, and when the natural fluctuations

of the biomarker are understood, a detailed evaluation of the extent of contamination may be possible. The use of acetylcholinesterase (AChE) as a biomarker for exposure to organophosphorus (OP) and carbamate insecticides is among the most understood biomarkers.

Organophosphorus and carbamate insecticides are applied around the world and may enter surface waters through drift, runoff, spills or intentional application (Willis and McDowell, 1982; Ramade, 1987; Edwards and Fisher, 1991; Liess and Schulz, 1999). OP insecticides are the most widely used class of insecticides in the United States due to their short persistence in soil, low capacity for bioaccumulation and their wide range of activity (Hill, 1995).

Carbamate and OP insecticides bind to acetylcholinesterase and inhibit hydrolysis of the neurotransmitter acetylcholine. Accumulation of acetylcholine in the synaptic cleft leads to continuous stimulation of the nervous system. This excitation can interfere with normal biological functions and may lead to death. Most OP insecticides must undergo an oxidative desulfuration step by mixed function oxidase metabolism in the fat body, Malpighian tubules and digestive tract of invertebrates, or the liver of vertebrates to become an active anti-acetylcholinesterase agent (Hill, 1995). AChE inhibition by OP's is considered irreversible while carbamate inhibition may be reversible. Recovery from irreversible inhibition can only occur through synthesis of new AChE and may take one to three weeks (Fleming, 1981; Fleming and Grue, 1981).

Contamination by AChE inhibiting pesticides in aquatic ecosystems is typically brief and may be difficult to detect with chemical analyses. Carbamate and OP pesticides

hydrolyze rapidly, and metabolites with AChE inhibitory action may not be detected by chemical analysis. AChE inhibition is a potentially useful biomarker of OP and carbamate exposure because inhibition: (1) is relatively long lasting; (2) can be correlated to deleterious effects to the organism; (3) can indicate exposure to brief contamination events; and (4) shows action of undetected toxic OP and carbamate metabolites.

Most literature about AChE inhibition involves resistance to OP's and carbamates in pest species and "important" nontarget organisms. However, studies on nontarget organisms are becoming more common. Andersen (2002) reviews the important papers covering AChE inhibition in aquatic, nontarget organisms.

Effective biomarkers are useful for indicating exposure to one or a small number of pollutants at sublethal levels. However, their utility declines as organisms become exposed to greater numbers of pollutants and/or environmental concentrations that are acutely toxic to some species. Under these conditions community composition in streams may change. Detailed examination of the taxa present and their densities can be used to show these community-level changes. Biomonitoring does not offer the precision of chemical analysis or biomarkers, but it can detect alterations to community composition and structure. Pollution decreases the number of species present, and can create an environment favorable for only a few species, which may be present in large numbers. By comparison, in "clean" streams numerous species exist in moderate numbers (Hilsenhoff, 1977).

Many methods for evaluating impacted aquatic communities have been used. However, use of biomonitoring may be limited due to: (1) disagreement about accepted

standard methodology for quantitative measurement; (2) difficulty in establishing cause and effect relationships; (3) inadequate taxonomic knowledge; (4) lack of qualified manpower and money (Arthington, 1982); and (5) alteration of aquatic community, or loss/gain of a species does not necessarily indicate the type of pollutant (or other insult) involved (Benke, *et al.* 1981). Also, evaluation of disturbed streams relies on comparisons to the previous unimpacted state of the stream or reference sites above an impacted reach of stream. However, previous conditions are often unknown, and reference sites may not exist or adequately represent the disturbed site.

Most methods for analyzing biomonitoring data attempt to describe the state of pollution in terms of a representative number. Although summing up the condition of a stream in this fashion may overlook many details, it makes evaluation by governing bodies easier. One type of evaluation is the use of biotic indices. Biotic indices designate a score for each species (or taxon) relating to its tolerance to pollution. The sum or average of these scores (depending on the specific index used) at each site gives an index of pollution. Some important examples include the Hilsenhoff Biotic Index (Hilsenhoff, 1998), the Index of Biotic Integrity (Karr, 1981), Trent Biotic Index (Woodiwiss, 1964), and Chandler's Biotic Score (Chutter, 1972). Use of biotic indices are discussed in greater detail by Hilsenhoff (1982).

Another form of community assessment is the use of diversity indices. A diversity index considers the number of species present, evenness, and proportional abundances (community structure). However, all diversity indices have major drawbacks. They do not consider the kinds of species present (i.e. "clean water insects" vs. "dirty water

insects") or absolute abundances (Pontasch and Brusven, 1988). Also, when diversity indices are used to evaluate pollution, they cannot distinguish naturally low diversity from pollution-related low diversity (e.g. small, cold streams may have naturally low diversity values) (Hilsenhoff, 1977).

Community comparison indices (or similarity indices) are designed to compare community composition or structure between two sites. There are many ways to do this and many versions of this method including the Bray and Curtis index (Bray and Curtis, 1957), Jaccard's coefficient of similarity (Jaccard, 1902), Canberra's metric (Lance and Williams, 1967), Morisita index (Morisita, 1959), Simplified Morisita index, also known as the Morisita-Horn index (Horn, 1966), and percentage similarity (Renkonen, 1938). For further discussion of diversity indices and community comparison indices see Pontasch and Brusven (1988), Smith, *et al.* (1990), and Pontasch, *et al.* (1989).

Objectives

This thesis consists of two different studies of pollution in streams. The first major study explores the use of AChE activities in the stream mayfly *Isonychia bicolor* as a biomarker for cholinesterase inhibiting insecticides in Northeast Iowa (and potentially Eastern United States) streams. This study was initiated by Andersen (2002) who first developed and optimized a modification of the Ellman assay (Ellman, *et al.* 1961) for use with *I. bicolor*. Andersen then established baseline AChE activity in lab and field settings, temperature and nutrient effects on AChE in stream microcosms, and investigated AChE levels following exposure to the OP insecticide chlorpyrifos in stream microcosms. The

research reported in Chapters One, Two and Three of this thesis attempts to answer some remaining questions from Andersen's (2002) work.

The objective of research reported in Chapter One was to determine AChE activity of *I. bicolor* in four streams in Northeast Iowa throughout 2002 and 2003. The objective of work reported in Chapter Two was to determine the effects of photoperiod and *I. bicolor* nymph body size on baseline AChE activities The objective for Chapter Three was to determine the effects of the OP insecticide terbufos on AChE activity in stream microcosms.

The second major study looked at McLoud Run, an urban, cold-water stream. The Iowa Department of Natural Resources stocks this stream with trout but it has been plagued with fish kills, and trout were found to contain the organochlorine insecticide chlordane. This study was undertaken in order to develop a better understanding of the insect communities in McLoud Run, their trophic dynamics and to potentially determine the cause(s) of fish kills.

The objectives of the McLoud run study were: (1) to quantify and identify benthic macroinvertebrate taxa present at three sites in McLoud Run; (2) to determine the trophic status of periphytic communities by calculating the Autotrophic Index (APHA, *et al.* 1989), a ratio of autotrophs (algae) to heterotrophs (organisms that consume autotrophs), at the three sites; and (3) to determine the amount of chlordane present in benthic macro-invertebrates present in McLoud Run. The results of this research are reported in Chapter Four.

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CHAPTER 1

MONITORING *ISONYCHIA BICOLOR* (EPHEMEROPTERA: ISONYCHIIDAE) ACETYLCHOLINESTERASE ACTIVITY IN FOUR NORTHEAST IOWA, USA RIVERS

Abstract

During May, July and September 2002 and 2003 *Isonychia bicolor* (Ephemeroptera: Isonychiidae) head capsule acetylcholinesterase (AChE) activity was determined at ten sites on four streams in northeast Iowa, USA, to determine potential exposure to organophosphorus insecticides. Also, in 2002, AChE activities were monitored weekly throughout May and June at three sites on the Volga River. Similarly, during 2003, three sites on both the Volga River and the Upper Iowa River were sampled weekly from May 21 until July 14. In addition, the Cedar River in Cedar Falls was sampled monthly from April to September 2002 and from April to December 2003 to monitor seasonal fluctuations in AChE activity.

During 2002, weekly monitoring on the Volga River indicated relatively low AChE activities, and few significant ($p \leq 0.05$) changes over time. Significant differences ($p \leq 0.05$) were found among the ten sites on all sampling dates (per mg protein) and in July (per g tissue). Monthly sampling of the Cedar River found no significant ($p > 0.05$) differences.

The 2003 weekly sampling on the Volga and Upper Iowa Rivers indicated a significant ($p \leq 0.05$) decrease in activities on one Volga site and two Upper Iowa sites that coincided with a major rainfall event between July 2 and July 14 that may have carried

pesticide runoff into these streams. Sampling of all ten sites in 2003 yielded no significant ($p>0.05$) differences among sites on any dates. Again, monthly sampling at Cedar Falls showed no significant ($p>0.05$) seasonal differences.

This field study illustrates that *I. bicolor* AChE activity fluctuates within sites over time, as well as among sites within a sampling date. Such fluctuations make interpretation of data difficult. However, the July, 2003 rainfall event on the Volga and Upper Iowa Rivers may illustrate correlation between decreased AChE activities and a possible insecticide runoff event.

Keywords: stream insect, acetylcholinesterase activity, organophosphorus insecticides, biomonitoring, biomarker

Introduction

Organophosphorus (OP) insecticides are the most widely used insecticides for corn rootworm control in Iowa. During 1995, 2.749 million pounds of OP insecticides were applied to corn in Iowa (Hartzler, *et al.* 1997). In areas where corn is planted in consecutive years (corn-on-corn planting) heavy insecticide usage may be necessary. Accidental spills, spray drift or runoff may expose nontarget aquatic organisms to lethal or sublethal levels of OP insecticides. However, these contamination events are brief and may be missed by chemical sampling methods.

OP as well as carbamate insecticides bind to acetylcholinesterase and inhibit hydrolysis of the neurotransmitter acetylcholine. Accumulation of acetylcholine in the synaptic cleft leads to repeated stimulation of neurons, which can interfere with normal biological functions and may lead to death. Acetylcholinesterase inhibition is a potentially useful biomarker of OP and carbamate exposure because inhibition: (1) is relatively long lasting; (2) can be correlated to deleterious effects on the organisms of interest; (3) can indicate exposure to brief contamination events, and; (4) indicates presence of unmeasured toxic OP and carbamate metabolites.

AChE inhibition as a potential biomarker has been studied extensively in terrestrial wildlife. Many of these studies focused on nontarget avian species because birds tend to ingest OP and carbamate pesticides. Fewer studies focus on mammals and other vertebrates, as they seldom ingest pesticides and are therefore less likely to become exposed. Melancon (1995) provides a review of terrestrial vertebrate AChE inhibition

studies. Research into insect AChE inhibition has focused on efficacy, mode of action, and resistance to OP and carbamate insecticides in target species.

Studies of AChE inhibition in aquatic systems have focused on fish (e.g., Coppage and Matthews, 1974; Richmonds and Dutta, 1992). However, benthic macroinvertebrates are better suited for such studies because of their relative immobility. AChE inhibition in freshwater aquatic macroinvertebrates has been investigated by a number of authors (e.g., Coppage and Mathews, 1974; Galgani and Bocquene, 1990; Abdullah, *et al.* 1994; Moulton, *et al.* 1996; Fornstrom, *et al.* 1997). Several studies have measured macroinvertebrate AChE inhibition in the field (e.g., Parker and Callaghan, 1997; Olsen, *et al.* 2001) and in the laboratory (e.g., Beauvais, *et al.* 1999; Callaghan, *et al.* 2002). However, none have validated laboratory findings with field monitoring. Schulz (2004) stresses that the ultimate scientific goal in ecological risk assessment of pesticides is to understand and assess potential effects under field conditions. Schulz (2004) also suggests the need for studies of exposure and effect conducted in natural surface waters affected by normal farming practices. Most AChE biomarker field studies using aquatic invertebrates have focused on mollusk and crustacean species, which tend to have a limited and patchy geographic distribution, and often occur in low abundance. In contrast aquatic insects are the most abundant and species rich macroinvertebrates in most stream ecosystems and some species may have a wide geographic distribution. These qualities make aquatic insects useful for AChE biomonitoring.

A study by Andersen (2002) established and optimized a modified version of the

Ellman AChE assay (Ellman, *et al.* 1961) for the common mayfly *Isonychia bicolor* (Ephemeroptera: Isonychiidae) using a plate reading spectrophotometer. Andersen (2002) also studied baseline *I. bicolor* AChE activities in seven streams in northeast Iowa. He initially determined that field populations from the Volga and Upper Iowa Rivers had significantly ($p \leq 0.05$) lower AChE activities than that in the Cedar River when analyzed immediately following collection However, when insects from these streams were kept in artificial streams for 30 days the AChE activity increased in all groups with the Volga and Upper Iowa *I. bicolor* AChE activities increasing dramatically. In addition, the Volga and Cedar River activities were significantly ($p \leq 0.05$) higher than those of the Upper Iowa after 30 days in the artificial stream.

During July, September and November, 2000 Andersen (2002) sampled *I.bicolor* from fifteen sites on seven streams (Cedar, Volga, Upper Iowa, Wapsipinicon, Little Wapsipinicon, Turkey and Little Cedar Rivers). Results from this study indicated the Volga, Upper Iowa and the Little Cedar Rivers had relatively low AChE activities and the Cedar River was relatively high. Other streams had intermediate AChE activities. Also, Cedar River AChE activities were significantly ($p \leq 0.05$) lower in the winter months compared to summer, suggesting some seasonal effects on AChE activity.

During 2001 Andersen (2002) studied 10 sites on four rivers in northeast Iowa (Cedar, Little Cedar, Volga and Upper Iowa Rivers) during May, July and September. In addition, he sampled three sites on the Volga River weekly from May to July 2001 as well as immediately prior to and following three storm events in 2001. Sampling of all ten

sites indicated relatively low AChE activities at all sites and found no significant differences during May and September. However, during July the Little Cedar River had significantly ($p \leq 0.05$) higher AChE activity than all others. The Volga River had relatively low AChE activities during all 2001 sampling. Andersen (2002) found no significant differences in AChE activity following any of the rain events.

Andersen (2002) also preformed several laboratory-based microcosm experiments. The first study investigated nutrition effects on baseline AChE activity of field collected *I. bicolor*. This study utilized four treatments: (1) a periphyton slurry; (2) artificial food; (3) periphyton slurry and artificial food; and (4) no nutrients. No significant differences were found among treatments following 10 or 20 day exposures. Another study investigated the effects of temperature on baseline activity. In this study field collected *I. bicolor* were exposed to four constant temperature treatments (5, 10, 19 and 25^{0}C) and one fluctuating temperature treatment (15-22^{0}C) reflecting typical diel fluctuations in the source stream. It was found that AChE activities in the 19 and 25^{0}C streams were significantly ($p \leq 0.05$) higher than other treatments and similar to activities found in the source riffle.

Andersen (2002) also exposed field collected *I. bicolor* to the OP insecticide chlorpyrifos for 48 h in two separate experiments. Stream microcosms were dosed with nominal chlorpyrifos concentrations of 0.0, 0.01, 0.1, 1.0 and 10.0 μg/L (11/2000) and 0.0, 0.37, 1.11, 3.33 and 10.0 μg/L (9/2001). In the first study, AChE activities were significantly ($p \leq 0.05$) inhibited after 48 h of exposure in the 10 μg/L treatments. After 48 h streams were purged of chlorpyrifos and clean water was allowed to continuously

flow through the microcosms. After seven days of clean water all *I. bicolor* were dead in the 10 μg/L treatments. However, AChE activities in the other treatments rose slowly throughout the experiment until day 35 when they were near that of "normal" high summer activities. During the later study, AChE activities were significantly ($p \leq 0.05$) different from controls in the 1.11 and 10.0 μg/L treatments following 24 hr exposure. The 10.0 μg/L treatment was still significantly lower than the other treatments following 48 h exposure. Again, after 48 h streams were purged of chlorpyrifos and clean water was allowed to continuously flow through the microcosms. After clean water for seven days, AChE activity in 10.0 μg/L treatment had risen and was not significantly different from other treatments.

The present study monitored *I. bicolor* AChE activity in four northeast Iowa, USA, streams. *Isonychia bicolor* was chosen due to the species' wide distribution, and abundance throughout the midwestern and eastern regions of the United States (Kondratieff and Voshell, 1984; Pontasch and Cairns, 1989; Breneman and Pontasch, 1994; Merritt and Cummins, 1996) and has been maintained for extended periods in stream microcosms (e.g., Breneman and Pontasch, 1994). Previous research with *I. bicolor* found relatively large fluctuations in AChE activity/g tissue within a sampling site on a given date (Andersen, 2002). This study attempted to eliminate some of this variability by reporting AChE activity as activity/gram tissue as well as activity/mg total protein. The Bradford protein assay was utilized to find the total protein of the tissue homogenate prepared for AChE activity.

Materials and Methods

Experimental Chronology

Isonychia bicolor were collected at ten sites in four rivers once during May, July and September, 2002 and 2003 for analysis of AChE activity (Ten Sites Sampling). During 2002 and 2003 three sites on the Volga River were sampled once weekly throughout May and June (Volga Intensive Sampling) and in 2003 three sites on the Upper Iowa River were sampled weekly in May and June (Upper Iowa Intensive Sampling). Also, the Cedar River in Cedar Falls, Iowa was sampled monthly from April to September 2002 and from April to December 2003 to monitor seasonal fluctuations in AChE activity (Cedar River Sampling).

Field Site Locations

The ten riffle sites sampled in 2002 and 2003 were located in four northeast Iowa, USA rivers: the Volga, Upper Iowa, Little Cedar and Cedar. Three sites were located on the Volga River. The farthest upstream site was located north of the confluence of the North and South Branches of the Volga River in the North Branch (VRNB). The second wass located in Twin Bridges Park (VRTB) near Randalia, Iowa and the third (VRSP) was located in the Volga State Recreation Area north of Fayette, Iowa. Sites on the Upper Iowa River (listed from upstream to downstream) were located in Kendallville, Iowa (UIKV), in the Chimney Rock Campground (UICR) near Bluffton, Iowa and in Decorah, Iowa (UIDE). One site was located on the Little Cedar River at Chickasaw Park (LCCP) near Ionia, Iowa. The three sites on the Cedar River (listed from upstream to

downstream) were located in the towns of Janesville (CRJV), Cedar Falls (CRCF) and near Evansdale (CRED). Appendix A contains descriptions of each site, and a map of site locations.

Collection and Maintenance of Test Organisms

At each riffle site *I. bicolor* were collected with kick nets (mesh size ~1mm). Fifteen late instar nymphs were added to each of three coolers (7-L capacity) filled with source water for transportation back to the laboratory. During transportation from the Volga and Upper Iowa Rivers, or when travel times exceeded one hour, temperature and dissolved oxygen were maintained near ambient stream conditions by pumping air through a small radiator placed in a cooler of ice; the cooled air was then shunted to each cooler through an airstone. Transporting insects in this manner results in few, if any, mortalities (Pontasch and Cairns, 1989; Pontasch and Cairns, 1991; Andersen, 2002). Upon arrival at the laboratory each cooler was aerated for approximately 24 h and brought to 20°C. Five randomly selected *I. bicolor* were then taken from each cooler and their head capsules removed and homogenized for AChE analysis (see below).

During the May, July and September "Ten Sites" sampling three Hess samples were also taken at each sampling location to determine *I. bicolor* densities. Each sample was preserved in 70% ethanol for later enumeration. The density data from 2002 and 2003 are located in Appendix B.

At each sampling site and date, standard water chemistry measurements were recorded and are reported in Appendix C. Dissolved oxygen, temperature, conductivity,

and pH were measured with a pre-calibrated SONDE 3800 Water Quality Logger (Yellow Springs Instruments, Inc., Ohio, USA). Hardness and alkalinity were determined with water quality test kits (Hach Chemical Co., Loveland, Colorado, USA). Daily hydrologic (streamflow) data from the U.S. Geological Survey were recorded for the "Volga Intensive Study" in 2002 and the Volga and Upper Iowa "intensive studies" in 2003 These data are reported in Appendix D

Determination of AChE activity

Acetylcholinesterase activity was quantified using the Ellman method (Ellman, *et al.* 1961) optimized for *I. bicolor* by Anderson (2002). In the lab five randomly selected nymphs were taken from each of 3 coolers per site and decapitated. Head capsules were blotted with tissue paper to remove excess water and weighed. The five head capsules from each cooler were pooled and homogenized in a glass tissue grinder with 0.5% Triton X-100 detergent in Tris pH 7.4 buffer solution to give a 200:1 dilution factor. Homogenate from each replicate was added to a microcentrifuge tube and centrifuged (10 min; 14,000 rpm). Supernatant aliquots were separated into two cryovials then frozen (-80°C) until analyzed for AChE activity. Aliquots were separated to create a spare that remained frozen in case of sampling error, spillage, etc.

AChE activity was quantified in a microplate-reading spectrophotometer (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA, USA). Each well contained an aliquot of *I. bicolor* head capsule supernatant (30 μl), acetylthiocholine iodide (AThChI) solution (0.0418M; 30 μl), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) colorimetric reagent

solution (0.442 M; 20 μl), and Tris pH 8.0 buffer solution (170 μl). Acetylthiocholine iodide (AThChI) is similar in structure to acetylcholine and acetylcholinesterase hydrolyzes AThChI in a similar manner to acetylcholine. This hydrolysis results in a positively charged acetate ion and a negatively charged thiocholine complex. The thiocholine complex reacts with DTNB to create 5-thio-2-nitrobenzoate, a stable, yellow colored anion that absorbs light most strongly at 412 nm. For each replicate sample, AChE activity was measured in triplicate as the rate of increase in absorbance at 412 nm. Tris buffers, AThChI and DTNB were obtained from Sigma Chemical (St. Louis, MO, USA). Tris buffer solutions were refrigerated at 4°C for no longer than 1 month and were adjusted to appropriate pH values prior to each analysis. DTNB and AThChI were prepared immediately prior to spectrophotometric analysis. The optimum reaction temperature (~32°C) was achieved by maintaining DTNB and AThChI at room temperature, placing thawed cryovials of tissue homogenate in ice water and heating the Tris pH 8.0 buffer to ~80°C before pipetting. A blank (consisting of 200 μl Tris pH 8.0 buffer solution, 30 μl AThChI solution, 20 μl DTNB solution and no tissue homogenate) and a check standard (consisting of pooled homogenate supernatant from approximately 200 *I. bicolor* head capsules) were also analyzed in triplicate on each plate. If the check standard deviated from normal values the analysis was repeated. Care was taken to prevent bubble formation because bubbles cause inaccurate readings by the spectrophotometer.

Spectrophotometer software (SoftMAX PRO, Molecular Devices, Sunnyvale, CA, USA) was used to determine the highest AChE activity. Absorbance measurements

for all trials were read in all wells every twelve seconds for five minutes after an initial one-minute lag time. The following formula was used to convert mOD (milli-optical density) output units into international units of enzyme activity:

{[(enzyme mOD/min)-(blank mOD/min)]/1000} x 0.817 x dilution factor =

(μmoles AThChI hydrolyzed/min)/gram tissue

Determination of Total Protein Content

In this study, AChE activity is expressed in nmoles AThChI hydrolyzed/min/mg protein as well as μmoles AThChI hydrolyzed/min/g tissue. Andersen (2002) expressed activity only as μmoles AThChI hydrolyzed/min/g tissue. In order to find the protein content of the AChE tissue homogenate, the Bradford protein assay was optimized for use with *I. bicolor* tissue homogenates. Quantifying AChE activity relative to total protein content potentially minimizes the variation caused by differing amounts of other substances such as chitin or fat in the head capsules. A standard curve using bovine serum albumin was created to quantify the amount of protein in a solution of known concentration. The amount of protein (μg protein/μg tissue) in each sample was calculated using the formula (12{{Bradford assay O.D.} - 0.017}/48.775). From this the AChE activity per mg protein was calculated.

The Bradford assay utilized a 96 well microplate reading spectrophotometer. Each sample was analyzed in triplicate using 60 µl Bradford's reagent (Coomassie® Brilliant Blue G-250 dye; Bio Rad, Hercules, California, USA), 5 µl tissue homogenate, and 235 µl

twice distilled, reverse osmosis water in each well. Each sample was then mixed by repeated suction/expulsion through a micropipetter to ensure even mixture of the solution. Care was taken to prevent formation of bubbles during this process as bubbles create inaccurate spectrophotometric readings. Each microplate contained one blank (240 µl water and 60 µl Bradford reagent), and one check standard, as well as unknowns (each in triplicate). Microplates were allowed to sit approximately 5 minutes before analysis in the spectrophotometer.

Statistical Analyses

AChE activity data for 2002 and 2003 monitoring were statistically analyzed by a one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test for the separation of means using SAS® software (SAS Institute, 1999). "Ten Sites Sampling" data were analyzed both within a site over time and among sites within a sampling period. "Intensive Sampling" data were analyzed within a sampling site over time. Lack of *I. bicolor* or high water occasionally prevented sampling. When "Ten Sites Sampling" locations had samples from only two dates, AChE activities were analyzed using a t-test. Results from all statistical analyses were considered significant at the $\alpha = 0.05$ level.

Results and Discussion

Ten Sites Sampling 2002

Results from the May 2002 AChE activity analyses indicated that there were no significant ($p \geq 0.05$) differences among sites in activities per gram of tissue (Figure 1.1),

but that there were significant differences among the sites in activities per mg of protein (Figure 1.2). At VRNB and LCCP there were not enough organisms to conduct the analyses. The lower activity at the CRCF site relative to the two other Cedar River sites may be due to sampling two weeks before the other sites when water temperatures were considerably lower. The relatively high activity at the downstream CRED site in May suggests that there are no substantial effects from the Cedar Falls-Waterloo metropolitan area. The activities at the two sites sampled on the Volga River were nearly identical to the CRCF site, which had been sampled the previous day. In May the significant ($p \leq 0.05$) drop in activities at UICR and UIDE relative to UIKV suggests the possible presence of anticholinesterase agents downstream from UIKV.

During July 2002 sampling, there were not enough organisms at CRJV and CRED to permit analyses. At the remaining eight sites there were significant differences among sites in AChE activities per g tissue (Figure 1.3) and per mg protein (Figure 1.4). As was the case during July, 2001 sampling (Andersen, 2002), LCCP had the highest activity among sites. Previous sampling by Andersen (2002) found AChE activity of ~ 17 μmoles AThChI hydrolyzed/min/g tissue during summer months. This number was considered the peak natural activity rate in northeast Iowa rivers; however the true peak may, in fact, be higher. July, 2002 activities at VRNB and VRTB were near "peak" activity and higher than their July, 2001 activities per g tissue. These data mark the first time that Volga River sites had "peak" AChE activities in this study or that of Andersen (2002). Activities at the remaining sites were below "peak" activity but similar to July, 2001 activities per g tissue. As in May, activities per mg protein at two sites on the Upper Iowa River were

significantly ($p \leq 0.05$) lower relative to other sites in July. However, in July activities at sites upstream (UIKV) and downstream (UIDE) from UICR were significantly lower than UICR. This pattern differs from that of May, when both downstream sites (UICR and UIDE) appeared impacted.

During September, 2002 sampling, there were no significant ($p>0.05$) differences among sites in AChE activities per g tissue (Figure 1.5), but there were significant differences in activities per mg protein (Figure 1.6). Similar to the July results, the AChE activities at the Volga River sites were near "peak" activity and were, in fact, higher than at any other site. Once again, this is in contrast with previous research (Andersen, 2002) when the Volga River sites typically had the lowest activities. However, as was the case in both May and July, two sites on the Upper Iowa River had the lowest AChE activities. In September, as in May, the activities were lowest at UICR and UIDE downstream from UIKV. During 2002 the Cedar River sites, with the exception of CRED, were below "peak" activities, but they were higher than in September, 2001. The relatively higher activity at CRED compared to sites upstream (CRJV and CRCF) once again suggests that there is no "urban effect" on AChE activities below the Cedar Falls-Waterloo metropolitan area. Analyses of AChE activities within a site over time (Appendix E) in most cases did not result in significant differences over time. However, activities were usually higher in September compared to July and, especially, May.

The Hess sample data show some trends in *I. bicolor* densities during 2002 (Appendix B). The patchy distribution of *I. bicolor* within streams resulted in high

standard deviations for all samples. The Cedar River sites had high densities of small, early instar nymphs in May, but those densities decreased in July and September when larger organisms were collected. A study of *I. bicolor* voltinism by Sweeney (1978) found that in a Pennsylvania creek, *I. bicolor* was bivoltine. Sweeney (1978) found that one generation overwinters as relatively large nymphs that emerge, mate and oviposit in early June. These eggs hatch in one to four weeks. The adults of this generation emerge and oviposit from early August to September. Their eggs hatch through autumn and nymphs grow until water temperatures drop below 15°C.

It may be that in northeast Iowa the "winter generation" develops somewhat earlier than Sweeney's (1978) Pennsylvania population, hence the presence of small nymphs in late May. Also, few nymphs, of any size, were found in the Upper Iowa or Volga River sites on May 14-15. This may represent the period immediately following emergence of the winter generation. Following such an emergence, larvae would be too small to collect and large larvae would be absent. The Cedar River sites were sampled on May 29 and contained many "very small" nymphs. This size class is big enough to be sampled with a Hess sampler, but too small for capture in kick screens and use in the AChE assay.

During July, *I. bicolor* size distribution was relatively similar at all sites. All size classes were present in moderate numbers at all sites. The same pattern occurred at all sites during September. However, at most sites more "very small" *I. bicolor* were present, indicating a fall oviposition congruent with Sweeney's (1978) work.

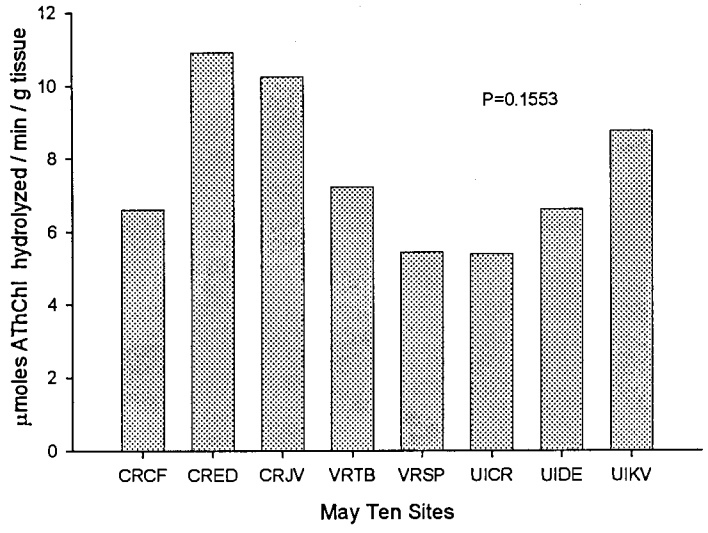

Figure 1.1 *Isonychia bicolor* AChE activities (per g tissue) during May 2002. P value is from a one-way ANOVA among sites.

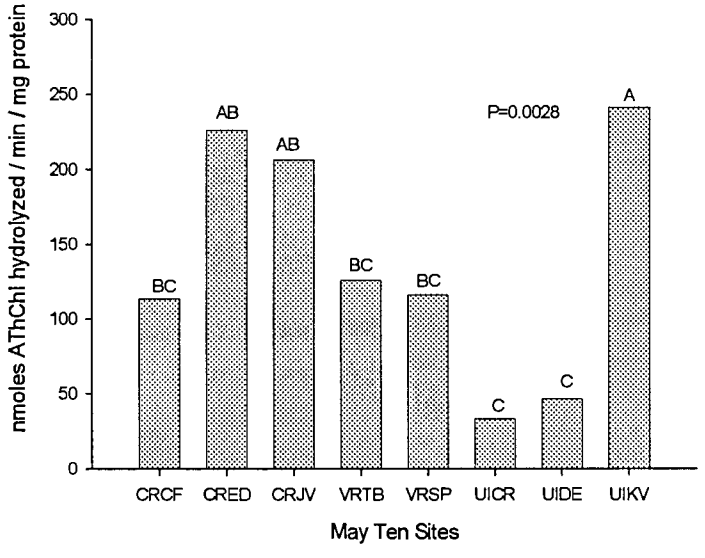

Figure 1.2 *Isonychia bicolor* AChE activities (per mg protein) during May 2002. P value is from a one-way ANOVA among sites. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

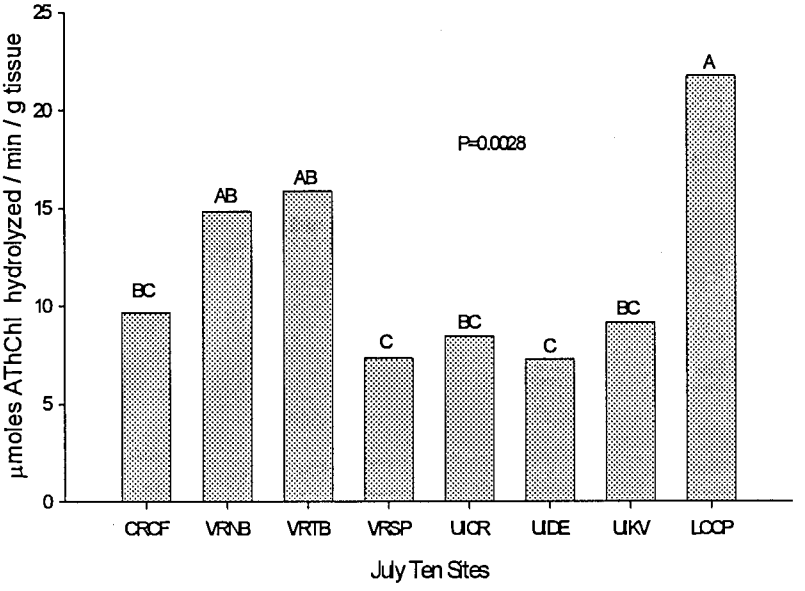

Figure 1.3 *Isonychia bicolor* AChE activities (per g tissue) during July 2002. P value is from a one-way ANOVA among sites. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

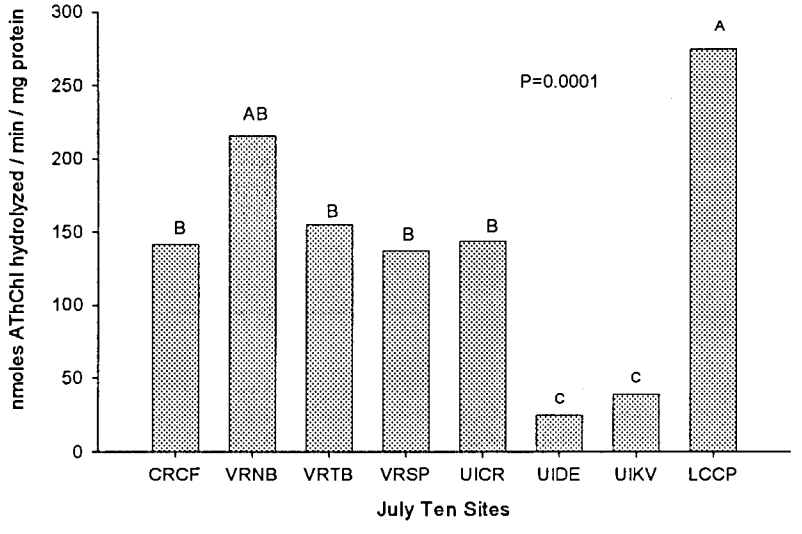

Figure 1.4 *Isonychia bicolor* AChE activities (per mg protein) during July 2002. P value is from a one-way ANOVA among sites. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

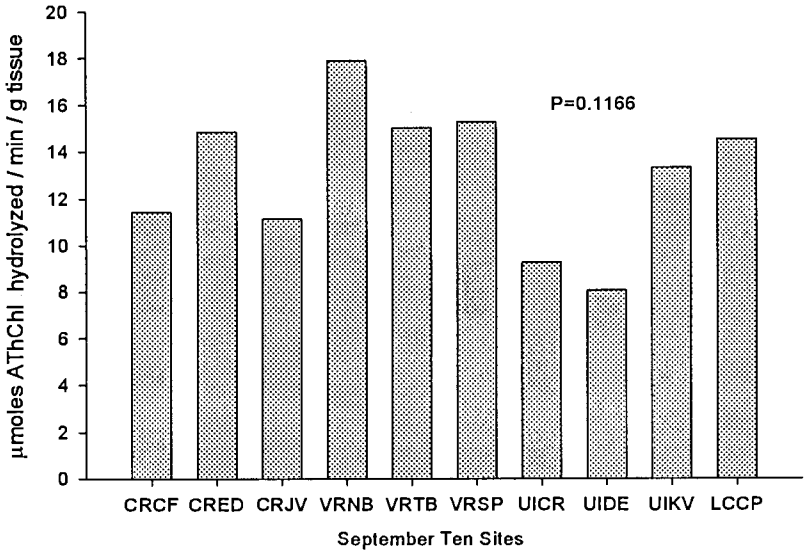

Figure 1.5 *Isonychia bicolor* AChE activities (per g tissue) during September 2002. P value is from a one-way ANOVA among sites.

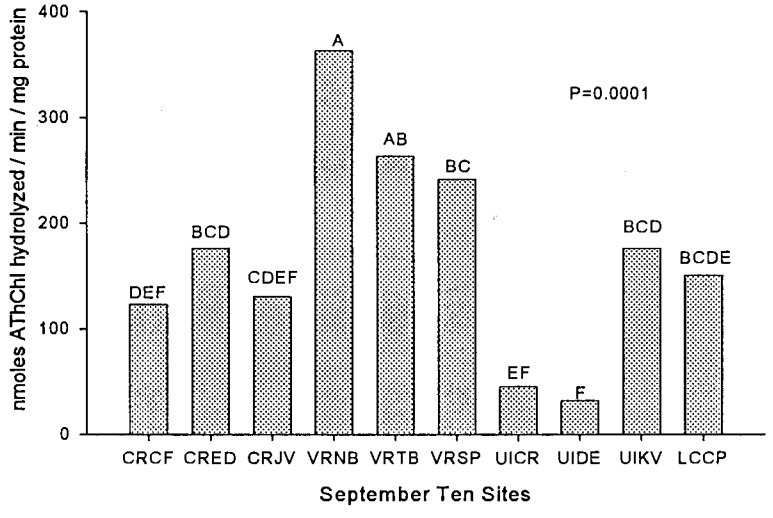

Figure 1.6 *Isonychia bicolor* AChE activities (per mg protein) during September 2002. P value is from a one-way ANOVA among sites. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

Ten Sites Sampling 2003

Results from the May 2003 AChE activity analyses indicated that there were no significant differences among sites in activity per gram of tissue (Figure 1.7) or in activity per mg of protein (Figure 1.8). At VRNB, VRTB, UIKV and LCCP there were not enough organisms to conduct the analyses. The similar activities at CRCF and CRED suggest that there is no "urban effect" occurring below the Cedar Falls-Waterloo metropolitan area. These results are similar to those from the previous May.

During July all ten sites were sampled, but there were no significant differences in AChE activities among the sites per g tissue (Figure 1.9) or per mg protein (Figure 1.10). As was the case during July 2001 (Andersen, 2002) and 2002 sampling LCCP had one of the highest activities among sites even though insufficient organisms were present in May at that site. July 2003 activity at UIKV was the highest among the ten sites and near "peak," but the AChE activity at the two downstream sites (UICR and UIDE) on the Upper Iowa River were the lowest among the sites. This pattern is similar to results from the Upper Iowa River during May 2002, and may indicate the continued presence of cholinesterase inhibiting agents downstream from UIKV. However it should be noted that mean *I. bicolor* densities at UIDE during July 2003 were higher than densities at any other site sampled during 2003. This was the result of high densities of small and "very" small organisms at UIDE (see Appendix B).

A similar pattern was observed in July 2003 on the Volga River with the downstream VRSP site having a lower mean activity than VRNB and VRTB. Activity at

CRED was similar to that at CRCF suggesting, once again, that there is no "urban effect" from the Cedar Falls-Waterloo metropolitan area. It must be remembered that, overall, there were no significant differences in activities among the ten sites, so the above observations can only be considered as trends. As was the case during 2002 (see "Intensive Sampling" below), low flow conditions existed, with few major storms, and activities on the Volga and Upper Iowa Rivers were higher than those reported in July 2001 by Andersen (2002).

During September, 2003 sampling there were insufficient organisms at VRNB to conduct an analysis. Similarly, during September 2001 only five insects (one sample) could be collected at that site (Andersen, 2002), but during September 2002 VRNB had the highest activity among the ten sites. Among the other nine sites analyzed there were no significant ($p>0.05$) differences among sites in AChE activity per g tissue (Figure 1.11) or per mg protein (Figure 1.12). In addition, the activities were similar, at most sites, to those found in September 2002, but appeared higher than those found in Andersen's (2002) study during September 2001. However, September,2002 and September 2003 were not compared statistically. In contrast to the July results, VRSP had a higher activity than VRTB, and UICR and UIDE were more similar to UIKV. However, in the latter case the increased similarity among sites on the Upper Iowa River was due to a slight decrease in activity at UIKV in combination with slightly increased (UICR) or similar (UIDE) activities at the downstream sites relative to July. Activities at VRSP in September, 2002 and 2003 were similar and relatively higher than most other sites. In

addition, they were approximately twice the rate of activity measured at VRSP by Andersen (2002) in September 2001.

Analyses of AChE activities within a site over time (Appendix F) in most cases did not result in significant differences over time (an exception is VRSP). However, as was the case in 2002, AChE activities were typically higher in September compared to July and in particular, May.

May Hess sampling resulted in few nymphs collected, especially large nymphs. This may indicate the post-emergent period of the winter generation. It is interesting to note that all sites were sampled at the end of May and few large insects were present. In 2002, the Volga and Upper Iowa sites were sampled in the middle of May and relatively high numbers of large nymphs were present. This further suggests that a large proportion of *I. bicolor* may emerge in mid to late May in Northeast Iowa. In July 2003 *I. bicolor* were relatively evenly distributed across the size classes. In September, again, distribution was relatively even across size classes, however total numbers tended to be higher in July than at most sites.

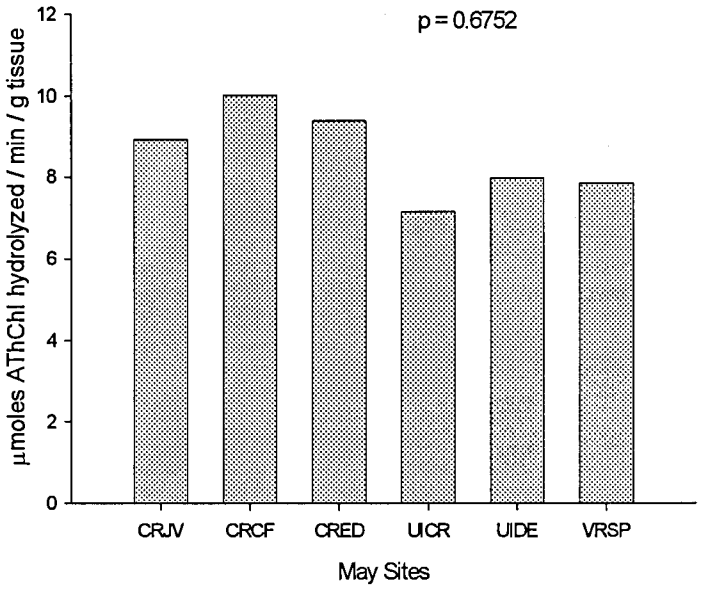

Figure 1.7. *Isonychia bicolor* AChE activities (per g tissue) during May 2003. P value is from a one-way ANOVA.

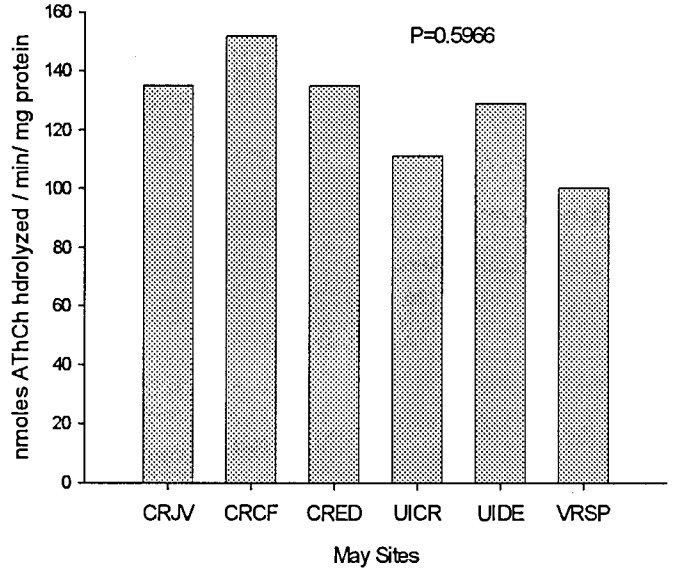

Figure 1.8 *Isonychia bicolor* AChE activities (per mg protein) during May 2003. P value is from a one-way ANOVA among sites.

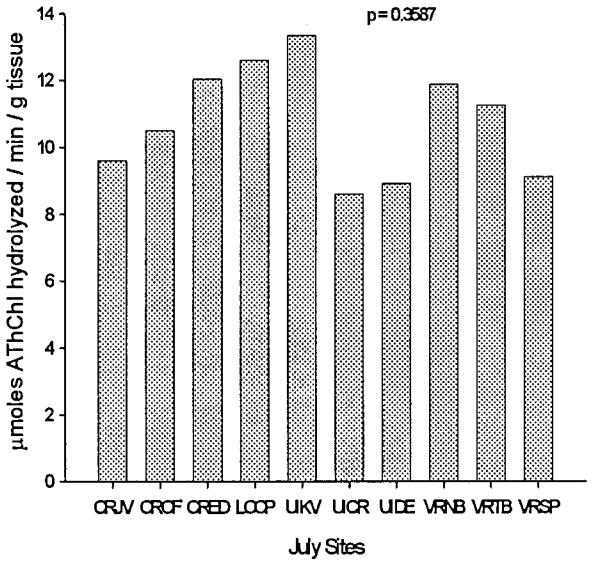

Figure 1.9 *Isonychia bicolor* AChE activities (per g tissue) during July 2003. P value is from a one-way ANOVA among sites.

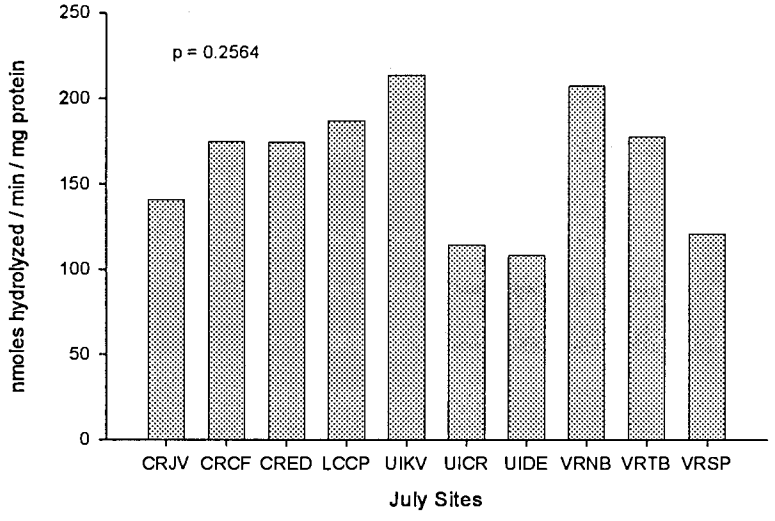

Figure 1.10 *Isonychia bicolor* AChE activities (per mg protein) during July 2003. P value is from a one-way ANOVA among sites.

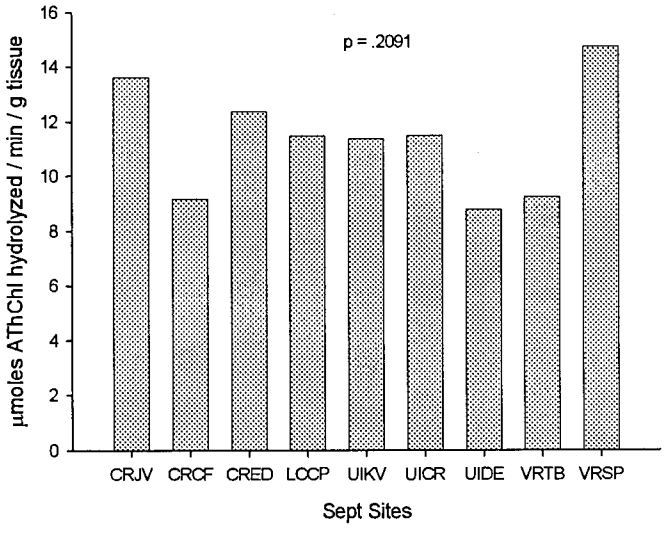

Figure 1.11 *Isonychia bicolor* AChE activities (per g tissue) during September 2003. P value is from a one-way ANOVA among sites.

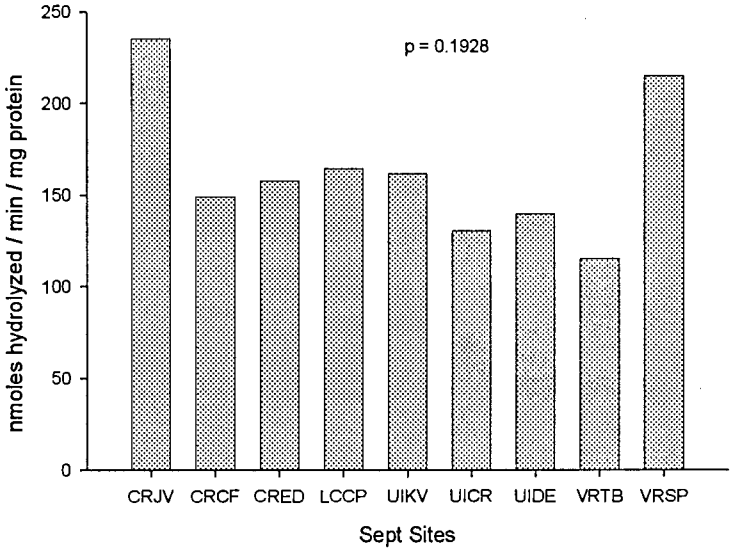

Figure 1.12 *Isonychia bicolor* AChE activities (per mg protein) during September 2003. P value is from a one-way ANOVA among sites.

Volga River Intensive Sampling, 2002

Three sites on the Volga River (from upstream: North Branch, Twin Bridges and State Park) were sampled weekly from May 14 through July 10, 2002. Standard physical/chemical water quality measurements from each sampling trip are in Appendix C. On several sampling trips there were insufficient organisms to conduct AChE analyses at one or more sites, and those dates are indicated in the figure captions for each site.

At VRNB, AChE activities (per g tissue) were not significantly ($p>0.05$) different among June sampling dates and were below "peak" rate (Figure 1.13 and 1.14). However, July AChE activities per g tissue and per g protein were significantly ($p\leq0.05$) higher than the June dates and near "peak". No significant ($p>0.05$) differences in AChE activities per g tissue or per mg protein were found at VRTB during the intensive study (Figure 1.15 and 1.16). At VRSP there were no significant differences among dates in AChE activities (per g tissue) during the period of intensive sampling and activities were well below "peak" activity rate (Figure 1.17). However, AChE activities (per mg protein) at VRSP were significantly ($p \leq0.05$) different during 2002 intensive sampling. AChE activities (per mg of protein) peaked on 6/3/02 then decreased following a major flood event (see Appendix D) which occurred between 6/3/02 and 6/5/02. AChE activities subsequently peaked again on 7/10/02.

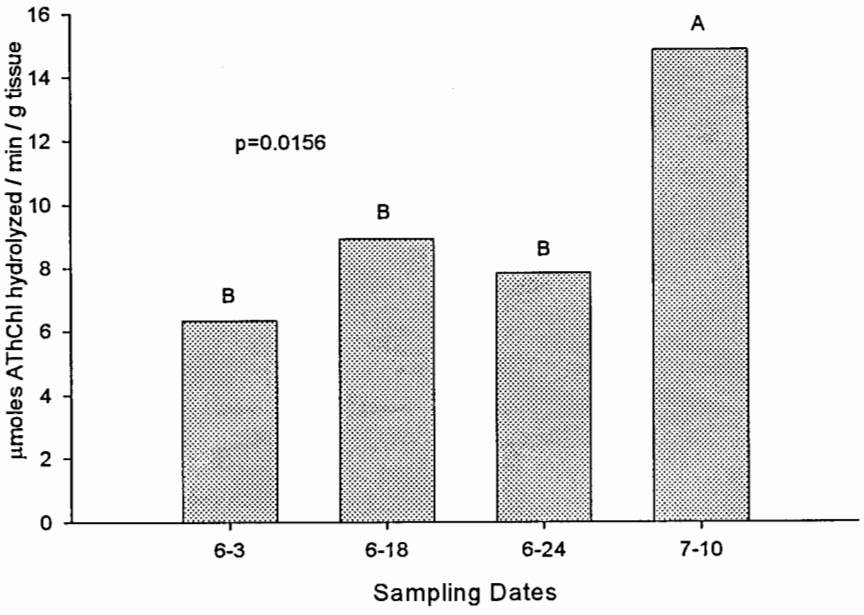

Figure 1.13 *Isonychia bicolor* AChE activities (per g tissue) at VRNB during 2002 intensive sampling. There were insufficient organisms on 5/14, 5/20, 5/28, 6/11 and 6/18. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different (p>0.05) based on Duncan's Multiple Range Test.

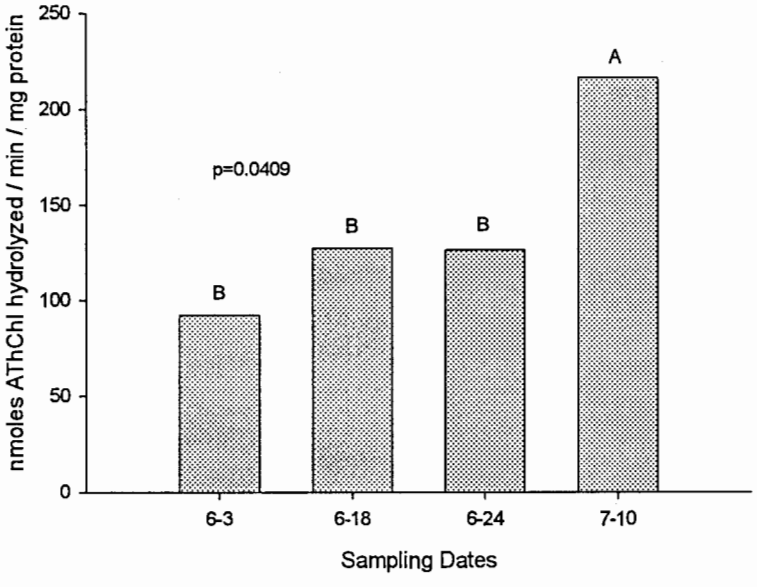

Figure 1.14 *Isonychia bicolor* AChE activities (per mg protein) at VRNB during 2002 intensive sampling. There were insufficient organisms on 5/14, 5/20, 5/28, 6/11 and 6/18. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different (p>0.05) based on Duncan's Multiple Range Test.

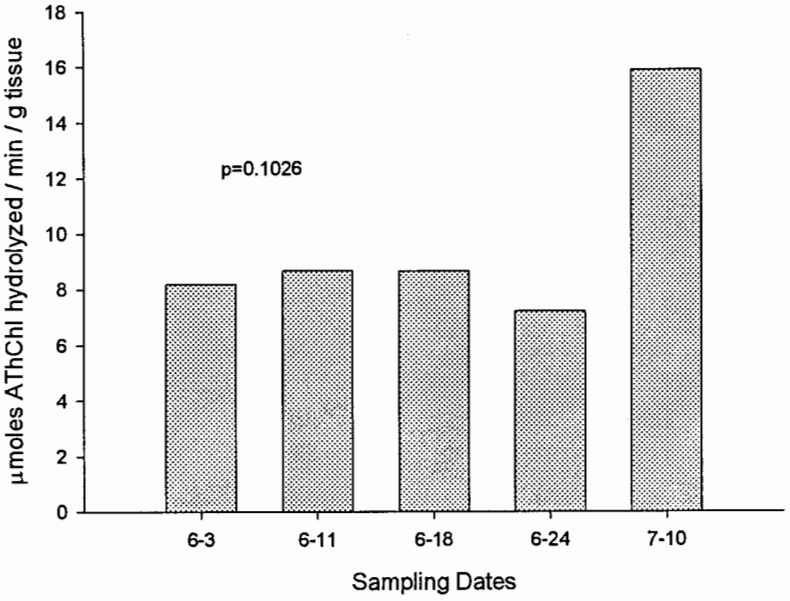

Figure 1.15 *Isonychia bicolor* AChE activities (per g tissue) at VRTB during 2002 intensive sampling. There were insufficient organisms on 5/20 and 5/28. P value is from a one-way ANOVA among dates.

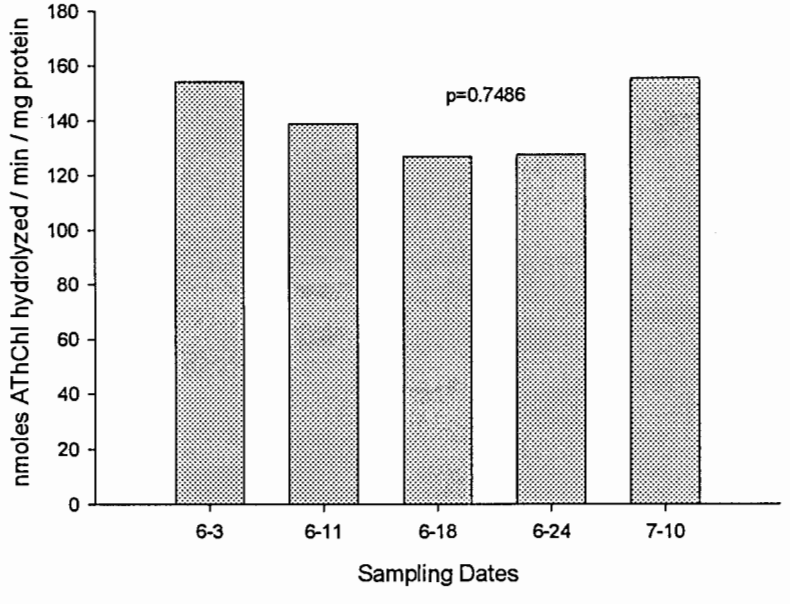

Figure 1.16 *Isonychia bicolor* AChE activities (per mg protein) at VRTB during 2002 intensive sampling. There were insufficient organisms on 5/20 and 5/28. P value is from a one-way ANOVA among dates.

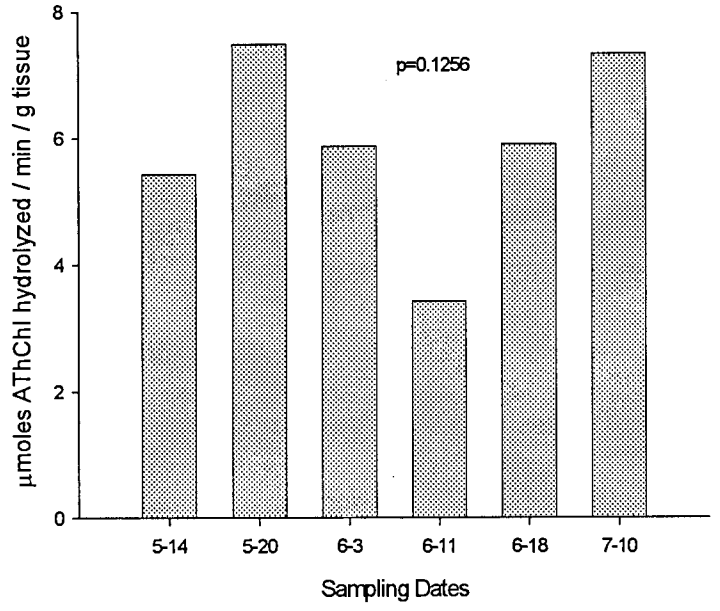

Figure 1.17 *Isonychia bicolor* AChE activities (per g tissue) at VRSP during 2002 intensive sampling. There were insufficient organisms on 5/28 and 6/24. P value is from a one-way ANOVA among dates.

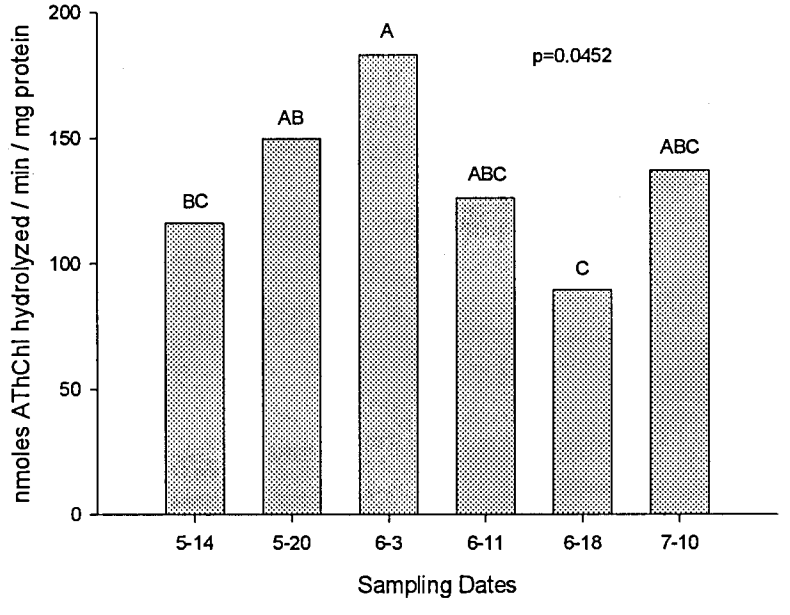

Figure 1.18 *Isonychia bicolor* AChE activities (per mg protein) at VRSP during 2002 intensive sampling. There were insufficient organisms on 5/28 and 6/24. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

Volga and Upper Iowa Rivers Intensive Sampling, 2003

Three sites on the Volga River (from upstream: VRNB, VRTB and VRSP) and three sites on the Upper Iowa River (from upstream: UIKV, UICR and UIDE) were visited weekly from May 21, 2003 through July 14, 2003. High water during the second week in May prevented sampling at all locations. The three sites on the Volga River were also sampled on April 23, 2003. Standard physical/chemical water quality measurements are provided in Appendix C. However, on May 21, 2003 water chemistry was not measured at UICR and UIDE because high water prevented sampling, and at VRNB and VRTB because of a lack of organisms to sample. On several other sampling trips there were insufficient organisms to conduct AChE analyses at one or more sites. These dates are noted in their respective figure captions.

No significant ($p>0.05$) differences in AChE activities were found at VRNB (Figures 1.19 and 1.20) or at VRTB (Figures 1.21 and 1.22). However, activity rates were generally higher than those from the same sampling periods in 2002. At VRSP there were significant ($p \leq 0.05$) differences among dates in AChE activities per g tissue (Figure 1.23) and per mg protein (Figure 1.24) during the period of intensive sampling. Early samples at VRSP had low AChE activities. Although activities at VRSP increased significantly ($p \leq 0.05$) over time, they never reached "peak" levels, and were lower than those upstream at VRNB and VRTB. It is interesting to note that the significant ($p \leq 0.05$) decrease in AChE activities between July 2 and July 14 at VRSP in AChE activity per mg protein was not as marked at the upstream Volga River sites. This drop in AChE

activity may have been caused by rainfall that caused discharge in the Volga River (downstream from VRSP at Littleport) to rise from ~300 cfs on July 7 to ~ 1600 cfs on July 8, preventing sampling on July 9 at all sites (see Appendix D). During periods of heavy rain (measured as increased discharge) field runoff may carry increased loads of insecticides into streams, inhibiting AChE activities.

Activities did not change significantly ($p>0.05$) at UIKV throughout the duration of the 2003 intensive study (Figures 1.25 and 1.26). Heavy rainfall between July 2 and July 9 sampling dates caused discharge to increase from ~ 200 cfs on July 7 to ~375 cfs on July 9 at Decorah, Iowa. Activity at UICR showed no significant ($p>0.05$) change per g of tissue, but showed a significant ($p\leq0.05$) increase on July 2, followed by a significant ($p \leq0.05$) decrease on July 14 in activity per mg protein (Figures 1.27 and 1.28). This drop in AChE activity once again coincided with the July 7-8 rainfall event that may have affected AChE activities at VRSP on the Volga River (see above). Similarly, activities per g tissue at UIDE increased significantly ($p\leq0.05$) between June 27 and July 2 and then dropped significantly ($p\leq0.05$) by July 14 (Figure 1.29). Activities per mg protein fluctuated significantly ($p\leq0.05$) throughout the study at UIDE with a dramatic increase between June 27 and July 2 followed by a significant ($p\leq0.05$) decrease on July 14 following the rainfall event (Figure 1.30).

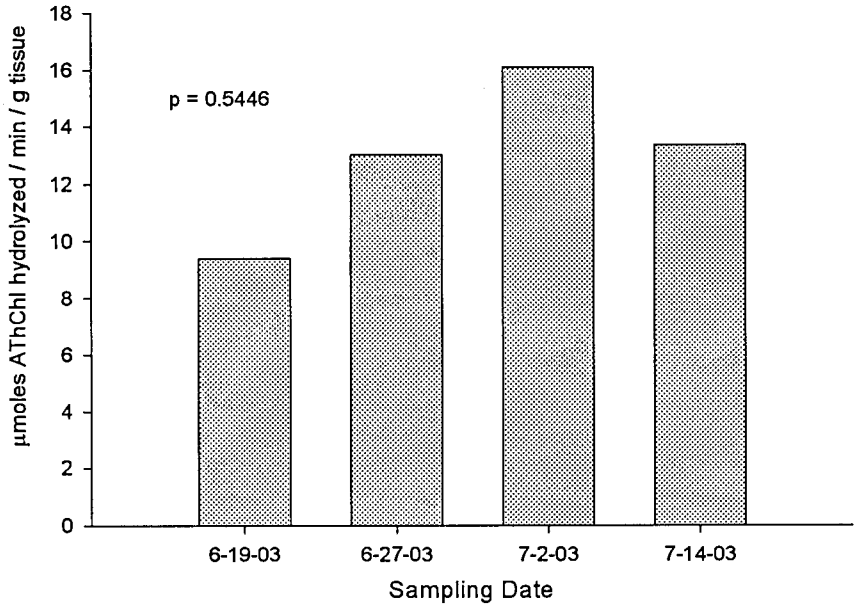

Figure 1.19 *Isonychia bicolor* AChE activities (per g tissue) at VRNB during 2003 intensive sampling. There were insufficient organisms on 5/21, 5/29, 6/6 and 6/13. P value is from a one-way ANOVA among dates.

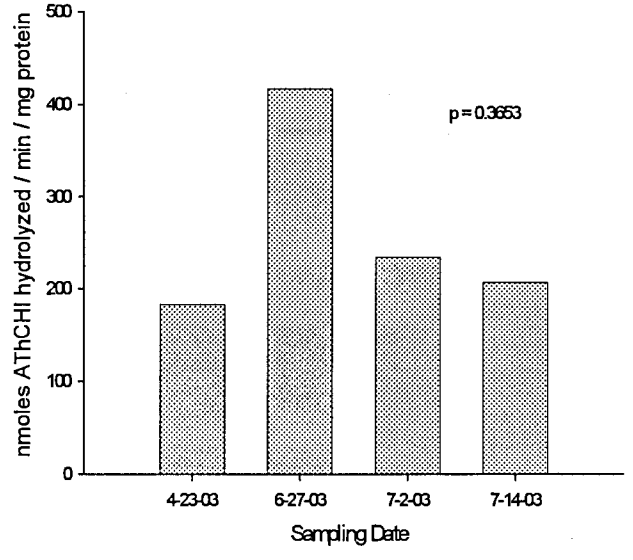

Figure 1.20 *Isonychia bicolor* AChE activities (per mg protein) at VRNB during 2003 intensive sampling. There were insufficient organisms on 5/21, 5/29, 6/6 and 6/13. P value is from a one-way ANOVA among dates.

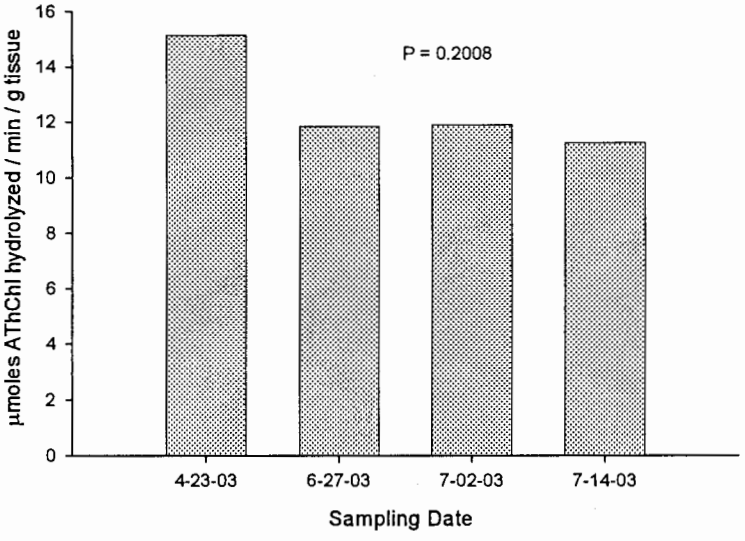

Figure 1.21 *Isonychia bicolor* AChE activities (per g tissue) at VRTB during 2003 intensive sampling. There were insufficient organisms on 5/21, 5/29, 6/6, 6/13 and 6/19. P value is from a one-way ANOVA among dates.

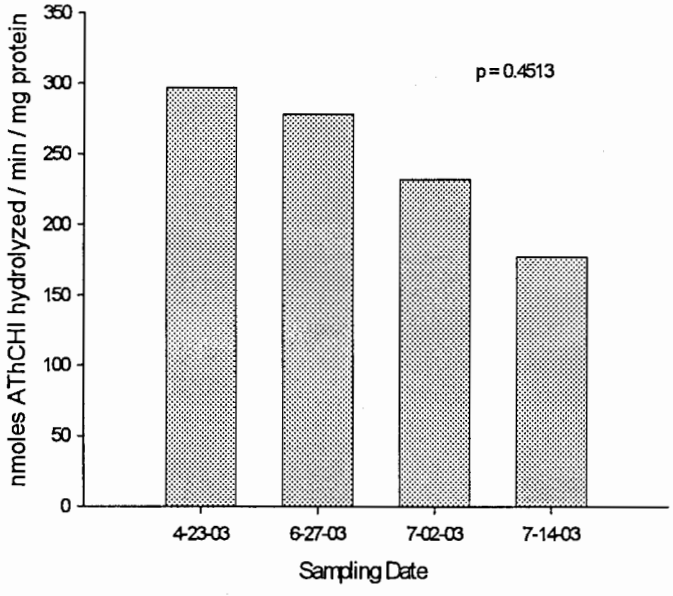

Figure 1.22 *Isonychia bicolor* AChE activities (per mg protein) at VRTB during 2003 intensive sampling. There were insufficient organisms on 5/21, 5/29, 6/6, 6/13 and 6/19. P value is from a one-way ANOVA among dates.

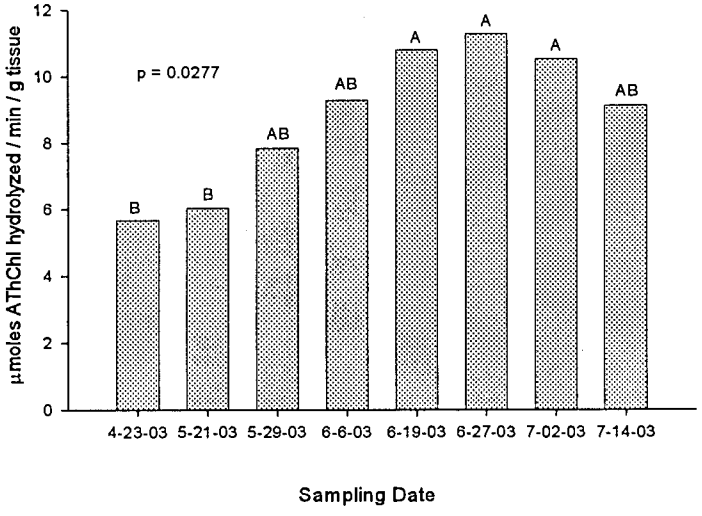

Figure 1.23 *Isonychia bicolor* AChE activities (per g tissue) at VRSP during 2003 intensive sampling. There were insufficient organisms present on 6/13. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

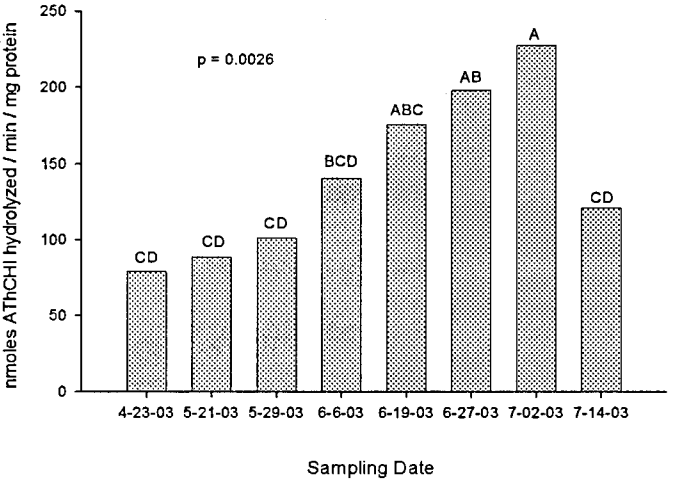

Figure 1.24 *Isonychia bicolor* AChE activities (per mg protein) at VRSP during 2003 intensive sampling. There were insufficient organisms present on 6/13. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

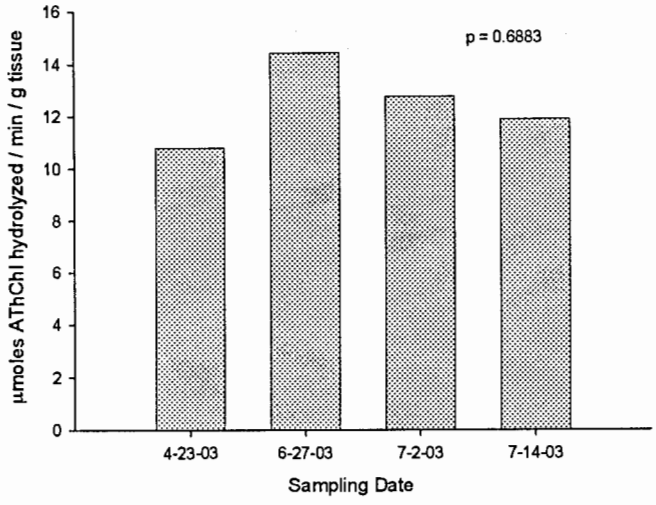

Figure 1.25 *Isonychia bicolor* AChE activities (per g tissue) at UIKV during 2003 intensive sampling. 4/23, 5/21, 5/29, 6/6 and 6/13. P value is from a one-way ANOVA among dates.

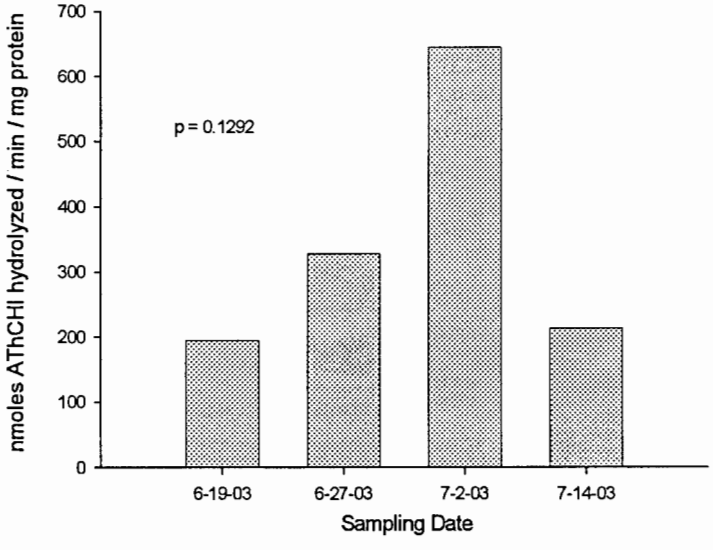

Figure 1.26 *Isonychia bicolor* AChE activities (per mg protein) at UIKV during 2003 intensive sampling. There were insufficient organisms present on 4/23, 5/21, 5/29, 6/6 and 6/13. P value is from a one-way ANOVA among dates.

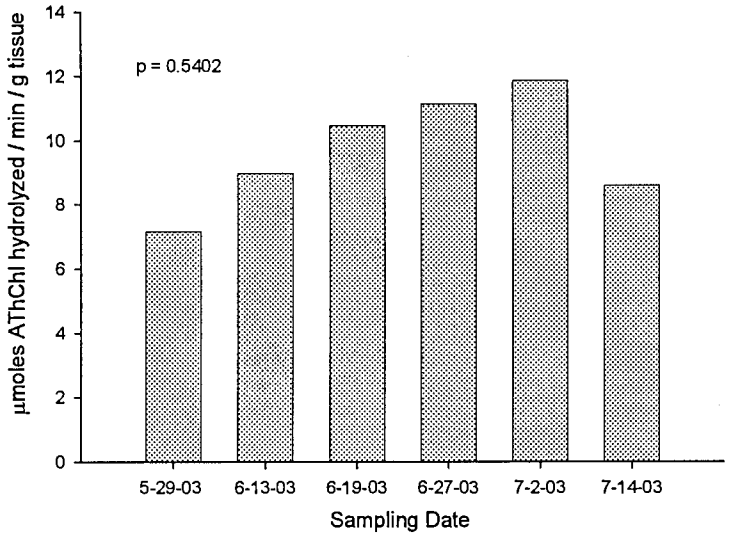

Figure 1.27 *Isonychia bicolor* AChE activities (per g tissue) at UICR during 2003 intensive sampling. There were insufficient organisms present on 4/23, 5/21, and 6/6. P value is from a one-way ANOVA among dates.

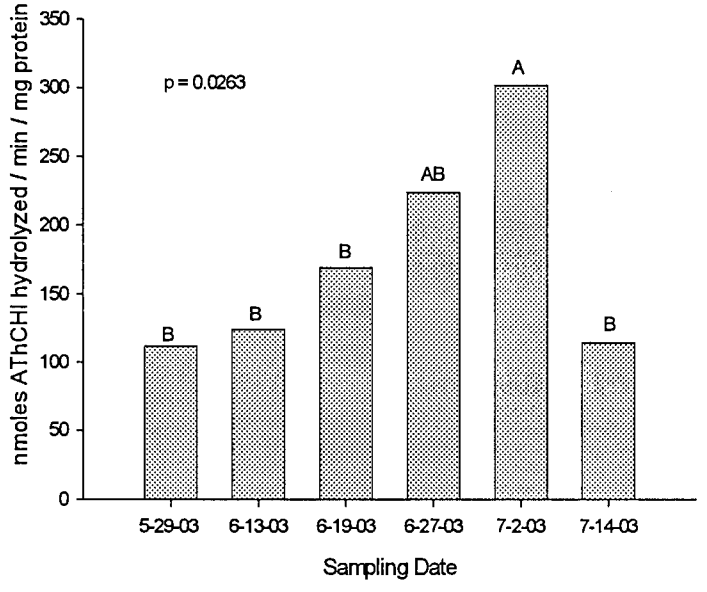

Figure 1.28 *Isonychia bicolor* AChE activities (per mg protein) at UICR during 2003 intensive sampling. There were insufficient organisms present on 4/23, 5/21, and 6/6. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different ($p>0.05$) based on Duncan's Multiple Range Test.

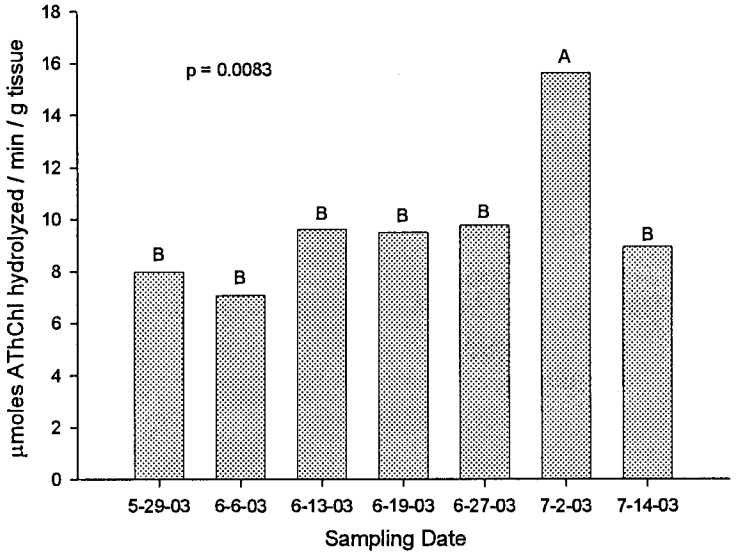

Figure 1.29 *Isonychia bicolor* AChE activities (per g tissue) at UIDE during 2003 intensive sampling. There were insufficient organisms present on 4/23 and 5/21. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different (p>0.05) based on Duncan's Multiple Range Test.

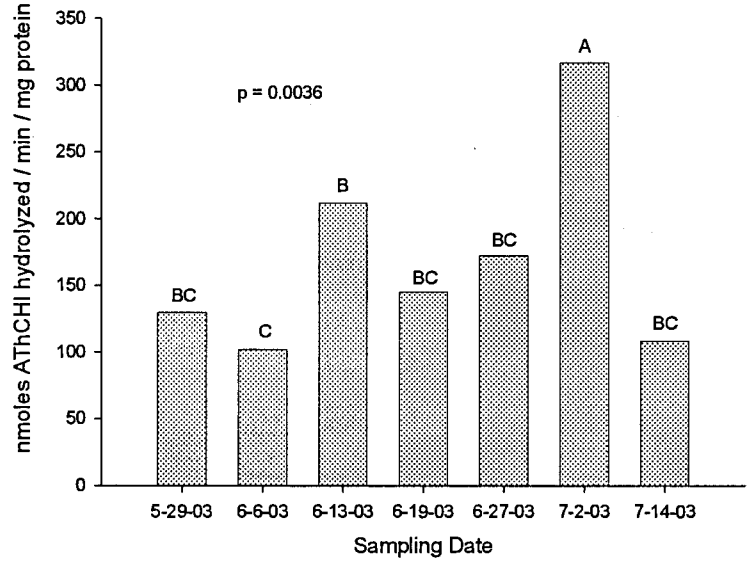

Figure 1.30 *Isonychia bicolor* AChE activities (per mg protein) at UIDE during 2003 intensive sampling. There were insufficient organisms present on 4/23 and 5/21. P value is from a one-way ANOVA among dates. Bars with the same letter are not significantly different (p>0.05) based on Duncan's Multiple Range Test.

Cedar River Sampling, 2002

Results from the AChE activity analyses indicated that there were no significant ($p>0.05$) differences among the seven monthly sampling dates (Figures 1.31 and 1.32), and that AChE activities never reached "peak" summer levels during 2002. In addition, the December, 2001 AChE activity appeared higher than previous winter levels (Andersen, 2002), however these data were not statistically analyzed. Based on water temperature data from this and previous years, it appears that the relatively warmer (by 2°C) water temperature in December 2001 in combination with relatively cooler (by 4°C) summer water temperatures may have resulted in the lack of significant differences in AChE activities among dates. However, the extremely low flow conditions throughout spring and summer 2002 may have also been a factor. Results of the standard water chemistry analyses conducted on each sampling trip are provided in Appendix C.

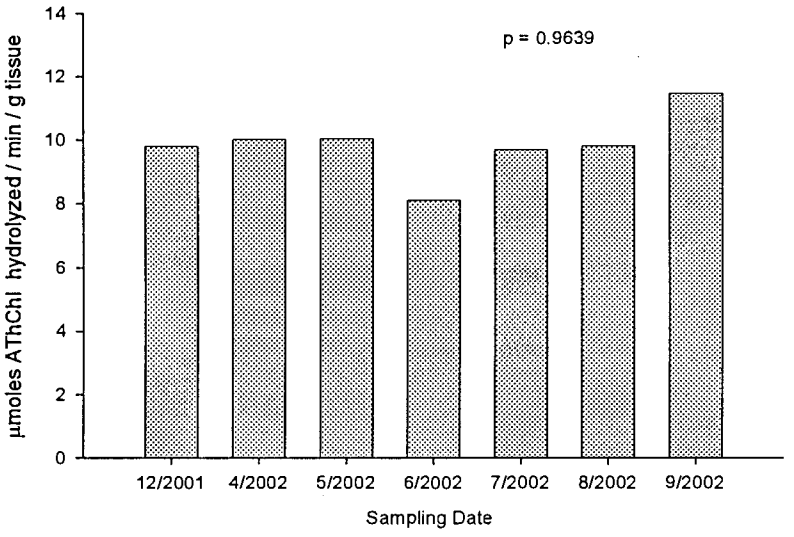

Figure 1.31 *Isonychia bicolor* AChE activities (per gram of tissue) in the Cedar River at Cedar Falls during sampling December 2001- September 2002. P value is from a one-way ANOVA among dates.

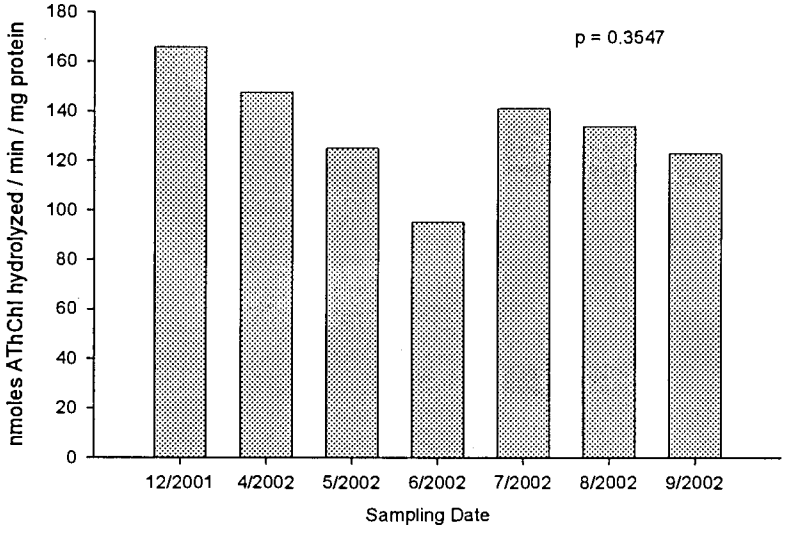

Figure 1.32 *Isonychia bicolor* AChE activities (per mg of protein) in the Cedar River at Cedar Falls during sampling December 2001- September 2002. P value is from a one-way ANOVA among dates.

Cedar River Sampling, 2003:

Results from the AChE activity analyses indicated that, once again, there were no significant ($p>0.05$) differences among the six Cedar River sampling dates (Figures 1.33 and 1.34), and that AChE activities never reached "peak" summer levels during 2003. However, the mean AChE activity in August 2003 was the highest value obtained, and the water temperature was also the highest during that month. As was the case during 2002, the water temperature in July, 2003 was relatively cooler (by 4°C) than that in Andersen's (2002) study during 2001. In addition, the extremely low flow conditions throughout spring and summer, 2002 continued through summer 2003. These or other unknown variables may have caused the lack of significant differences in AChE activities during spring and summer, 2003, and the failure to attain what had been considered "peak" (~17 umoles ATCI hydrolyzed/min/g tissue) summer levels. Results of the standard water chemistry analyses conducted on each sampling trip are provided in Appendix C.

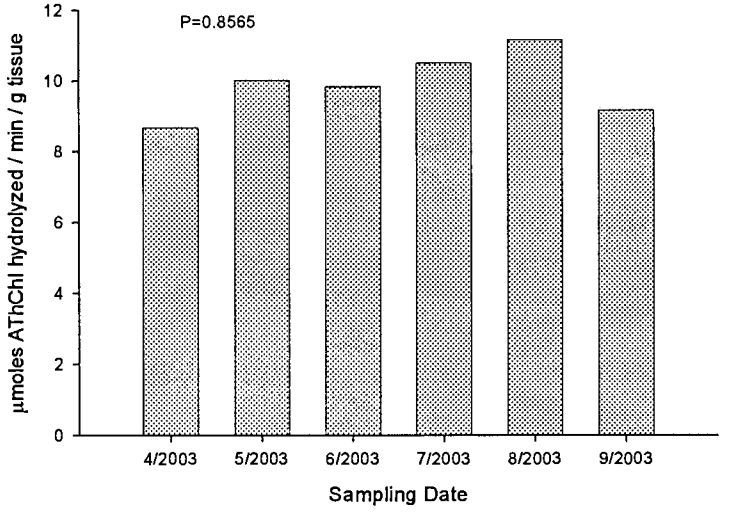

Figure 1.33 *Isonychia bicolor* AChE activities (per gram of tissue) in the Cedar River at Cedar Falls during sampling April - September 2003. P value is from a one-way ANOVA among dates.

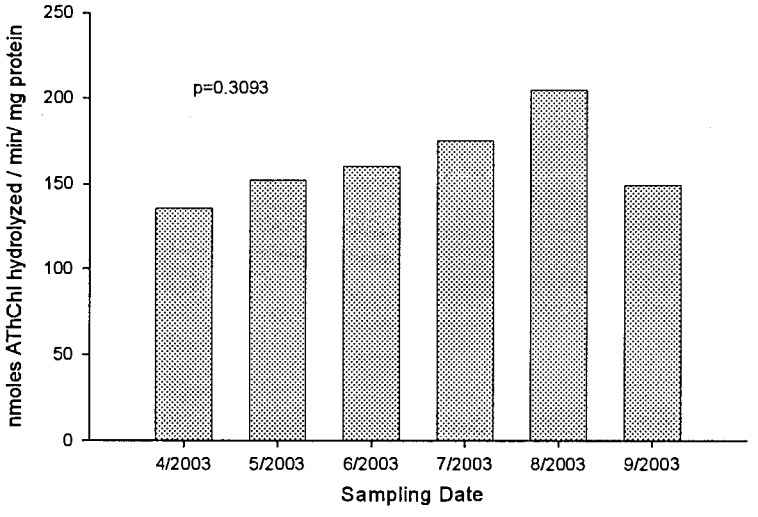

Figure 1.34 *Isonychia bicolor* AChE activities (per mg of protein) in the Cedar River at Cedar Falls during sampling April - September 2003. P value is from a one-way ANOVA among dates.

Conclusions

"Ten Sites" sampling in 2002 indicated few discernable trends. Likewise 2003 sampling yielded no significant ($p>0.05$) differences among sites on most sampling dates. However, apparent decreases in AChE activities from upstream to downstream sites occurred on the Upper Iowa in May, 2002 and July, 2003 as well as on the Volga in September, 2002 and June, 2003. It is possible that OP and carbamate pesticide concentrations are higher at downstream sites due to increased exposure to runoff.

Intensive sampling on the Volga River in 2002 and the Volga and Upper Iowa Rivers in 2003 indicated significant ($p\leq0.05$) decreases in AChE activities which coincided with large rainfall events. Such events may have washed insecticides into these streams causing a decrease in AChE activities. However, no chemical analyses were performed at the time so causes of decreased activity are only speculation. The Cedar River had relatively stable AChE activities with no significant ($p>0.05$) differences in either 2002 or 2003.

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CHAPTER 2

ISONYCHIA BICOLOR (EPHEMEROPTERA: ISONYCHIIDAE) ACETYL-CHOLINESTERASE ACTIVITY: INVESTIGATING THE EFFECTS OF PHOTOPERIOD AND LARVAL BODY SIZE ON BASELINE ACTIVITY

Abstract

Previous field sampling suggested that *Isonychia bicolor* (Ephemeroptera: Isonychiidae) head capsule acetylcholinesterase (AChE) activities may naturally fluctuate throughout the year. In order to use AChE activity from field collected insects as a biomarker of organophosphorus insecticide exposure, such baseline fluctuations must be understood. These studies investigated the effects of larval body size and photoperiod on *I. bicolor*. During June and August, 2002, *I. bicolor* were sampled from the Cedar River, Cedar Falls, Iowa, USA. Insects were divided into three size classes: small, medium, and large, and analyzed for AChE activity. No significant ($p>0.05$) differences were found among size classes in either June or August.

During July, 2002, 540 *I. bicolor* were sampled from the Cedar River in Cedar Falls. 60 insects were transferred to each of 9 stream microcosms and acclimated to one of three photoperiod regimes (18:6, 12:12, and 6:18 L:D). AChE activity was monitored weekly for three weeks. No significant ($p>0.05$) differences were found, suggesting photoperiod is not an important factor affecting AChE activity in *I. bicolor*.

Results of these studies suggest that any size *I. bicolor* larvae can be used for AChE analysis, and that photoperiod is not an important factor influencing AChE activity in future microcosm or field studies with *I. bicolor*.

Keywords: baseline acetylcholinesterase activity, stream insects, photoperiod effects, size effects

Introduction

The development of useful bioindicators has been hampered by inability to discern xenobiotic-induced effects from natural variation. Conclusive results are impossible without adequate understanding of natural field fluctuations. Such fluctuations may occur in a regular, periodic manner such as seasonal or daily rhythms or occur irregularly.

Without knowledge of the cause and temporal scale of such variability, little confidence can be placed in the validity of a bioindicator. Callaghan, *et al.* (2002) suggest that "if acetylcholinesterase is to be useful as a real tool to detect low levels of organophosphorus pollution, it should be very specific to pesticide poisoning but relatively insensitive to environmental variation." Understanding natural changes in AChE activity in the absence of cholinesterase inhibitors is necessary to increase confidence in interpretation. This concept has been recognized by many authors (e.g., Day and Scott, 1990; Melancon, 1995; Beauvais, *et al.* 1999), but few studies have attempted to pinpoint sources of natural variation.

Moulton, *et al.* (1996) and Callaghan, *et al.* (2002) studied naturally occurring factors that may affect AChE activity in aquatic organisms. Of these, the most often studied variable was temperature. Temperature affects larval growth by influencing rates of feeding, assimilation, and respiration; food conversion efficiencies; enzymatic kinetics and endocrine processes; or indirectly by altering the quantity and quality of available food material (Sweeney 1984). Moulton, *et al.* (1996) hypothesized that increased temperature can affect AChE activity in 3 ways: (1) endogenous levels increase with temperature;

(2) increased metabolism may affect pesticide toxicity; and (3) rate of reactivation of inhibited cholinesterase may be higher.

Moulton, *et al.* (1996) exposed the freshwater mussel *Elliptio complanata* and the asiatic clam *Corbicula fluminea* to aldicarb (a carbamate insecticide) and acephate (an organophosphate insectide), assaying cholinesterase of the adductor muscle in *E. complanata* and whole bodies of *C. fluminea*. In two experiments they exposed *E. complanata* for 96 hours to 5 mg/L aldicarb and 5 mg/L acephate while maintaining a control for each. Treatments were exposed to four different temperatures (21°C, 24°C, 27°C and 30°C). Analysis revealed no significant ($p>0.05$) differences among temperature treatments or in controls.

The midge *Chironomus riparius* is a commonly reared toxicity test organism used in several laboratory and field-based studies of AChE activity (Detra and Collins 1986; Sturm and Hansen 1999; Beauvais *et al.*, 1999; Fisher, *et al.*, 2000; Callaghan, *et al.*, 2001; Olsen, *et al.*, 2001; Callaghan, *et al.*, 2002;). Callaghan, *et al.* (2002) looked at *C. riparius* AChE activities after exposure to the OP insecticide pirimiphos at different temperature, dose and time regimes. They exposed *C. riparius* to 0, 0.1, 1.0, and 10μg/L pirimiphos at 3, 12, and 22°C for 48, 72 or 96 h and found the dose response curve to be similar at all temperatures. Natural fluctuations in temperature occur rapidly during the diel cycle as well as more slowly in the yearly cycle. Unless laboratory based experiments attempt to duplicate these patterns, they may not adequately illustrate enzyme activity fluctuations.

Beauvais, *et al.* (1999) studied the effects of hypoxia on *C. riparius* AChE activity. They found no hypoxia effects on larval *C. riparius* AChE activity. Hypoxia is a condition prevalent in wetlands but seldom found in healthy streams. Hypoxia would likely kill most sensitive stream insects before AChE inhibition occurred. Midges of the genus *Chironomus* have oxygen binding compounds in their hemolymph that allows them to survive in low oxygen conditions. Such conditions are uncommon in "healthy" streams. However, the importance of this study is that it eliminated hypoxia as a complicating factor in future AChE biomarker studies using *C. riparius.*

Another complicating factor affecting AChE activity is the potential for natural cholinesterase inhibiting compounds. Monserrat, *et al.* (2001) discuss the cyanobacteria *Anabaena spiroides,* which is known to produce a number of cholinesterase inhibiting compounds. In eutrophic conditions cyanobacteria blooms may be common. These cyanobactera can produce neurotoxins including the acetylcholine analog anatoxin-a, sodium channel blockers saxitoxin and neosaxitoxin and the acetylcholinesterase inhibitor anatoxin-a[s]. Other unknown natural AChE inhibitors may be present in streams, further complicating the understanding of "normal" cholinesterase levels.

Previous work using *Isonychia bicolor* acetylcholinesterase (AChE) activity as a biomarker has shown periodic patterns of AChE activity fluctuation. Monthly sampling of the Cedar River, in Cedar Falls has shown a trend of lower AChE activity during winter months and peak activity during the summer months (Andersen, 2002). This seasonal effect may be due to one or a number of natural or unnatural variables. Also, previous

research has shown greatly differing AChE activity among sites within a sampling date indicating substantial differences within a relatively small geographic area (Andersen, 2002).

Andersen (2002) utilized field-collected *I. bicolor* in artificial streams to look at effects of different temperature and nutrition treatments on AChE activity. In the temperature study 60 *I. bicolor* were added to each of fifteen artificial streams and acclimated to one of four constant temperature treatments (5, 20, 19, and 25°C) and one fluctuating temperature treatment (15-22°C) that simulated diel temperature changes in the source riffle. Each treatment was replicated in triplicate. Ten days after the acclimation temperature was reached, five randomly selected *I. bicolor* were removed from each stream and processed in the manner described below (see Materials and Methods) for examination of AChE activity. Andersen (2002) found that the 19 and 25°C treatments were significantly ($p \leq 0.05$) higher than the other treatments, nearing 25 µmol acetylthiocholine iodide (AThChI) hydrolyzed/min/g tissue, while the 10 and 5°C treatments were lower, near 15 and 12 µmol AThChI hydrolyzed/min/g tissue respectively. The 10-25°C treatment was lowest nearing 10 µmol AThChI hydrolyzed/min/g tissue. This illustrates the importance of temperature effects on AChE activity. However, these results did not account for protein content of the samples. Higher temperatures may have caused fat deposits to be reduced, causing AChE activity to appear higher than in other treatments.

Andersen (2002) also used field collected *I. bicolor* in artificial streams to examine the effects of four nutrition regimes on AChE activity. The treatments consisted of: (1) a natural periphyton (colonized on foam cubes in a natural stream, then squeezed into artificial streams); (2) artificial food (ground "trout-chow", yeast and alfalfa); (3) both periphyton and artificial food; and (4) no nutrients. Nutrient treatments were added every 72 h. He found that AChE activity was not significantly different ($p>0.05$) among treatments after 20 days. This suggests that nutrition is not an important variable affecting natural fluctuations in streams, and that nutrient additions are unnecessary in stream microcosm studies of *I. bicolor* AChE activity. Callaghan, *et al.* (2002) also found that food amendments had no significant ($p>0.05$) effect on *C. riparius*.

Many papers investigating organophosphorus and carbamate insecticide exposure to different larval instars exist (e.g., Koziol and Witkowski, 1981; Balasubramanian and Balasubramanian, 1984; Christie, *et al.* 1991). However, most papers studied mortality or susceptibility of insects at different instars from an agricultural standpoint (concerned with lethal dosages to an organism at a specific point in the life cycle). Few studies explore the AChE activity in non-target organisms. A study by Ibrahim and Ottea (1995) has shown that developmental stages can affect the AChE activity of insects. They found that the AChE activity of the adult tobacco budworm, *Heliothis virescens*, to be 10 to 18 times higher than in late instar larvae. The authors attribute this to the generalization that more active insects have higher AChE activity. This position is supported by Day and Scott (1990) who discuss the observation that insects that are physically active tend to

have higher AChE activity than less active species (e.g., adult houseflies vs. lepidopteran larvae).

The following study attempts to clarify the effects of two variables that may affect AChE activity in *I. bicolor*, nymphal body size and photoperiod. When collecting throughout the year, many different sized *I. bicolor* nymphs are encountered. Occasionally only small or large individuals are available. If differences in AChE activity exist between different nymphal instars, results will be difficult to interpret. Similarly, photoperiod effects (if any) on AChE activity may confound results from nymphs collected at different times of the year.

Materials and Methods

Determination of AChE activity

Preparation and analysis of acetylcholinesterase activity was performed using the methods of Ellman, *et al.* (1961) optimized for *I. bicolor* by Anderson (2002). Head capsules were homogenized in a glass tissue grinder with 0.5% Triton X-100 detergent in Tris pH 7.4 buffer solution. For every mg of tissue, 200 μl of buffer solution was added resulting in a 200:1 dilution factor. Homogenate from each replicate was added to a micro-centrifuge tube and centrifuged (10 min; 14,000 rpm). Supernatant aliquots were separated into two cryovials then frozen (-80°C) until analyzed for AChE activity. Aliquots are separated in order to create a spare that remained frozen in case of sampling error, spillage, etc.

Acetylcholinesterase activity was quantified in a microplate-reading spectrophotometer (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA, USA). Each well contained an aliquot of *I. bicolor* head capsule supernatant (30μl), acetylthiocholine iodide (AThChI) solution (0.0418M; 30μl), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) colorimetric reagent solution (0.442 M; 20 μl), and Tris pH 8.0 buffer solution (170 μl). Acetylthiocholine iodide (AThChI) is similar in structure to acetylcholine and acetylcholinesterase hydrolyzes it in a similar manner to acetylcholine. This hydrolysis results in a positively charged acetate ion and a negatively charged thiocholine complex. The thiocholine complex reacts with DTNB to create 5-thio-2-nitrobenzoate a stable, yellow colored anion that absorbs light most strongly at 412 nm. For each replicate sample, AChE activity was measured in triplicate as the rate of increase in absorbance at 412 nm. Tris buffers, AThChI and DTNB were obtained from Sigma Chemical (St. Louis, MO, USA). Tris buffer solutions were refrigerated at 4°C for no longer than one month and were adjusted to appropriate pH values prior to each analysis. DTNB and AThChI were prepared immediately prior to spectrophotometric analysis.

The optimum reaction temperature (~32°C) was achieved by maintaining DTNB and AThChI at room temperature, placing thawed cryovials of tissue homogenate in ice water and heating Tris pH 8.0 buffer to ~80°C before pipetting. A blank (consisting of 200 μl Tris pH 8.0 buffer solution, 30 μl AThChI solution, 20 μl DTNB solution but no tissue homogenate), and a check standard (consisting of pooled homogenate supernatant from approximately 200 *I. bicolor* head capsules) were also analyzed in triplicate on each

plate. Care was taken to prevent formation of any bubbles during the entire process as bubbles cause inaccurate readings by the spectrophotometer.

Spectrophotometer software (SoftMAX PRO, Molecular Devices, Sunnyvale, CA, USA) was used to determine the highest AChE activity (Vmax). Absorbance measurements for all trials were read in all wells every twelve seconds for five minutes after an initial one-minute lag time. The following formula was used to convert mOD (milli-optical density) output units into international units of enzyme activity:

{[(enzyme mOD/min)-(blank mOD/min)]/1000} x 0.817 x dilution factor =

(μmoles AThChI hydrolyzed/min)/gram tissue

Determination of Total Protein Content

In this study, AChE activity is expressed in nmoles AThChI hydrolyzed/min/mg protein as well as μmoles AThChI hydrolyzed/min/g tissue. Andersen (2002) expressed activity only as μmoles AThChI hydrolyzed/min/g tissue. In order to find the protein content of the AChE tissue homogenate, the Bradford protein assay was optimized for use with *I. bicolor* tissue homogenates. Quantifying AChE activity relative to total protein content potentially minimizes the variation caused by differing amounts of other substances such as chitin or fat in the head capsules. A standard curve using bovine serum albumin was created to quantify the amount of protein in a solution of known concentration. The amount of protein (μg protein/μg tissue) in each sample was calculated using the formula (12((Bradford assay O.D.) - 0.017)/48.775). From this the AChE activity per mg protein is calculated.

The Bradford assay utilized a 96 well microplate reading spectrophotometer. Each sample was analyzed in triplicate using 60 µl Bradford's reagent (Coomassie® Brilliant Blue G-250 dye; Bio Rad, Hercules, California, USA), 5 µl tissue homogenate, and 235 µl twice distilled, reverse osmosis water in each well. Each sample was then mixed by repeated suction/expulsion through a micropipetter to ensure even mixture of the solution. Care was taken to prevent formation of bubbles during this process as bubbles create inaccurate spectrophotometric readings. Each microplate contained one blank (240 µl water and 60 µl Bradford reagent), and one check standard, as well as unknowns (each in triplicate). Microplates were allowed to sit approximately 5 minutes before analysis in the spectrophotometer.

Investigating Possible Differences in AChE Activities Due to Size

On both June 20 and August 7, 2002 50 *I. bicolor* were taken from the Cedar River in Cedar Falls, Iowa, using kick-nets and transferred to each of three coolers (7-L capacity), containing two artificial substrates and filled with river water, for transportation back to the laboratory. At the laboratory, the insects were held in aerated coolers for 24 h and brought to the optimal temperature of 20° C. After acclimation, the organisms in each cooler were divided into three size classes based on their length and head capsule widths During the June study "large" *I. bicolor* nymphs had body lengths and head widths of $\geq$12.5 mm and $\geq$ 1.7 mm, respectively. The "medium" *I. bicolor* nymphs had body lengths and head widths of 7.8-10mm and 1.0-1.5mm, respectively. The "small" *I. bicolor* nymphs had body lengths and head widths of <7.8 mm and <1.2 mm, respectively. During

the August study, the "large" *I. bicolor* nymphs had body lengths and head widths of ≥11.0 mm and ≥ 1.6 mm, respectively. The "medium" *I. bicolor* nymphs had body lengths and head widths of 8.3-<11mm and 1.2-<1.6 mm, respectively. The "small" *I. bicolor* nymphs had body lengths and head widths of <8.3 mm and <1.2 mm, respectively. Size differences between June and August exist because nymphs were generally smaller in June. From each of the three coolers, five randomly selected head capsules from each size class were then homogenized (see above) with the resulting supernatants placed in cryovials and stored in a low temperature (-80° C) freezer for subsequent AChE analysis (see above). The data for each size class from each month were statistically analyzed by a one-way Analysis of Variance (ANOVA).

Photoperiod Effects on AChE Activity

On July 23, 2002 *I. bicolor* (540) from the Cedar River in Cedar Falls were sampled with D-nets and 30 were placed in each of 18 coolers containing two rock-filled artificial substrates for transportation to the Ecosystem Simulation Laboratory at the University of Northern Iowa. The contents of two coolers (60 insects and four substrates) were transferred to each of 9 artificial streams and acclimated for 24 h to a temperature of 20° C. Three photoperiods (18:6, 12:12 and 6:18 L:D) were established in triplicate using automatic timers connected to the daylight equivalent lights above each stream. Each stream was enclosed in a curtain of black plastic sheeting to prevent external light from entering. AChE activities were then monitored weekly for three weeks in an attempt to

quantify any possible photoperiod effects. The weekly AChE activity data were statistically analyzed by a one-way Analysis of Variance (ANOVA).

Results

Size Effects on AChE Activities

Results from the June study indicated no significant ($p>0.05$) differences among the three size classes for AChE activity/g tissue (Figure 2.1) or AChE activity/mg protein (Figure 2.2). Similarly, results from the August study indicated no significant differences among the three size classes for AChE activity/g tissue (Figure 2.3) or AChE activity/mg protein (Figure 2.4).

Photoperiod Effects on AChE Activities

Samples taken one week after the experiment began (7/30/02) indicated no significant differences among the three treatments for AChE activity/g tissue (Figure 2.5) or AChE activity/mg protein (Figure 2.6). Two weeks after the experiment began (8/6/02) once again there were no significant differences among the three treatments for AChE activity/g tissue (Figure 2.7) or AChE activity/mg protein (Figure 2.8). Similarly, three weeks after the experiment began (8/13/02) there were no significant differences among the treatments (Figures 2.9 and 2.10). The experiment could not be continued beyond three weeks because of a lack of *I. bicolor* in the artificial streams.

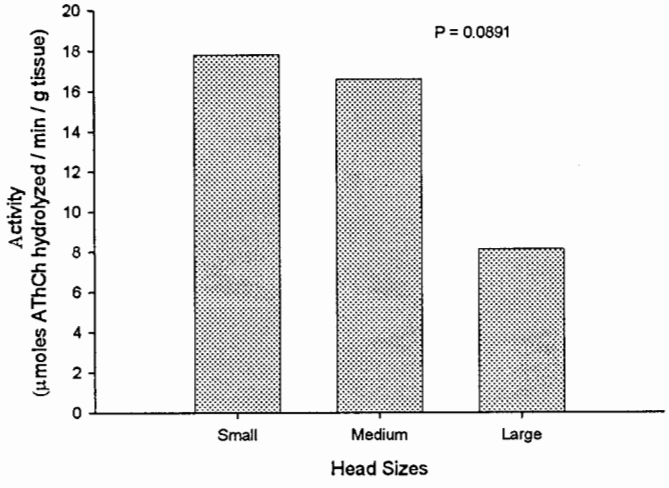

Figure 2.1 *Isonychia bicolor* AChE activities (per gram of tissue) during the June study of possible size effects. P value is from a one-way ANOVA among sizes.

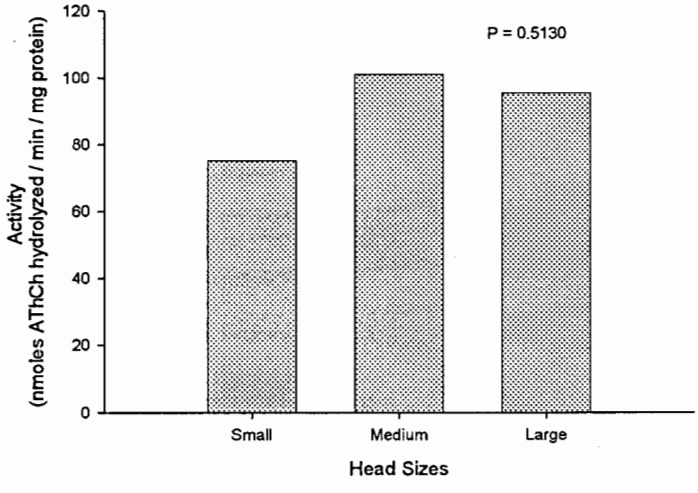

Figure 2.2 *Isonychia bicolor* AChE activities (per mg of protein) during the June study of possible size effects. P value is from a one-way ANOVA among sizes.

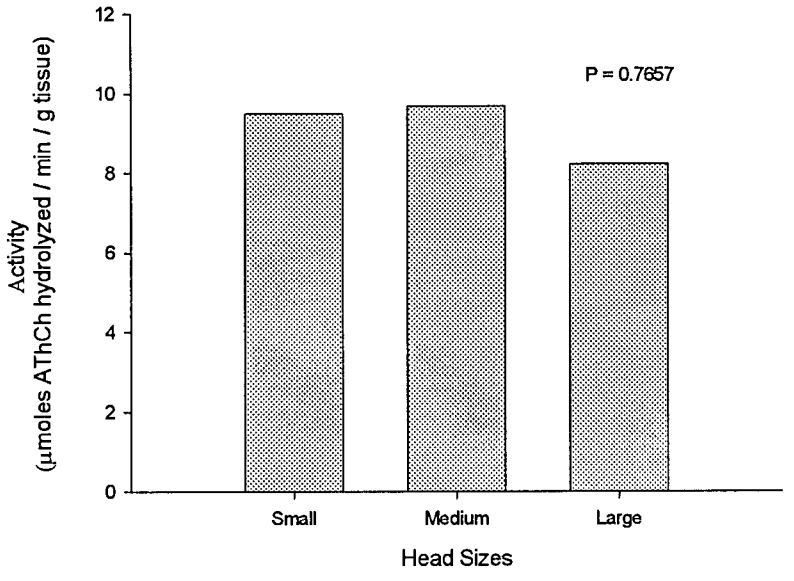

Figure 2.3 *Isonychia bicolor* AChE activities (per gram of tissue) during the August study of possible size effects. P value is from a one-way ANOVA among sizes.

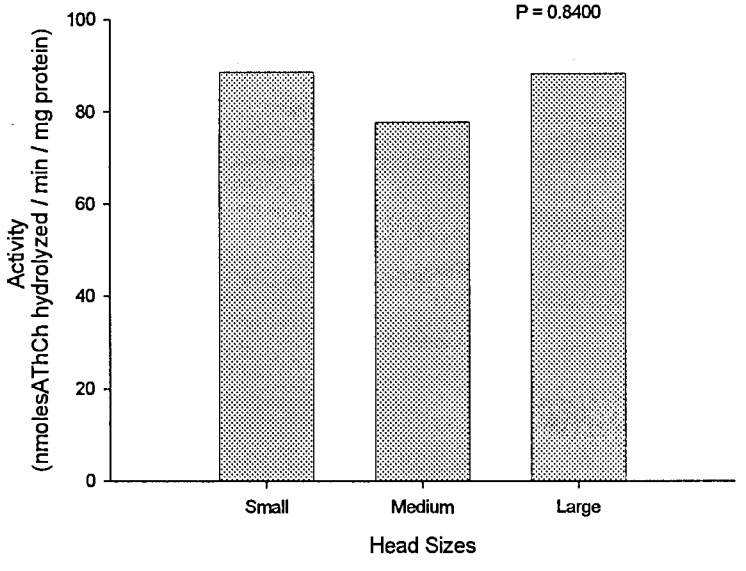

Figure 2.4 *Isonychia bicolor* AChE activities (per mg of protein) during the August study of possible size effects. P value is from a one-way ANOVA among sizes.

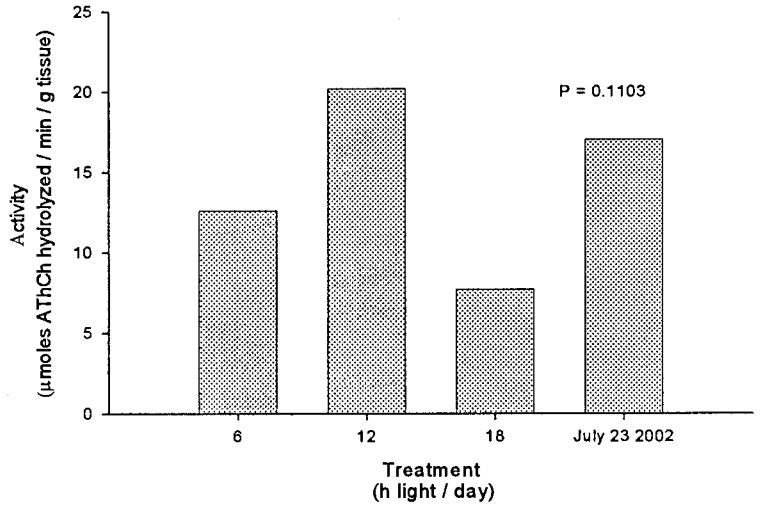

Figure 2.5 *Isonychia bicolor* AChE activities (per gram of tissue) after a one week exposure to three different photoperiods. P value is from a one-way ANOVA among treatments. The July 23 bar represents AChE activity at time of collection from Cedar River and was not included in statistical analysis.

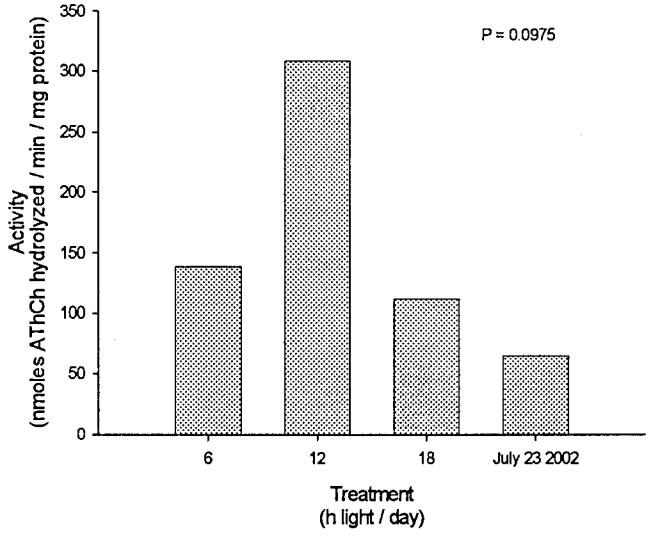

Figure 2.6 *Isonychia bicolor* AChE activities (per mg of protein) after a one week exposure to three different photoperiods. P value is from a one-way ANOVA among treatments. The July 23 bar represents AChE activity at time of collection from Cedar River and was not included in statistical analysis.

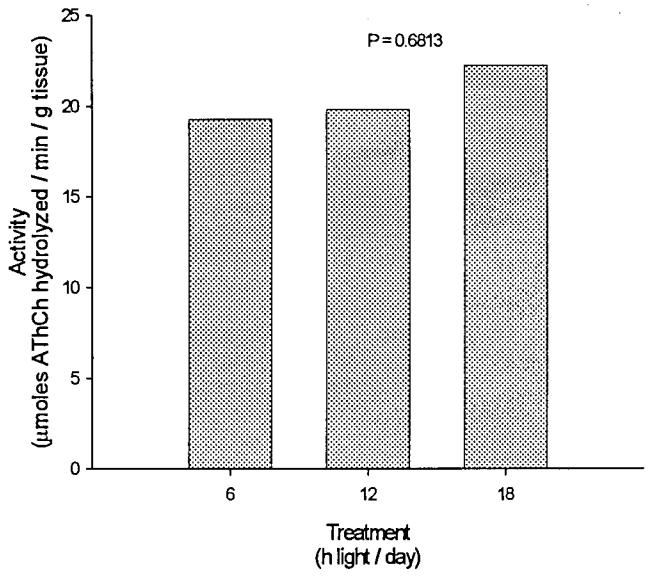

Figure 2.7 *Isonychia bicolor* AChE activities (per gram of tissue) after a two week exposure to three different photoperiods. P value is from a one-way ANOVA among treatments.

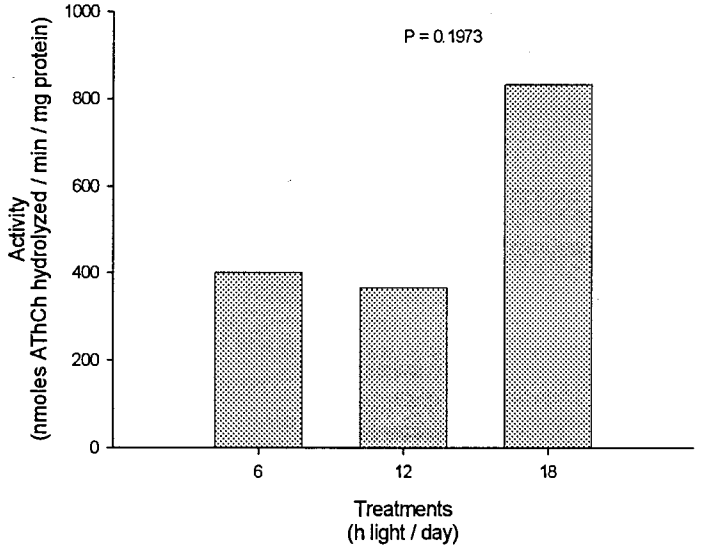

Figure 2.8 *Isonychia bicolor* AChE activities (per mg of protein) after a two week exposure to three different photoperiods. P value is from a one-way ANOVA among treatments.

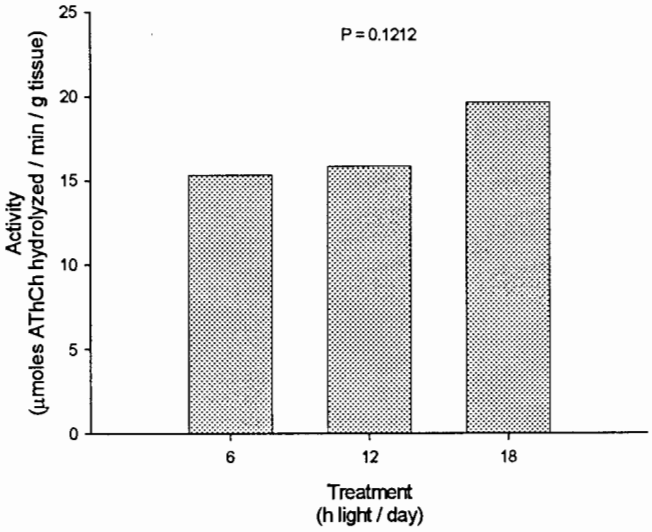

Figure 2.9 *Isonychia bicolor* AChE activities (per gram of tissue) after a three week exposure to three different photoperiods. P value is from a one-way ANOVA among treatments.

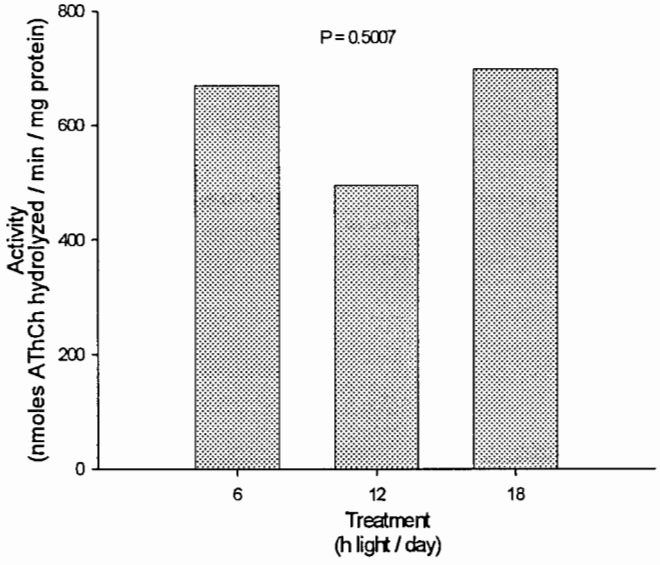

Figure 2.10 *Isonychia bicolor* AChE activities (per mg of protein) after a three week exposure to three different photoperiods. P value is from a one-way ANOVA among treatments.

Discussion

In the future, *Isonychia bicolor* of any size may be used in this assay because no significant differences were found among different sized nymphs. However, when using smaller larvae, limited amounts of homogenate are produced. If smaller insects are to be used, more nymphs may need to be pooled in order to provide enough homogenate to perform the AChE and Bradford assays.

It appears that differences in photoperiod do not affect *I. bicolor* AChE activity. This allows more confidence in future microcosm studies using *I. bicolor* where lab photoperiod does not exactly match field conditions. This photoperiod study does not conclusively show that daylight is not an important natural factor affecting AChE activity patterns. Changes in photoperiod coupled with certain other seasonal cues such as changing temperatures may create significant changes in AChE activity. Such combinations of environmental changes were not part of this study. Also, diel photoperiod rhythms may produce significant changes in AChE activity. In this study *I. bicolor* were sampled at approximately the same time each day; if cyclical, daily changes are present, this study would not reveal them.

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CHAPTER 3

ISONYCHIA BICOLOR (EPHEMEROPTERA: ISONYCHIIDAE) ACETYLCHOLINESTERASE ACTIVITY FOLLOWING EXPOSURE TO TERBUFOS

Abstract

Stream microcosms were used to expose late instar *Isonychia bicolor* (Ephemeroptera: Isonychiidae) to the organophosphorus insecticide terbufos for 24 h at nominal concentrations of 0.0, 2.5, 5.0, 10.0 and 20.0 µg/L and then purged with 200 ml/min of clean water. Streams were sampled prior to dosing, after 24 h exposure, 24 h after purging and 8 d after purging. This study was conducted to correlate terbufos concentrations with *I. bicolor* acetylcholinesterae (AChE) activity inhibition.

Following 24 h exposure, AChE activity per g tissue in the 20 μg/L treatment was significantly different from the control. Following 24 h purging, AChE activities per g tissue and per mg protein were significantly lower in the 20 μg/l treatment. By the eighth day after purging, all organisms in the 20 μg/L treatment were dead, and among the remaining treatments there were no significant differences from the control in AChE activities per g tissue or per mg protein. An analysis of AChE activities within a treatment over time indicated that in the 10 μg/L treatment activity per g tissue was significantly higher ($p \leq 0.05$) on day 9 than on day 2, but the increase was not significant ($p > 0.05$) for activity per mg protein. Overall the data suggest that *I. bicolor* AChE activities can rebound from exposures to 10 μg/L but not 20 μg/L.

This study indicated that terbufos concentrations near 20 µg/L significantly inhibit *I. bicolor* AChE activity and may lead to mortality following 24 h exposure. Also, *I. bicolor* exposed to 10 µg/L or less rebounded to pre-exposure AChE activity levels within 9 days.

Keywords: Stream insect, acetylcholinesterase activity, organophosphorus insecticide, terbufos

Introduction

Organophosphorus (OP) and carbamate insecticides are used extensively worldwide. These pesticides may enter aquatic ecosystems by aerial drift or surface runoff, potentially affecting non-target organisms. These contamination events may be brief and missed by chemical monitoring. Also, chemical analysis may be too expensive for regular sampling.

Organophosphorus insecticides kill by binding to acetylcholinesterase (AChE) thus preventing hydrolysis of the neurotransmitter acetylcholine. The accumulation of acetylcholine in the synaptic cleft causes repeated stimulation of nerves, interfering with normal biological functions. Binding of OP insecticides to AChE may last for many days following initial exposure and is typically irreversible; subsequent increases in AChE activity come from new enzyme synthesis (Habig and DiGiulio,1991; Wilson, *et al.* 1992; Ecobichon, 1996).

The insecticide terbufos (S-[[(1,1-dimethylethyl)thio]methyl] O,O-diethyl phosphorodithioate) is manufactured by American Cyanamid and sold under the trade names AC 92100®, Aragran®, Contraven®, Counter®, and Plydox®. Terbufos rapidly hydrolyzes in water (USEPA, 1999). A number of studies have dealt with terbufos toxicity to freshwater aquatic organisms (Mayer and Ellersieck, 1986; Howe, *et al.* 1994) and numerous fish kills have been reported (USEPA, 1999). Terbufos is considered highly toxic to fish and aquatic invertebrates with a 96-hour LC50 of 3.1 μg/L and 0.2 μg/L in *Daphnia magna* and *Gammarus psuedolimnaeus*, respectively (Mayer and Ellersieck,

1986). Reported 96-hour LC50 values for the technical material (88% active ingredient) are 0.8 to1.3 μg/L in rainbow trout, 390.0 μg/L in fathead minnow and 1.7 to 2.4 μg/L in bluegills (Mayer and Ellersieck, 1986).

Counter® is a clay based (15% active ingredient) or polymer based (20% active ingredient) granular formula applied to corn, sugar beets, and sorghum crops in the United States (USEPA, 1999) and is used extensively in Iowa (Hartzler, *et al.* 1997). This product, when used to control soil pests of corn (primarily corn rootworm), constitutes 90% of terbufos usage (by weight) in the United States. Terbufos is typically applied once yearly at a rate of 1.3 lb of active ingredient per acre in field corn applications when corn is planted in the same fields for successive years. Less terbufos is used when planting corn following crop rotations (0.75 lb active ingredient/acre for "first year" corn). Terbufos is applied to the soil surface either at planting, on post-emergent corn, or post cultivation. Approximately 95% of Counter® is applied at planting and 85% of this is deposited on the soil surface reducing soil incorporation (USEPA, 1999).

Hydrolysis and biodegradation are the primary modes of terbufos dissipation in soil (USEPA, 1999). Volatilization may be a major route of dissipation of Terbufos due to relatively high vapor pressure (3.16×10^{-4} mm Hg). Granular application reduces potential for aerial drift, while increasing chances of surface runoff, especially in areas of high rainfall. Terbufos has a log K_{ow} of 4.52 and water solubility of 15 mg/L. Photolysis is not an important means of dissipation in fields when incorporated in the soil (USEPA, 1999). However, photolysis may be an important means of degradation in surface water

where terbufos is unstable, with a half-life of one day. The terbufos metabolites terbufos sulfoxide and terbufos sulfone are more persistent and mobile in soil and water than terbufos and are assumed by the USEPA (1999) to have similar toxicity. Bioconcentration factors for terbufos range from 320 to 940 indicating that terbufos has a moderate potential for bioaccumulation (USEPA, 1999).

Most studies investigating AChE inhibition in nontarget aquatic organisms involve fish (e.g., Coppage and Mathews, 1974; Coppage and Mathews, 1975; Zinkl, *et al.* 1987; Richmonds and Dutta, 1992), crustaceans (e.g., Kobayashi, *et al.* 1986; Bocquene and Galgani, 1991; Abdullah, *et al.* 1994; Crane, *et al.* 1995; Fornstrom, *et al.* 1997) or mollusks (e.g., Fleming, *et al.* 1995; Moulton, *et al.* 1996). While fish, crustaceans and mollusks can be used as bioindicators, they do not inhabit most interior streams in abundance and are not widely distributed. Also, fish may swim long distance which renders them useless in site-specific bioindicator studies.

In contrast, aquatic insects are relatively abundant, have wide geographic distributions and tend to move less than fish. These features make aquatic insects potentially useful as bioindicators. A number of studies have illustrated AChE inhibition in stream insects following exposure to OP insecticides (Karnak and Collins, 1974; Flannagan, *et al.* 1978; Day and Scott, 1990; Detra and Collins, 1991; Ibrahim, *et al.* 1998; Beauvais, *et al.* 1999; Fisher, *et al.* 2000; Callaghan, *et al.* 2001; and Crane, *et al.* 2002) and others have shown that OP exposure can affect macroinvertebrate densities (Pusey, *et al.* 1994; Crane, *et al.* 1995; Fleming, *et al.* 1995; Van den Brink, *et al.* 1995).

A large number of studies have used *Chironomus riparius* as a bioindicator of OP exposure (Detra and Collins, 1991; Beauvais, *et al.* 1999; Sturm and Hansen, 1999; Fisher, *et al.* 2000; Callaghan, *et al.* 2001; Olsen, *et al.* 2001; Callaghan, *et al.* 2002). This midge is a common toxicity test organism, easily maintained and reared in the lab. However, *C. riparius* is uncommon in "healthy" streams and therefore may not be useful as an instream bioindicator of OP exposure in streams.

Isonychia bicolor was chosen for these experiments because of its wide distribution and year-round presence in most streams (Kondratieff and Voshell, 1984). Previous studies (Sweeney, 1978; Kondratieff and Voshell, 1984; Pontasch and Cairns, 1989; Breneman and Pontasch, 1994; Andersen, 2002) have shown that *I. bicolor* can be maintained for extended periods in stream microcosms.

The AChE inhibiting pesticides chlorpyrifos and terbufos are the most widely used insecticides in row crop applications in Iowa. Figure 3.1 illustrates terbufos use in the United States. It is important to establish the AChE inhibition from OP's and carbamate pesticides in the laboratory, if AChE inhibition is to have a diagnostic value in the field. When possible test organisms should be derived from field populations of interest. A study by Hoffman and Fisher (1994) illustrated biochemical and fitness differences in sensitivity to insecticides in field and laboratory-derived populations of *C. riparius*. Such differences may complicate translation of experimental results with laboratory-derived populations to field studies with natural populations and vice-versa (Schulz, 2004).

The following research was conducted to correlate terbufos concentrations with *I. bicolor* AChE activity inhibition. Using methods similar to those of Andersen (2002), this research investigated: (1) *I. bicolor* AChE inhibition following a 24 hour exposure to terbufos; and (2) the changes in AChE inhibition following the 24 hour exposure.

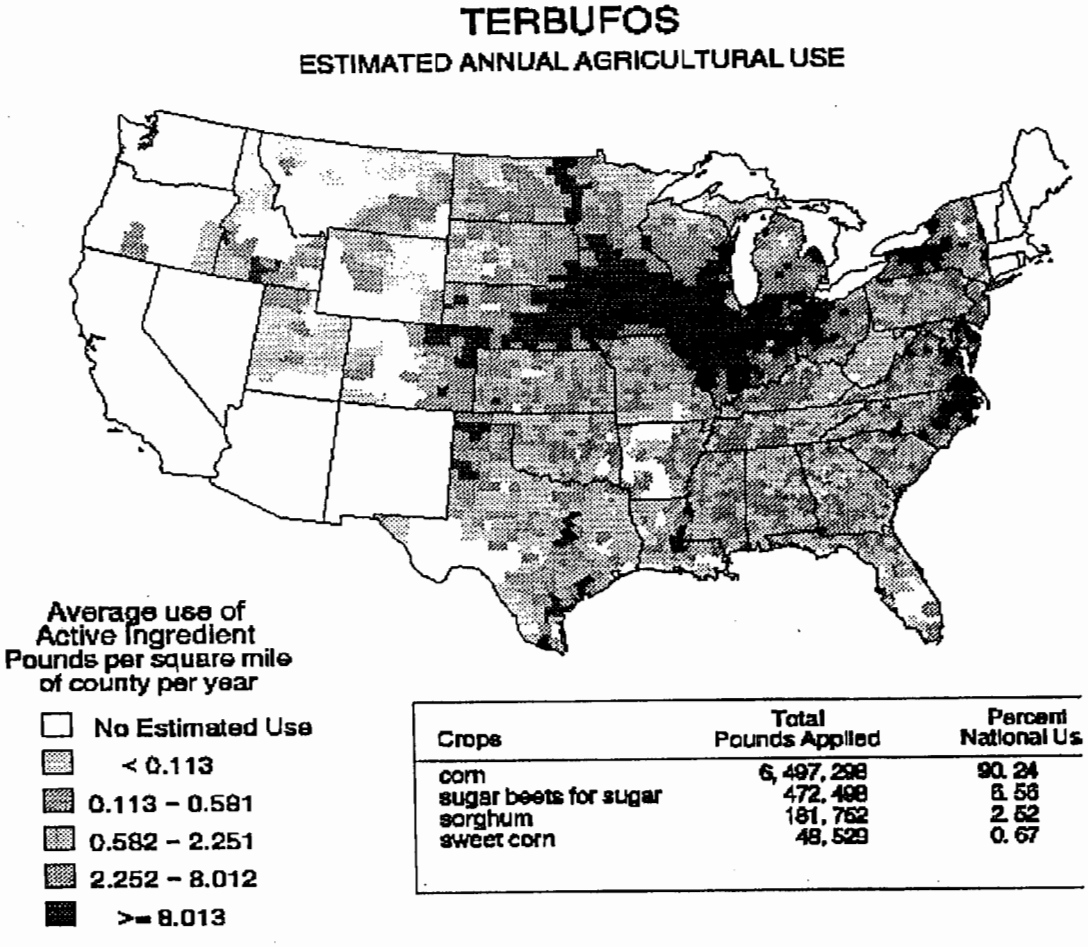

Figure 3.1. Terbufos use in the United States in 1998. (United States Geological Survey, 1998)

Materials and Methods

Stream Microcosms

This laboratory study employed 15 oval artificial streams (1.7 X 0.24 X 0.13 m channel) constructed of molded fiberglass. A standpipe in each stream maintained depth at 13 cm and volume at 55 L. Dechlorinated tap water was supplied to each stream to provide flow through conditions. This tap water has similar physical/chemical characteristics to Northeast Iowa's streams. Current (25 cm/sec) was provided by paddle wheels attached to an iron rod (1-cm diameter) turned by a 0.25 hp electric motor. Two 120-cm Durotest Vita-Lites® over each stream provided daylight equivalent light, and the photoperiod corresponded to the ambient photoperiod during the test. Each stream was covered by a 1.00 X 0.75 X 0.30 m emergence trap (mesh size ~1.0 mm).

Insect Collection, Transportation and Sampling

During September, 2003 *I. bicolor* (900) from the Cedar River in Cedar Falls were collected with D-nets and 30 placed in each of 30 coolers containing two rock-filled artificial substrates for transportation to the Ecosystem Simulation Laboratory at the University of Northern Iowa. The contents of two coolers (60 insects and four substrates) were transferred to each of 15 artificial streams. Five insects from each of the 15 artificial streams were immediately analyzed for AChE activity to determine pre-exposure activities. Following introduction to the microcosms, *I. bicolor* were acclimated for 48 h to a temperature of 20° C.

Technical grade terbufos was then added to the artificial streams to achieve triplicate nominal concentrations of 0.0, 2.5, 5, 10, and 20 ug/L terbufos. Previous research had suggested that *I. bicolor* can survive a 30 d exposure to 10 ug/L terbufos, but "quivering" of appendages was observed at that concentration (Smith, 1996). Dechlorinated tap water was shut off immediately prior to terbufos addition to prevent flow through conditions. AChE activities were determined from each stream 24 h after the initial pulse dose. Then 200 ml/min of dechlorinated tap water was continuously supplied to each artificial stream to purge the terbufos. AChE activities were then monitored 24 h after purging began and again eight days after purging. Following eight days of purging, there were insufficient organisms in most artificial streams for continued sampling, primarily because of adult emergence.

Determination of AChE Activity

Preparation and analysis of acetylcholinesterase activity was performed by the methods of Ellman, *et al.* (1961) optimized for *I. bicolor* by Anderson (2002). Each sample consisted of five *I. bicolor* head capsules. Head capsules were homogenized in a glass tissue grinder with 0.5% Triton X-100 detergent in Tris pH 7.4 buffer solution. For every mg of tissue, 200 μl of buffer solution was added resulting in a 200:1 dilution factor. Homogenate from each replicate was added to a micro-centrifuge tube and centrifuged (10 min; 14,000 rpm). Supernatant aliquots were separated into two cryovials then frozen (-80°C) until analyzed for AChE activity. Aliquots were separated in order to create a spare that remains frozen in case of sampling error, spillage, etc.

Acetylcholinesterase activity was quantified in a microplate-reading spectrophotometer (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA, USA). For each sample, AChE activity was measured in triplicate as the rate of increase in a absorbance at 412 nm. Each well contained an aliquot of *I. bicolor* head capsule supernatant (30 μl), acetylthiocholine iodide (AThChI) solution (0.0418 M; 30 μl), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) colorimetric reagent solution (0.442 M; 20 μl), and Tris pH 8.0 buffer solution (170 μl). Acetylthiocholine iodide (AThChI) is similar in structure to acetylcholine and acetylcholinesterase hydrolyzes AThChI in a similar manner to acetylcholine hydrolysis. This reaction results in a positively charged acetate ion and a negatively charged thiocholine complex. The thiocholine complex reacts with DTNB to create 5-thio-2-nitrobenzoate a stable, yellow colored anion that absorbs light most strongly at 412 nm. Tris buffers, AThChI and DTNB were obtained from Sigma Chemical (St. Louis, MO, USA). Tris buffer solutions were refrigerated at 4°C for no longer than one month and were adjusted to appropriate pH values prior to each analysis. DTNB and AThChI were prepared immediately prior to spectrophotometric analysis.

The optimum reaction temperature (~32°C) was achieved by maintaining DTNB and AThChI at room temperature, placing thawed cryovials of tissue homogenate in ice water and heating tris 8.0 buffer to ~80°C before pipetting. A blank (consisting of 200 μl Tris pH 8.0 buffer solution, 30 μl AThChI solution, 20 μl DTNB solution but no tissue homogenate) and a check standard (consisting of pooled homogenate supernatant from approximately 200 *I. bicolor* head capsules) were also analyzed in triplicate on each plate.

Care was taken to prevent formation of any bubbles during the entire process as bubbles cause inaccurate readings by the spectrophotometer.

Spectrophotometer software (SoftMAX PRO, Molecular Devices, Sunnyvale, CA, USA) was used to determine the highest AChE activity (Vmax). Absorbance measurements for all trials were read in all wells every twelve seconds for five minutes after an initial one-minute lag time. The following formula was used to convert mOD (milli-optical density) output units into international units of enzyme activity:

{[(enzyme mOD/min)-(blank mOD/min)]/1000} x 0.817 x dilution factor =

(μmoles AThChI hydrolyzed/min)/gram tissue

Determination of Total Protein Content

In this study, AChE activity is expressed in nmoles AThChI hydrolyzed/min/mg protein as well as μmoles AThChI hydrolyzed/min/g tissue. Andersen (2002) expressed activity only as μmoles AThChI hydrolyzed/min/g tissue. In order to find the protein content of the AChE tissue homogenate, the Bradford protein assay was optimized for use with *I. bicolor* tissue homogenate. Quantifying AChE activity relative to total protein content potentially minimizes the variation caused by differing amounts of other substances such as chitin or fat in the head capsules. A standard curve using bovine serum albumin was created to quantify the amount of protein in a solution of known concentration. The amount of protein (μg protein/μg tissue) in each sample was calculated using the formula (12((Bradford assay O.D.) - 0.017)/48.775). From this the AChE activity per mg protein was calculated.

The Bradford assay utilized a 96 well microplate reading spectrophotometer. Each sample was analyzed in triplicate using 60 µl Bradford's reagent (Coomassie® Brilliant Blue G-250 dye; Bio Rad, Hercules, CA, USA), 5 µl tissue homogenate, and 235 µl twice distilled, reverse osmosis water in each well. Each sample was then mixed by repeated suction/expulsion through a micropipetter to ensure even mixture of the solution. Care was taken to prevent formation of bubbles during this process as bubbles create inaccurate spectrophotometric readings. Each microplate contained one blank (240 µl water and 60 µl Bradford reagent), and one check standard, as well as unknowns (each in triplicate). Microplates were allowed to sit approximately 5 minutes before analysis in the spectrophotometer.

The AChE data (both µmoles AThChI hydrolyzed/ min /gram protein as well as µmoles AThChI hydrolyzed/min/ gram tissue) from each sampling date were statistically analyzed by a one-way Analysis of Variance (ANOVA) among treatments followed by Dunnett's Test for the comparison of means with a control. In addition, the data within each treatment were statistically analyzed by a one-way ANOVA among dates followed by Duncan's Multiple Range Test for the separation of means.

Results and Discussion

Water quality characteristics in the artificial stream microcosms and the Cedar River were similar (Table 3.1). Prior to acclimation, the treatments were not significantly ($p>0.05$) different in terms of AChE activities per g tissue (Figure 3.1) or per mg protein (Figure 3.2). Following 24 h terbufos exposure, AChE activity per g tissue in the 20

μg/L treatment was significantly ($p \leq 0.05$) different from the control (Figure 3.3), but no significant ($p > 0.05$) difference was found in AChE activity per mg protein in any treatment (Figure 3.4). Following 24 h purging, AChE activities (per g tissue and per mg protein) were significantly ($p \leq 0.05$) lower in the 20 μg/l treatment (Figure 3.5 and 3.6). By the eighth day after purging, all organisms in the 20 μg/L treatment were dead, and among the remaining treatments there were no significant ($p > 0.05$) differences in AChE activities per g tissue (Figure 3.7) or per mg protein (Figure 3.8). An analysis of AChE activities within a treatment over time indicated that in the 10 μg/L treatment activity (per g tissue) was significantly higher ($p \leq 0.05$) on day 9 than on day 2 (Figure 3.9), but the increase was not significant ($p > 0.05$) for activity per mg protein (Figure 3.10). Overall the data suggest that *I. bicolor* AChE activities can rebound from exposures to 10 μg/L but not 20 μg/L.

Table 3.1 Physical/Chemical characteristics of water in artificial stream microcosms and Cedar River.

Measurement	microcosms[a]	Cedar River
Alkalinity (mg $CaCO_3$/L)	158	160
Hardness (mg $CaCO_3$/L)	298	270
Conductivity (mS/cm)	0.406	0.436
pH	7.63	7.67
Temperature (°C)	18.7	21.3
Dissolved Oxygen (mg/L)	8.43	11.0

[a] Each value represents the mean from five microcosms

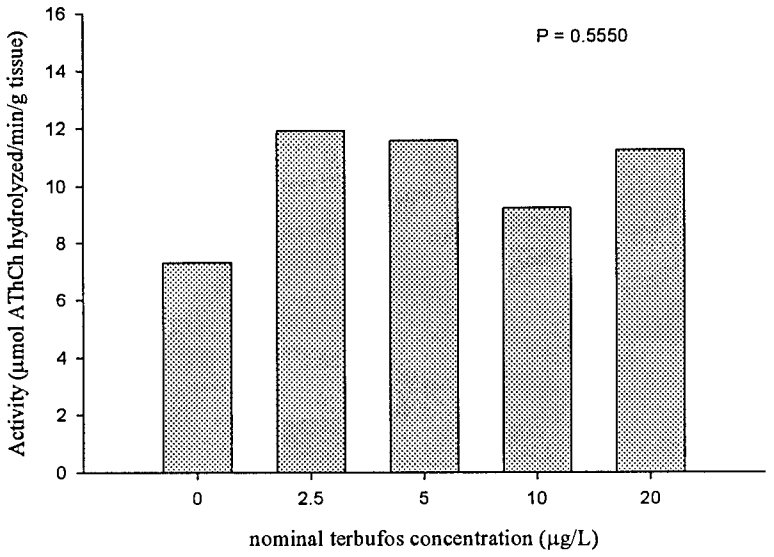

Figure 3.2 *Isonychia bicolor* AChE activities (per g tissue) in artificial stream microcosms prior to exposure to terbufos. P value is from a one-way ANOVA among treatments.

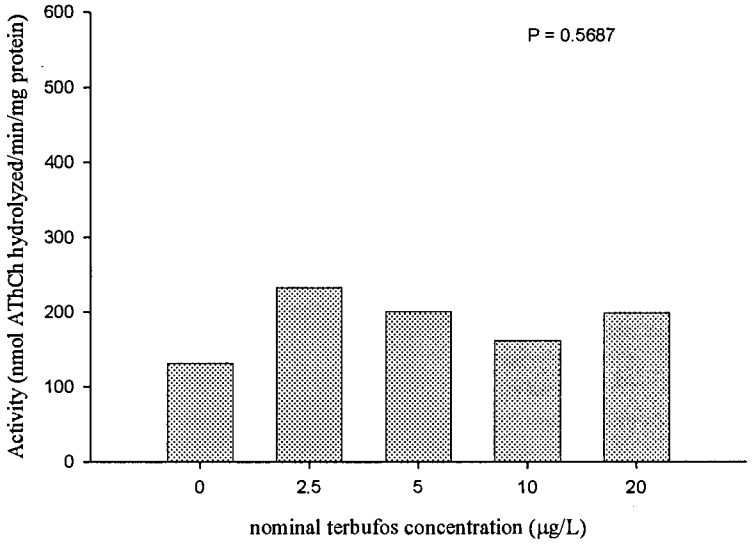

Figure 3.3 *Isonychia bicolor* AChE activities (per mg protein) in artificial stream microcosms prior to exposure to terbufos. P value is from a one-way ANOVA among treatments.

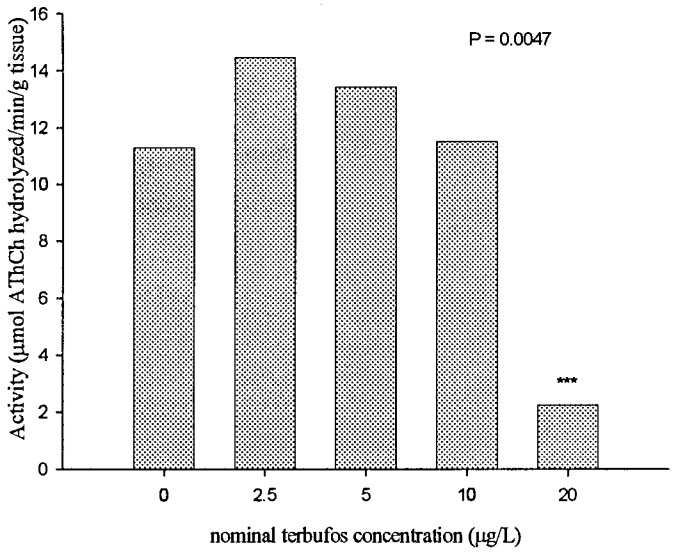

Figure 3.4 *Isonychia bicolor* AChE activities (per g tissue) in artificial stream microcosms after 24 h exposure to terbufos. P value is from a one-way ANOVA among treatments. ***significantly different ($p \leq 0.05$) from the control based on Dunnett's test

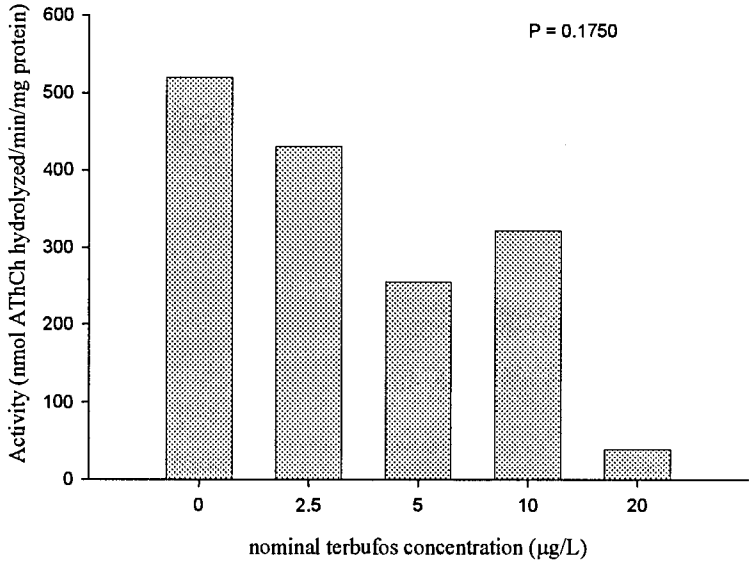

Figure 3.5 *Isonychia bicolor* AChE activities (per mg protein) in artificial stream microcosms after 24 h exposure to terbufos. P value is from a one-way ANOVA among treatments.

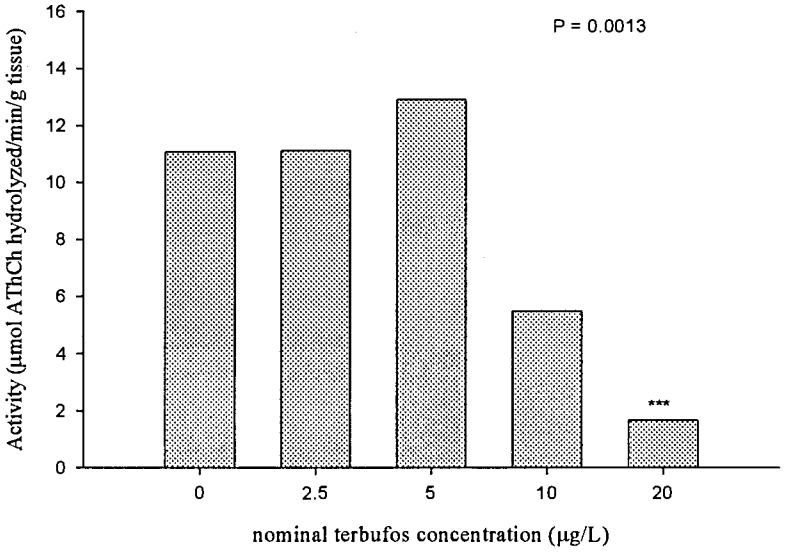

Figure 3.6
Isonychia bicolor AChE activities (per g tissue) in artificial stream microcosms exposed to terbufos 24 h after purging (48 h after initial exposure). P value is from a one-way ANOVA among treatments. ***significantly different ($p \leq 0.05$) from the control based on Dunnett's test.

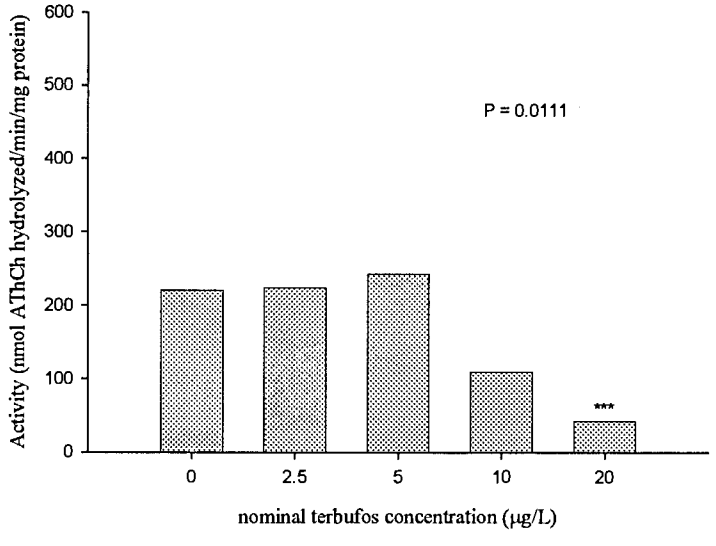

Figure 3.7 *Isonychia bicolor* AChE activities (per mg protein) in artificial stream microcosms exposed to terbufos 24 h after purging (48 h after initial exposure). P value is from a one-way ANOVA among treatments. ***significantly different ($p \leq 0.05$) from the control based on Dunnett's test.

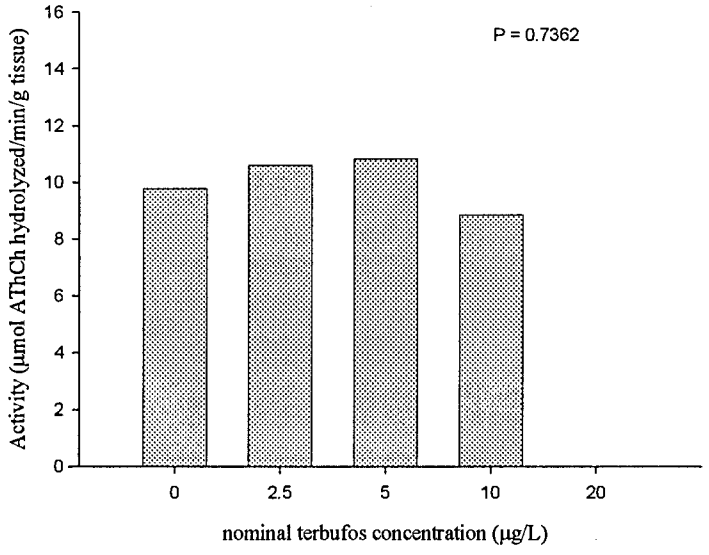

Figure 3.8 *Isonychia bicolor* AChE activities (per g tissue) in artificial stream microcosms exposed to terbufos 8 d after purging (9 d after initial exposure). P value is from a one-way ANOVA among treatments.

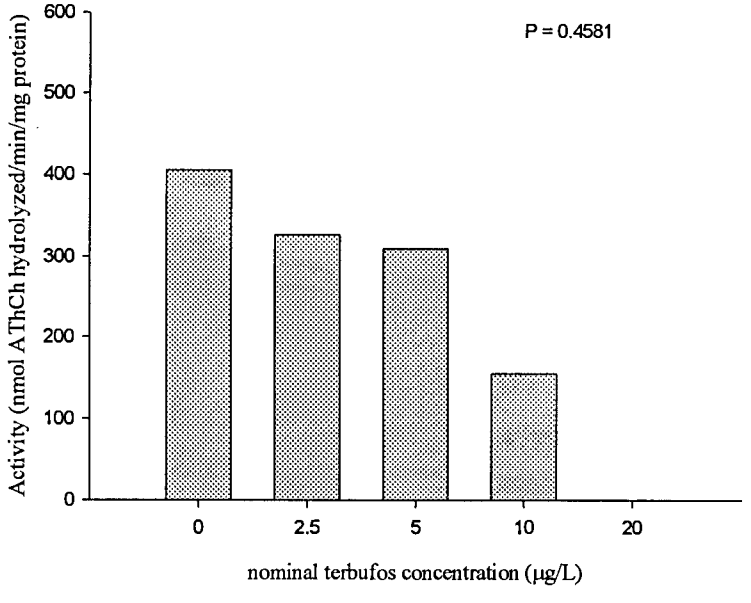

Figure 3.9 *Isonychia bicolor* AChE activities (per mg protein) in artificial stream microcosms exposed to terbufos 8 d after purging (9 d after initial exposure). P value is from a one-way ANOVA among treatments.

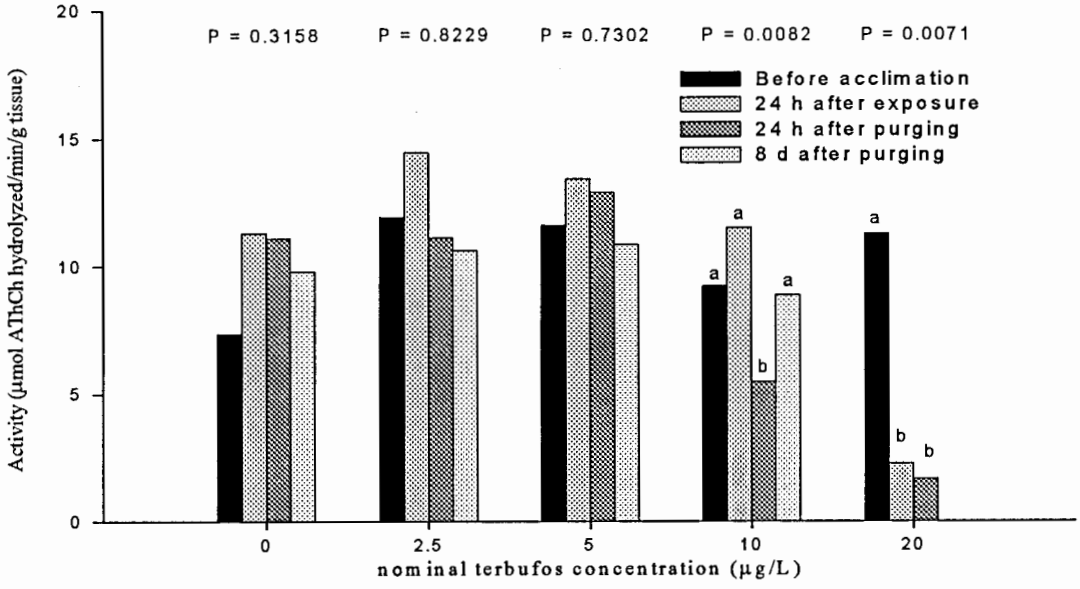

Figure 3.10 *Isonychia bicolor* AChE activities (per g tissue) in artificial stream microcosms exposed to terbufos. P values are from a one-way ANOVA for each treatment among days. Bars with the same letter are not significantly different ($p \geq 0.05$) based on Duncan's Multiple Range Test for the separation of means.

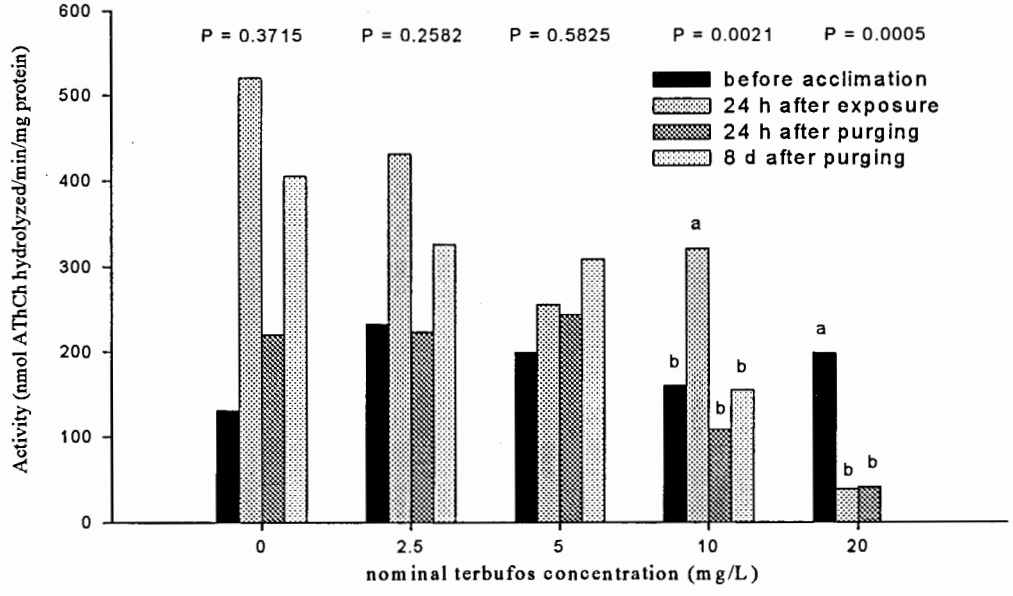

Figure 3.11 *Isonychia bicolor* AChE activities (per mg protein) in artificial stream microcosms exposed to terbufos. P values are from a one-way ANOVA for each treatment among days. Bars with the same letter are not significantly different ($p \geq 0.05$) based on Duncan's Multiple Range Test for the separation of means.

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CHAPTER 4

IN-STREAM BIOASSESSMENT OF AN URBAN, COLDWATER STREAM POLLUTED BY CHLORDANE

Abstract

Urban streams are subject to a wide array of anthropogenic insults. This study was undertaken to quantify possible impacts on the aquatic macroinvertebrate communities in McLoud Run, an urban, coldwater stream in Cedar Rapids, Iowa, USA. Aquatic macroinvertebrate communities were allowed to colonize rock-filled artificial substrates at three sites for one month before sampling. Sampling occurred monthly from August, 2002 until August, 2003. Three artificial substrates from each site were preserved in alcohol on each date. Monthly invertebrate samples were also collected for chlordane analysis. Chlordane is a persistent organochlorine pesticide previously found in trout from McLoud Run. Periphyton samples were also collected monthly for determination of chlorophyll *a*, biomass and the Autotrophic Index.

Invertebrate sampling indicated low numbers of "clean water" taxa such as Ephemeroptera, Trichoptera and Plecoptera (absent) as well as high numbers of "dirty water" taxa such as Chironomidae and Oligochaeta. In addition, collector/gatherers were predominant at all sites and mean Hilsenhoff Biotic Index values ranged between 5.5 and 6.1. These data suggest moderate organic enrichment at all sites.

Samples for chlordane analysis were pooled due to small macroinvertebrate biomass. However, chlordane levels were below detection limits (50 μg/l). Periphyton sampling was often hindered by sedimentation and spates. One site (McLoud Place) had

significantly higher ($p>0.05$) biomass and chlorophyll *a* on most dates than the remaining sites (J Avenue and 42nd Street). Autotrophic Index values were relatively high at all sites, indicating a high ratio of heterotrophs to autotrophs.

Keywords: urban streams, bioassessment, chlordane, functional feeding groups, Hilsenhoff Biotic Index, Autotrophic Index

Introduction

Urban streams throughout the world are frequently impaired. The cause of impairment may be due to many factors that complicate diagnoses of problems and their remediation. Several important sources of urban stream degradation are ubiquitous and difficult to rectify. These include increased runoff from impervious surfaces, non-point source pollution with complex pollutant mixtures, and loss of natural riparian vegetation. Degradation of streams in urban areas is often due to the synergistic interaction of these insults.

Streams in urban settings receive extreme volumes of runoff during rainfall events because impervious surfaces such as city streets, roofs and compacted soils coupled with efficient storm sewers and ditches cause sudden, high flows in urban streams. The amount of impervious surface in watersheds has been proposed as a key indicator of stream degradation by a number of authors. Stephenuck (2002) found that the Hilsenhoff Biotic Index (Hilsenhoff, 1987) increased dramatically when impervious surfaces of a watershed reached 8-12%. Schuler (1994) noted a sharp threshold in habitat quality existing with approximately 10-15% impervious surface areas. Schuler and Galli (1992; as cited by Schuler, 1994) found that trout and sculpin were excluded from Maryland piedmont streams at 10-12 % imperviousness. May, *et al.* (1997) found physical and biological measures generally changed most rapidly between 5 -10% imperviousness. Booth and Jackson (1997) found 10% effective imperviousness elicited demonstrable loss of aquatic system function.

With increased imperviousness, subsurface flow processes are diminished, causing water to quickly flow over the surface instead of infiltrating the soil (Booth and Jackson, 1997). These processes change the hydrologic regime of the stream. A study by Klein (1979) suggested that impervious surfaces result in decreased groundwater recharges and decreased low flow conditions in some physiographic provinces. However, in other provinces, baseflow may increase. In urban settings storm events also cause floods more frequently. Klein (1979) found that in rural or agricultural areas bankfull flows occur approximately once a year, in areas of 40 percent imperviousness bankfull flows occur 3 times yearly and in higher areas 5.6 times yearly.

Stream channels respond to frequent flooding by increasing their cross-sectional area either by down cutting of the streambed, widening of banks or both, to accommodate higher flows. Furthermore, loss of natural riparian vegetation in urban areas results in bank instability intensifying changes in channel morphology (Schuler, 1994). Also, in many streams humans employ artificial channelization to reduce meandering, which has detrimental results similar to, or greater than, the natural changes. These changes increase the hydraulic efficiency of the channel, transmitting flood waves downstream faster (Booth and Jackson, 1997). Increased flow causes modified stream discharge regimes, which result in excessive bank erosion, altered channel morphology and base flows, changes in bed composition and morphology, as well as degraded aquatic habitat (Stephenuck, 2002).

Klein (1979) suggests that flooding may have a rejuvenating effect on stream health. However, the resilience of stream communities may be strained as floods increase

in severity and frequency. Frequent flash flooding causes scouring of substrates and deposition of silt that can dramatically affect benthic macroinvertebrate communities and other aquatic life (May, *et al.*, 1997) especially in streams less than 4 meters wide (Lenat and Crawford, 1994). Rushing water may dislodge or bury invertebrates in sediment, causing severe decreases in numbers (Minshall, 1969). Fine sediments deposited over larger, solid substrates such as gravel and cobble create a "seal" that prevents colonization and attachment by many benthic macroinvertebrates (Brusven and Praether, 1974; Wiederholm, 1984). In these conditions burrowing and filter-feeding macroinvertebrates such as Oligochaeta and Chironomidae are favored (Wiederholm, 1984). If fine sediment doesn't exclude benthic insects it may affect feeding and respiration (Wiederholm, 1984). Furthermore, water flow to the intragravel region is reduced in the presence of silt and reduces intragravel dissolved oxygen (May, *et al.*, 1997). Fish egg development (especially in salmonids) depends on high water flow to provide intragravel dissolved oxygen and remove metabolic wastes.

Changes in the erosional-depositional characteristics of urban streams may favor development of a fauna adapted to extreme bed instability (Pedersen and Perkins, 1986). These changes may mask the effects of contamination from urban runoff. Pedersen and Perkins (1986) suggest that these processes may be a more fundamental determinant in the functioning of urban stream ecosystems than factors related to toxicity or organic loading.

Storm water runoff also carries many toxic compounds into streams. These may include heavy metals, pesticides, oil, road salt, detergents, and organic matter. Benke, *et*

al. (1981) noted that "pollutants stress aquatic communities in ways that affect species composition, productivity, trophic pathways, species interactions, and a host of other structural and functional aspects of ecosystems." A study by Bryan (1972) found that biochemical oxygen demand (i.e. the amount of oxygen needed to break down the organic load in water) and suspended solids in urban runoff to be comparable to that of secondary wastewater treatment effluent or even raw sewage, calling it "unsuitable dilution water for sewage". The biochemical oxygen demand needed for decomposition of this organic matter can cause anaerobic conditions that may eliminate many aquatic taxa. In these conditions red *Chironomus* spp. and Tubificidae may be the only fauna present. Hemoglobin in these species allows them to bind oxygen in anaerobic conditions. In cases where organic enrichment is less severe (i.e. when conditions aren't anaerobic) organisms such as grazing mayflies, Simuliidae and hydropsychid caddisflies may proliferate (Wiederholm 1984).

Temperature changes are a commonly encountered problem in urban streams because impervious areas can have local air and ground temperatures 10 to 12°C warmer than the fields and forests they replace (Schuler, 1994). Therefore, precipitation is warmed and rapidly carried into streams. Klein (1979) adds that further heat loss and gain is caused by widening of streams from increased runoff that causes stream depths to decrease and riparian vegetation to disappear. These changes cause increased daily and seasonal temperature maxima, decreased daily and seasonal minima, and changes in diurnal, seasonal and annual patterns of temperature fluctuation (Minshall, 1969;

Wiederholm, 1984). Heavily canopied streams typically warm more slowly in spring and cool less rapidly in fall. Temperature changes may have a number of detrimental effects on stream biota. These may include altering developmental time, body size, fecundity, as well as elimination or reduction of species and premature or delayed emergence patterns.

Loss of riparian vegetation is also a common problem in urban areas due to removal during development and losses from flooding and subsequent changes in stream morphology. Riparian vegetation has many important functions in maintaining stream quality, however it is difficult to ascertain how much and what type of vegetation is needed. May, *et al.* (1997) noted that not only the width of riparian corridors, but also the longitudinal continuity or connectivity are important factors in maintaining stream quality. Minshall (1969) found that the largest number of taxa existed in areas with the least disturbed forest nearby. Benke, *et al.* (1981) found that areas with >75% riparian cover have at least twice as many species as areas with <25% cover. May *et al.* (1997) also found that urban streams consistently had little mature riparian area (e.g., forest) and subsequently had little large woody debris. Large woody debris helps to dissipate flow energy, protects and stabilizes stream banks, provides instream cover and habitat diversity, and alters sediment deposition. Deep-rooted vegetation is typically replaced by grasses or ornamental plants that confer little resistance to stream widening (Booth and Jackson, 1997). In addition, loss of deciduous trees reduces the volume of leaves that enter a stream. Small, undisturbed streams are typically heterotrophic depending on allochthonous input (e.g. leaves) as the most important source of primary production

(Kaushik and Hynes, 1971; Wallace, *et al.*, 1977; Vannote, *et al.*, 1980; Wiederholm, 1984). Elimination of leaf packs due to loss of vegetation and frequent flooding may greatly change the trophic status of smaller streams.

McLoud Run is a small, urban, trout stream located entirely in Cedar Rapids, Iowa. Tissue analyses of (approximately) two year old trout from McLoud Run contained 0.39 ppm chlordane in the edible portions and 0.48 ppm in head and gut samples during 2001, exceeding the FDA action level of 0.3 ppm (John Olsen, Iowa Department of Natural Resources, personal communication), and there have been yearly trout kills during 2000-2002 related to elevated water temperatures from summer rains. A rain event on August 2, 2001 raised the temperature of McLoud run by 7°C in one hour, killing rainbow and brook trout. In response, the Iowa Department of Natural Resources (IADNR, 2005a) Fisheries Bureau has issued a catch and release only restriction on trout taken from McLoud Run. For purposes of Section 305(b) assessment, the general aquatic life uses of McLoud Run are considered "threatened" by the pesticide chlordane (John Olsen, IDNR, personal communication).

Chlordane (2,3,4,5,6,7,8,8-octochloro-2,3,3a,4,3,7a-hexahydro-4,7-methanoindene) is an organochlorine insecticide used from 1945 until it was banned by the EPA in 1988. Trade names for chlordane formulations included Velsicol 1068®, Velsicol 168®, M-410®, Belt®, Chlor-kil®, Chlortox®, Corodane®, Gold Crest C-100®, Gold Crest C-50®, Kilex®, Kypchlor®, Niran®, Synchlor®, Termi-ded®, Topichlor 20®, Chlordan®, Prentox®, and Penticklor®. Chlordane was manufactured by several

companies from 1945-1950, then exclusively by the Velsicol Corp. in Marshall, Illinois from 1951 until its use was banned. This chemical was used on row crops (especially corn) until 1983 and around house foundations for termite control until 1988 (ATSDR, 1994).

Chlordane is a nerve stimulant causing hypersensitivity, hyperactivity with violent bursts of convulsions and finally complete prostration with convulsive movements. The exact biochemical mode of action is unknown, however the site of disturbances is known to lie in the ganglia of the central nervous system. Target sites are presumed to be on proteins or phospholipids in nerve or muscle membranes (Eisler, 1990). The metabolites oxychlordane and heptachlor epoxides are also toxic. Oxychlordane is produced by both plants and animals and is typically more toxic than chlordane (Sittig, 1980)

Chlordane exists as two common isomers α-cis and β-trans, and a number of less common isomers with a molecular weight of 409.8. The technical product is a brownish liquid that contains varying amounts of chlordane and other active, closely related compounds. This mixture typically consisted of 24% trans-chlordane, 19% cis-chlordane, 10% heptachlor, 7% nonachlor, 21.5% other chlordane isomers and 18.5% closely-related chlorinated hydrocarbon compounds (Sittig, 1980) . From 1945-1950 a version of chlordane was produced under the trade names 1068 Chlordane® and Octa-Chlor® that contained an extremely toxic unreacted intermediate, hexachlorocyclopentadiene (Ingle, 1965). 70,000 tons of chlordane was used from 1946-1991 and 25-50% was estimated to still exist in the environment in 1991 (Pridmore, *et al.* 1992).

Numerous studies have measured concentrations of chlordane in various environmental compartments (Fox *et al.* 1983; Wood, *et al.* 1986; Erstfeld, *et al.* 1989; Pridmore, *et al.* 1991; Erstfeld, *et al.* 1996; Hoople and Foster, 1996; Periera, *et al.* 1996; Wright, *et al.* 1996; Erstfeld, *et al.* 1997; Park and Erstfeld, 1999; Rostad, *et al.* 1999; Rawn, *et al.* 2001). Chlordane is persistent in the environment with a half-life in soil of 3-14 years. Atmospheric transport can deliver chlordane great distances and all available evidence suggests that chlordane is ubiquitous in the environment (Eisler, 1990; Rawn, *et al.* 2001). A study by Stackelberg (1997) found chlordane in 80-100% of urban stream sediment samples and found a positive correlation between urban population and amounts of chlordane. Rostad, *et al.* (1999) also found total chlordane loads in Mississippi River sediment to be greatest near urban and industrialized areas.

Chlordane has a water solubility of 9 μg/L at 35°C. However, when chlordane is found in water samples concentrations typically occur in the ng/L range. Concentrations in suspended solids and sediment are higher, typically between <0.03-580 ppb (ATSDR, 1994). In water, chlordane tends to bind to biota, organic sediments or it volatilizes. Chlordane has an octanol/water partitioning coefficient of 6.04 (Wood, *et al.* 1986). Chemicals with octanol/water partitioning coefficients near 6 tend to bioaccumulate strongly in benthic biota (Oliver 1984). It appears that volatilization kinetics (volatilization half-life in lakes is estimated to be < 10 days) are faster than adsorption kinetics (ATSDR, 1994). The majority of chlordane enters water as runoff and is adsorbed to particulates before entry. Chlordane may desorb in the water and volatilize

rapidly near the surface. Wright (1996) suggests that soils with adsorbed chlordane tend to accumulate in depositional areas and affect Chironomidae (and potentially other invertebrates) there more so than those from erosional or transitional areas.

Chlordane is highly toxic to aquatic organisms and bioconcentrates in aquatic species. Acute toxicity of chlordane to freshwater fish and invertebrates occurs between 3 and 190 µg/L, with most values between 15 and 60 µg/L (Cardwell, *et al.* 1977). Cardwell, *et al.* (1977) found the freshwater chronic toxicity of chlordane for *Daphnia magna* and *Lepomis macrochirus* to be 16 and 1.6 µg/L, respectively. However, the fathead minnow (*Pimephales promelas*) test produced no significant differences ($p > 0.05$) from the control at the highest concentration tested (6.03 µg/L). A 13 month (Eisler, 1990) exposure to 0.32 µg/L caused reduced embryo vitality in brook trout (*Salvelinus fontinalis*). Concentrations as low as 1.7 µg/L caused mortality in *Chironomus* sp. in a 25 day exposure (Eisler, 1990).

Cardwell, *et al.* (1977) found technical chlordane bioconcentration factors (BCF) of 5,200 and 3,800 for *Hyallela azteca* and *Daphnia magna*, respectively. As technical chlordane is a mixture, these numbers were calculated by multiplying the arithmetic mean of each constituent's BCF by its percentage composition in technical chlordane then adding the products of each constituent. Cardwell, *et al.* (1977) also found the lipid normalized BCF (BCF value divided by lipid content) for *Pimephales promelas* to be 4,974. In a more recent study, Hoople and Foster (1996) found that the aquatic plant *Hydrilla verticillata* concentrated chlordane by foliar uptake from the water column.

The following is the current draft of the Section 305(b) water quality assessment for McLoud Run (John Olson, Iowa DNR, 2005b).

SUMMARY: The general (aquatic life) uses remained assessed (monitored) as "partially supported" due to the occurrence of a fish kill during the last three years (2000-2002) and the potential for similar kills to occur in the future. This kill involved temperature-sensitive fish species (trout) and was attributed to elevated water temperatures in runoff from this stream's urban watershed following summer rains. Results of ambient water quality monitoring conducted in summer 2002 for the Cedar Rapids intensive urban monitoring project indicate generally good chemical water quality in this stream.

EXPLANATION: The previous assessment of support for the general beneficial uses of this stream ("partially supporting" the general aquatic life uses) was based on the occurrence of two spill-related fish kills during the 1996-1997 biennial assessment period (see assessment developed for the 1998 and 2000 reporting cycles). The most recent fish kill, however, occurred on August 2, 2001 and was due to storm water runoff from urban watershed of this stream. The rainfall event occurred during very warm summer weather, and the relatively warm runoff water raised the temperature of McLoud Run by 19 degrees F in an hour. Temperature-sensitive species such as the (stocked) rainbow, brown, and brook trout were killed. According to DNR's assessment methodology for Section 305(b) reporting, occurrence of a single pollution-caused fish kill within the most recent three-year period (1997-1999) indicates that the aquatic life uses of a waterbody are only "partially supported." Thus, the general aquatic life uses of this stream remain assessed as "partially supported" due to thermal impacts from urban runoff.

Results of ambient water quality monitoring conducted in summer 2002 for the Cedar Rapids intensive urban monitoring project indicate good chemical water quality in this stream. The purpose of this project is to measure the daily variability of water quality through time in two urban streams in the Cedar Rapids area - McLoud Run and Indian Creek. Both streams are monitored by the City of Cedar Rapids Water Pollution Control as part of their storm water monitoring program. The daily monitoring is designed to supplement the storm water monitoring being conducted on these two streams by the City of Cedar Rapids. In 2002, sampling was conducted daily on McLoud Run from about mid-May to mid August. Parameters monitored include pesticides, toxic organics, conventional parameters (including nutrients), and bacterial indicators (e.g., fecal coliforms and *E. coli*). In general, results of this monitoring show good water quality. Although classified only for general uses, Iowa water quality criteria for either Class B(LR)

or Class B(CW) aquatic life uses were compared to results of monitoring as an indicator of the status of water quality in this stream. This comparison showed no violations of criteria for pH or ammonia in the 70+ samples collected during summer 2002. One of 83 samples contained a level of dissolved oxygen below the 7.0 mg/l water quality criterion for Class B(CW) coldwater streams: the sample collected on June 10, 2002 contained 6.4 mg/l of dissolved oxygen. Levels of metals, pesticides, and other toxic organic compounds did not exceed chronic aquatic life criteria . The only such parameter to approach a state criterion was pentachlorophenol (PCP). Fifteen of the 79 samples analyzed contained detectable levels of PCP; five of these samples were within 1 ug/l of the respective pH-dependent Class B(LR) criterion. None of the 81 samples analyzed contained detectable levels of either chlordane (detection level = 0.05 ug/l) or any of the seven PCB Aroclors analyzed (including Aroclors 1248, 1254, and 1260) (detection level = 0.5 ug/l). Both chlordane and PCBs have been found at elevated levels in fish from either McLoud Run or the adjacent Cedar Lake.

Fish tissue monitoring conducted in June 2001 following a mid-May fish kill showed that fillet samples of stocked trout (ages 1 and 2) contained levels of chlordane above the 0.300 ppm U.S. FDA action level. A second fish tissue sampling in July 2001 showed that levels were just below the FDA action level. In response, the IDNR Fisheries Bureau has issued a no-kill restriction on trout taken from McLoud Run. For purposes of Section 305(b) assessment, the general aquatic life uses of McLoud Run are considered "threatened" by the pesticide chlordane.

Due to the persistent nature of chlordane and McLoud Run's proximity to many older businesses and residences, aquatic macroinvertebrates may still be exposed to chlordane. If chlordane is present at sufficient levels, macroinvertebrate populations may decline, community composition may shift toward more tolerant species, and trout food species may become limited. Together with direct toxicity and bioaccumulation, these factors may contribute to a decline in trout health and, potentially, death. The objective of this research was to quantify macroinvertebrate population densities and chlordane levels

in McLoud Run. In addition, periphyton samples were taken to quantify chlorophyll *a* and biomass which were then used to calculate the Autotrophic Index

Materials and Methods

Study Area

McLoud Run is a small, cold-water, first-third order stream located in Cedar Rapids, Iowa. Post-World War II residences and small businesses constitute most of the drainage. Much of the stream is channelized and a portion of the stream flows through culverts beneath a residential neighborhood.

Three riffle sites were chosen for research. The upstream site is designated as 42nd Street, the intermediate site as McLoud Place and the downstream site as J Avenue. The 42nd Street site is located north of 42nd Street behind a photographer's studio. The riffle substrate is primarily sand and cobbles (10-15 cm diameter). Most of the riffle is completely shaded by riparian vegetation, and leaf packs were present from October to April. Apparently, during times of low flow this site has become dry. However, during this study the stream flowed continuously except for a period from January to mid-March when it was frozen solid. Subsurface sediments at this site were found to be black and smelled of sulfur throughout this study, suggesting anoxic conditions.

The second site is located south of the McLoud Place bridge. This riffle is composed of large cobbles (10-30 cm diameter) embedded in sand. Little overhanging

riparian vegetation exists on this portion of the stream, but a small area is shaded by the McLoud Place bridge.

The J Avenue riffle runs parallel to J Avenue beneath the I-380 overpass. This riffle is composed of cobbles (10-40 cm diameter) embedded in sand with exposed bedrock in some portions. Although the overpass shades most of this riffle, grassy, overhanging, riparian vegetation is abundant on the north side. The south bank is rip-rap for erosion control.

Macroinvertebrate Density Sampling

Artificial substrates were used to collect insects due to the relatively small riffles in McLoud Run that precluded the use of Hess or Surber samplers. Also, artificial substrates help to reduce community composition variability caused by natural substrate differences among sites. Macroinvertebrate communities were colonized in rock-filled plastic containers (10.6 X 10.6 X 8.3 cm) with six circular holes (12 mm dia) in each side. River rock (4-6 cm dia), purchased at a sand and gravel pit, was used to fill the plastic containers. Seven artificial substrates were secured monthly to each of two wooden frames that were anchored to the stream bottom at each site with iron rods and concrete blocks.

Artificial substrates were allowed to colonize for 30 days. Previous studies have shown that a 30 day colonization period is sufficient to ensure that macroinvertebrate species equilibrium has been achieved (Pontasch, 1995). After colonization a dip net was

placed behind each substrate during removal from the frame. The rocks from each substrate were then gently rubbed and rinsed to remove any attached organisms and the resulting slurry was rinsed through a 500 micron sieve. The contents of three randomly selected artificial substrates were preserved in three labeled jars containing 80% ethanol. Organisms captured in the dip net were added to the ethanol with the sample from each respective substrate. All colonized substrates were removed at the end of each sampling trip and replaced with new substrates.

Organisms were identified to the lowest practical taxonomic level. Chironomidae (Insecta: Diptera) were identified to genus when sufficient morphological features existed to allow positive identification under a dissecting scope. All other Chironomidae were identified to the subfamily level. Macroinvertebrate taxa were considered a "core" taxon and analyzed statistically if they contained a mean of four or more individuals at one site, on at least one sampling date, during the study. Remaining taxa were not statistically analyzed. The density of individuals in each "core" taxon were compared among sites on each sampling date (Figures 4.1-4.20). The data were analyzed by a one-way analysis of variance (ANOVA) in conjunction with Duncan's Multiple Range Test for the separation of means, or, when only two sites were compared, with a t-test.

Macroinvertebrate taxa were assigned to their respective functional feeding groups and these data were graphed for each site. The functional feeding group (FFG) approach was developed to illustrate trophic relationships in streams with greater clarity (Merritt and Cummins, 1996). By assigning insects to functional feeding groups researchers can

understand how organic matter is cycled within a stream and also the size and origin of the organic matter. Although these FFG data were not statistically analyzed, they help illustrate the trophic status of each stream reach. Also, data from this study were used to calculate the Hilsenhoff Biotic Index (HBI) (Hilsenhoff 1977; Hilsenhoff 1982; Hilsenhoff 1987; Hilsenhoff 1998). The HBI was developed to categorize streams according to levels of organic enrichment.

Temperature, dissolved oxygen, pH, hardness, alkalinity, and conductivity were monitored at each site on each sampling date (Appendix G). Substrates were introduced into McLoud Run on 7-16-02 and were visited monthly thereafter for one year. Heavy rains in early August 2002 washed out most substrates at all sites. Artificial substrates were then reinstalled on 8-15-02 and the study was reinitiated, running until 8-18-03.

Chlordane Analysis

During each collection trip, at each site, two colonized artificial substrates were removed and placed in a cooler filled with source riffle water for transportation to the laboratory. Upon arrival at the laboratory, insects were narcotized with carbonated water and removed from substrates. Insects from each site were placed in a small Nalgene container and frozen until analysis at the University Hygienic Laboratory. Initially, each sample was to be individually tested. However, the individual samples did not contain enough tissue to analyze for chlordane. The samples were then pooled into one sample to obtain enough tissue for analysis.

Periphyton Sampling

Periphyton was colonized on seven artificial substrates attached to the anchored macroinvertebrate substrate frames at each study site. The periphyton artificial substrates consisted of square (10.8 X 10.8 cm), high-density, plastic container lids roughened with coarse sandpaper. After the 30 d colonization, all seven periphyton substrates were removed and placed in a cooler filled with source riffle water for transportation to the laboratory.

Upon arrival at the laboratory a sample was taken from each of the substrates by scraping a delimited surface area with a bristle brush and filtering the scrapings onto Whatman GFC filter paper. Determination of chlorophyll *a* concentration was made with one half of the filter paper, and biomass (ash-free dry weight) was determined from the other half (APHA, *et al.* 1989). From this the Autotrophic Index (AI) can be calculated:

$$AI = \text{Biomass } (mg/m^2) \,/\, \text{Chlorophyll } a \ (mg/m^2)$$

The AI is a measure used to determine the trophic status of periphytic communities (i.e. heterotrophs versus autotrophs). Normal values range from 50-200 and values above 200 indicate possible organic enrichment (APHA, *et al.* 1989). Sedimentation at 42nd Street and J Avenue resulted in no more than two sites with Chlorophyll *a*, biomass and AI data on a given sampling date. Chlorophyll *a*, biomass and the AI values from each site were therefore analyzed by t-tests for comparisons between sites.

Results

Macroinvertebrate Density Sampling

Insects were the most diverse class of organisms found with 39 taxa. Sixteen of the twenty macroinvertebrate "core" taxa identified were from the orders Ephemeroptera, Trichoptera, and Diptera. The orders Hymenoptera, Hemiptera, Coleoptera, and Odonata were collected in numbers too low to be included in statistical analysis but are listed with the "core" taxa in Table 4.1.

Table 4.1. List of taxa identified in McLoud Run.

Insecta				
Ephemeroptera				
	Caenidae	*Caenis amica*		
	Baetidae	*Baetis flavistriga*		
	Ephemerellidae	*Ephemerella* sp.		
Trichoptera				
	Hydropsychidae	*Hydropsyche bronta*		
		Cheumatopsyche sp.		
	Hydroptilidae	*Hydroptila* sp.		
		Oxyethira sp.		
		Ochrotrichia sp.		
Coleoptera				
	Elmidae	*Stenelmis sandersoni*		
	Hydrophilidae	*Hydrobius* sp.		
	Dytiscidae	*Agabinus* sp.		
	Melyridae			
Diptera				
	Chironomidae			
		Tanypodinae	*Thienemannimyia* sp.	
			Larsia sp .*(or Krenopelopia* sp.*)*	
		Orthocladiinae	*Thienemanniella* sp.	
			Orthocladius sp.	
			Cricotopus sp.	
			Cricotopus trifascia group	
			Brillia sp.	
		Chironominae		
			Chironomini	*Dicrotendipes* sp.
				Polypedilum sp.
				Saetheria sp.
			Chironomini	*Phaenopsectra* sp.
				Paracladopelma sp.
				Synendotendipes sp.
			Tanytarsini	*Krenopsectra* sp.
				Rheotanytarsus sp
				Paratanytarsus sp

(table continues)

Table 4.1 (Continued). List of taxa identified in McLoud Run.

Diptera		
	Simuliidae	*Simulium vittatum*
		Simulium aureum
		Simulium tuberosum
	Empididae	
	Tabanidae	*Silvius* sp.
	Syrphidae	
	Psychodidae	*Telmatoscopus* sp. *or Pericoma* sp.
	Tipulidae	*Tipula* sp.
	Ceratopogonidae	*Culicoides* sp.
Hymenoptera		
	Diapriidae	*Trichopria* sp.
Hemiptera		
	Belostomatidae	*Abedus* sp.
Odonata		
	Libellulidae	*Erythemis* sp.
	Coenagrionidae	
Mollusca		
	Sphaeriidae	*Pisidium* sp.
	Planorbidae	*Helisoma* sp.
		Promenetus sp.
	Physidae	*Physella* sp.
	Ancylidae	*Ferrissia* sp.
Hydracarina		
		Sperchon sp.
Crustacea		
Amphipoda		*Gammarus fasciatus*
		Hyalella azteca
Annelida		
Oligochaeta		
	Naididae	*Stylaria* sp.*?*
	Tubificidae	*Limnodrilus* sp.*?*
Hirudinea		
	Glossiphoniidae	
	Erpobdellidae	
Turbellaria		

Mayflies (Ephemeroptera) included only one core taxon, *Baetis flavistriga* (Baetidae). This species was found only at 42nd Street and J Avenue and in relatively low numbers (Figure 4.1). Baetidae are not considered as sensitive to organic enrichment as other families of Ephemeroptera. In April two *Ephemerella* sp. (Ephemerellidae) were found; one each at both McLoud Place and J Avenue. The species *Caenis amica* (Caenidae) was also collected at 42nd Street in August 2002. However, samples from this date were heavily disrupted by flooding and not included in this study.

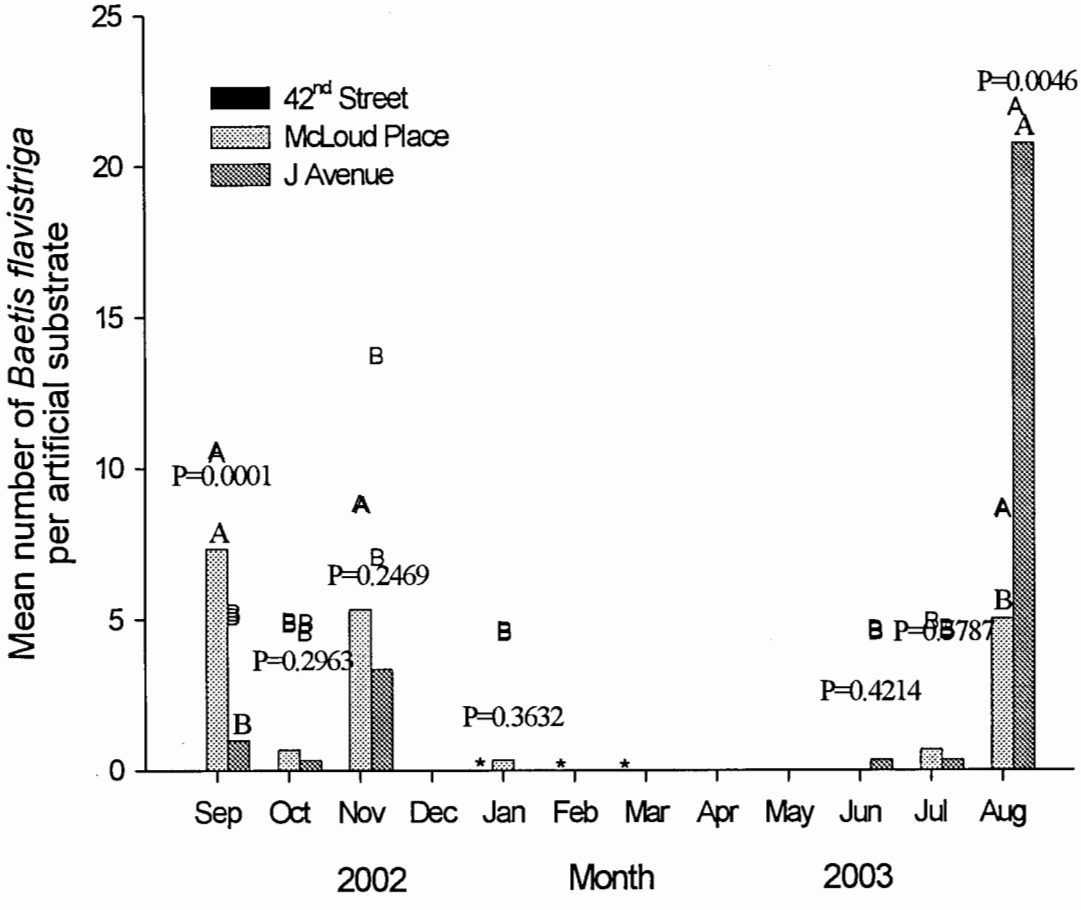

Fig 4.1. Mean number of *Baetis flavistriga* per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Baetis flavistriga* was not collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test in January.

The order Trichoptera contained two core taxa, *Hydropsyche bronta* (Hydropsychidae) and *Cheumatopsyche* sp. (Hydropsychidae). The family Hydropsychidae is considered one of the least sensitive Trichoptera families (Pontasch and Cairns 1991; Garie and McIntosh 1986) and are often among the most abundant insects in rivers with intermediate organic enrichment. Jones and Clark (1987) found that *Hydropsyche* were restricted to areas of low to moderate urbanization, and were not found in highly urbanized areas in Maryland streams. In McLoud Run, *Hydropsyche bronta* was found at all sites throughout the study period. The mean number of *H. bronta* per substrate was less than ten on each sampling date and site except in July and August, 2003 at J Avenue when the mean numbers were 14.7 and 47.7, respectively (Figure 4.2). The *Cheumatopsyche* sp. numbers were highest in July and August, 2003 as well, with peak mean numbers per substrate of 90.3 and 164.7, respectively (Figure 4.3). Jones and Clark (1987) found *Cheumatopsyche* in great numbers in low to moderate areas of urbanization in Maryland and high numbers in areas of high urbanization. The family Hydroptilidae was represented by three genera *Hydroptila*, *Oxyethira* and *Ochrotrichia*, none of which were "core" taxa.

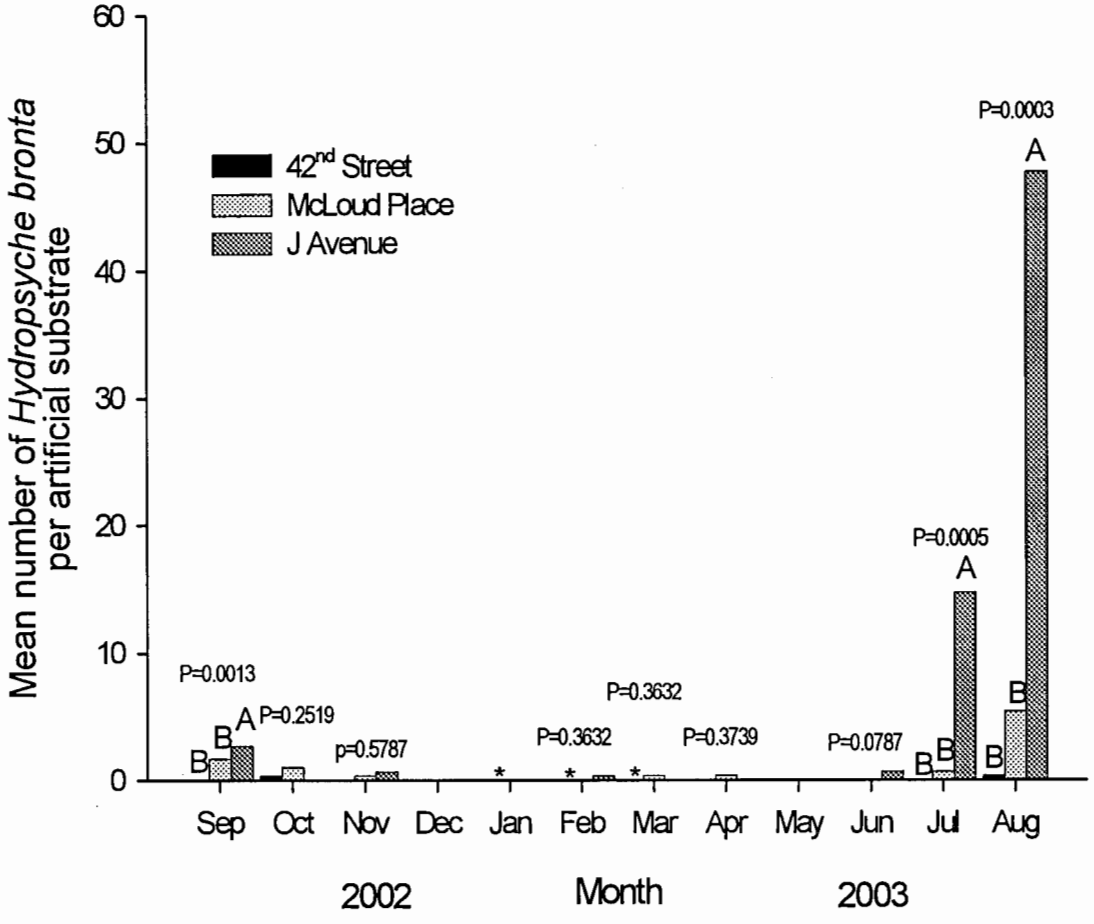

Fig 4.2. Mean number of *Hydropsyche bronta* per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Hydropsyche bronta* was not collected on dates with no P-values

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

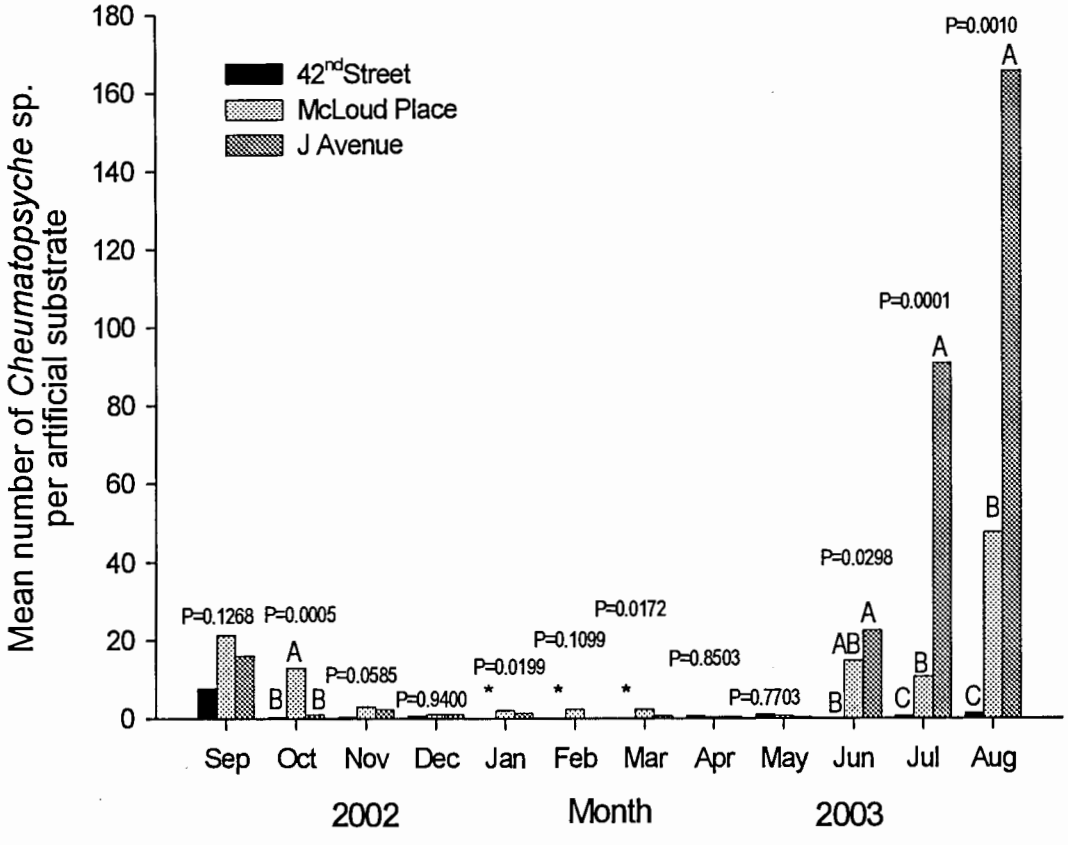

Fig 4.3. Mean number of *Cheumatopsyche* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

Diptera was the most species rich insect order collected in this study and included 13 "core" taxa. Nine of the core taxa belonged to the family Chironomidae, three to Simuliidae, and one to Psychodidae.

Chironomidae were the most often collected family of Diptera at McLoud Run. Pedersen and Perkins (1986) suggest that chironomids maintained relatively stable populations in Kelsey Creek (a degraded urban stream) as compared to other groups of insects due to their relatively short generation time and their rapid invasion potential. Many Chironomidae are considered to be relatively tolerant to poor water quality. Members of the Chironomidae subfamily Tanypodinae were not readily distinguishable with a dissecting microscope and, therefore, were considered one "core" taxon. However, several random slide mounts of Tanypodinae head capsules were prepared to identify which genera were present. Of these mounts approximately 90% belonged to the genus *Thienemannimyia*. The remaining individuals were from the genera *Larsia* and *Krenopelopia*. Tanypodinae were found at all sites on most sampling dates. However, mean numbers of Tanypodinae never exceeded ten individuals per substrate at any site (Figure 4.4).

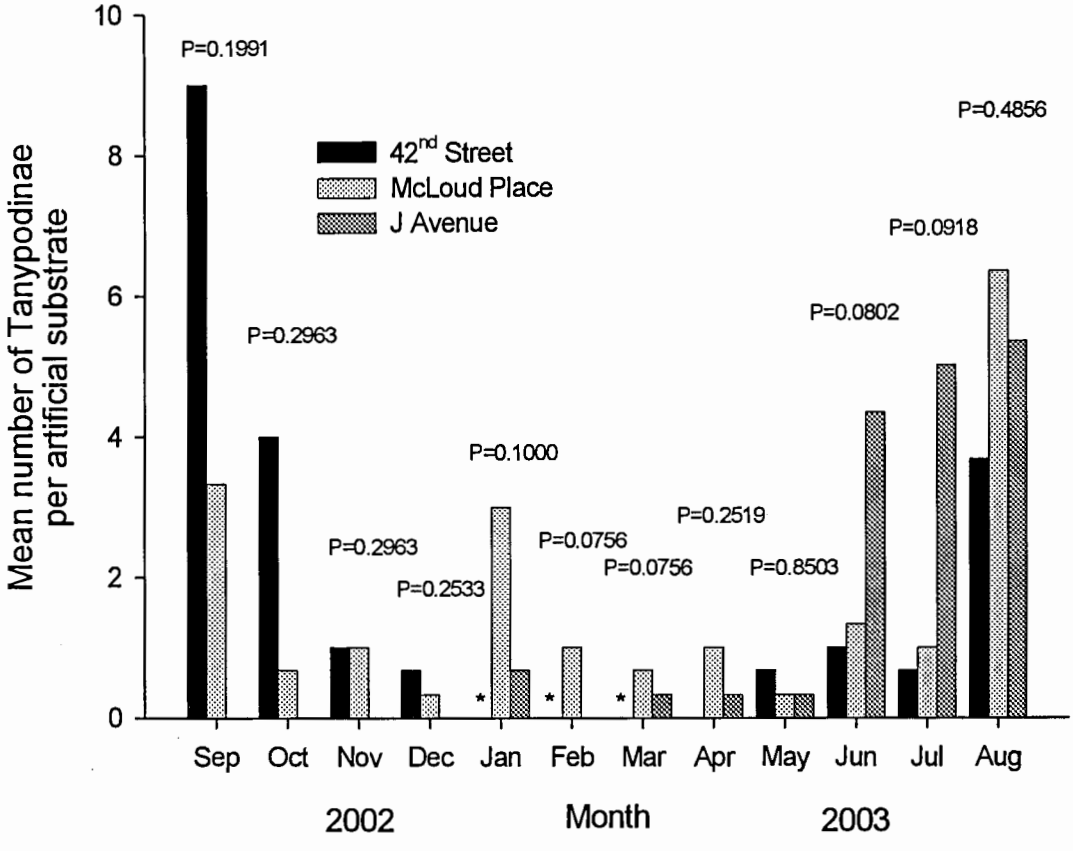

Fig 4.4. Mean number of Tanypodinae per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

Genera of the subfamily Orthocladiinae were also difficult to differentiate with a dissecting microscope. Only the genus *Thienemanniella* was easily separated. A number of headmounts were prepared from the remaining Orthocladiinae to ascertain the predominant genera. From this sample *Brillia*, *Orthocladius*, and *Cricotopus* were the only genera found. *Thienemanniella* was considered one "core" taxon and the remaining Orthocladiinae genera were considered another. *Thienemanniella* was found at all sites, on most sampling dates. This genus never exceeded a mean number of 25 individuals per substrate (Figure 4.5). The other genera of Orthocladiinae were collected on all sampling dates at all locations with relatively high densities (Figure 4.6). A study by Jones and Clark (1987) found numbers of *Cricotopus* and *Orthocladius* to be positively correlated with urbanization.

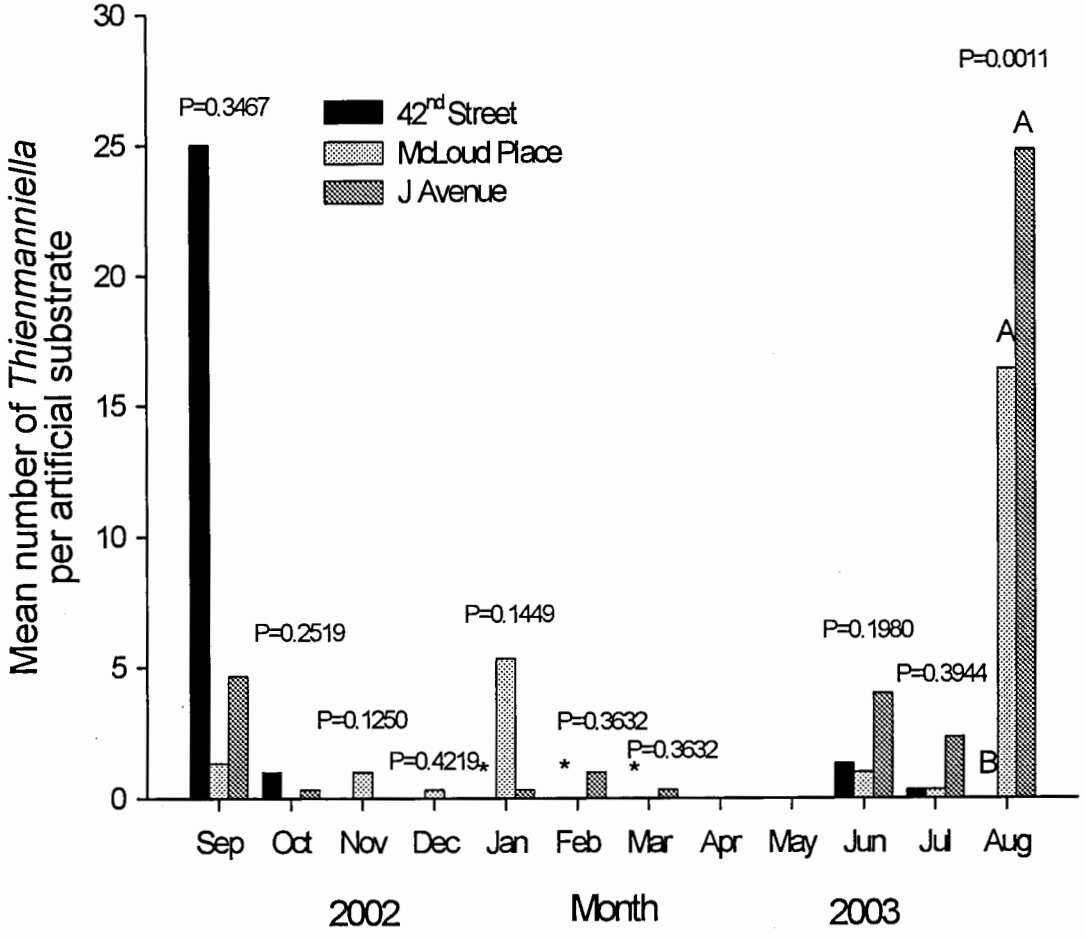

Fig 4.5. Mean number of *Thienemanniella* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Thienemanniella* sp. was not collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

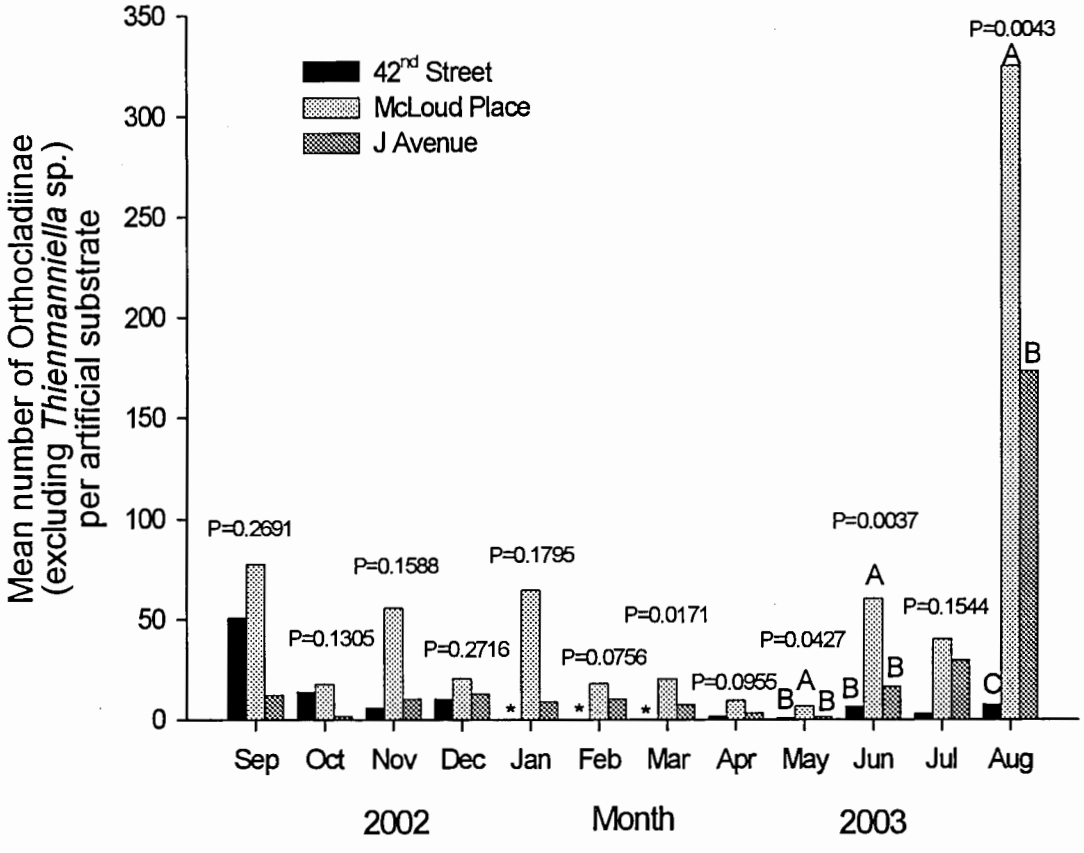

Fig 4.6. Mean number of Orthocladiinae (excluding *Thienemanniella* sp.) per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

All genera of the subfamily Chironominae were easily identified with the dissecting microscope. Of the six genera identified from the tribe Chironomini, *Dicrotendipes, Polypedilum, Saetheria,* and *Phaenopsectra* were "core" taxa. *Paracladopelma* and *Synendotendipes* were not analyzed statistically. *Dicrotendipes* was found at all locations on most sampling dates, however, it was typically found in low numbers (Figure 4.7). The genus *Polypedilum* was found at all sites but in the largest numbers at 42nd Street (Figure 4.8). Some species of *Polypedilum* are known to have oxygen binding proteins in their haemolymph (Watanabe, *et al.* 2002). This facilitates respiration in areas of depleted oxygen. The 42nd Street site appeared to have anoxic subsurface sediments and *Polypedilum* may have exploited this niche. *Polypedilum* are frequently found in sandy substrates (Barton and Smith 1984). Although they do not depend on sandy environments, they can utilize them when more favorable substrates are lacking (Barton and Smith, 1984). *Saetheria* also was most prevalent at 42nd Street, although it was found at the other sites (Figure 4.9). It is important to note that *Saetheria* (as well as at least two species of *Paracladopelma*) are known only from shifting-sand environments (Barton and Smith, 1984). The rock filled substrates utilized in this study occasionally became filled or partially filled with sand and silt. It is likely that all collections of these species are from silt laden substrates. The genus *Phaenopsectra* was found in limited numbers at all sites (Figure 4.10). Three genera from the tribe Tanytarsini were identified. *Krenopsectra* and *Rheotanytarsus* were "core" taxa; *Paratanytarsus* was not. The genus *Krenopsectra* was found at all sites in limited numbers (Figure 4.11). *Rheotanytarsus* was also found at

all sites and most dates (none were collected during May at any site) but was most abundant at 42nd Street and J Avenue in September 2002 (Figure 4.12).

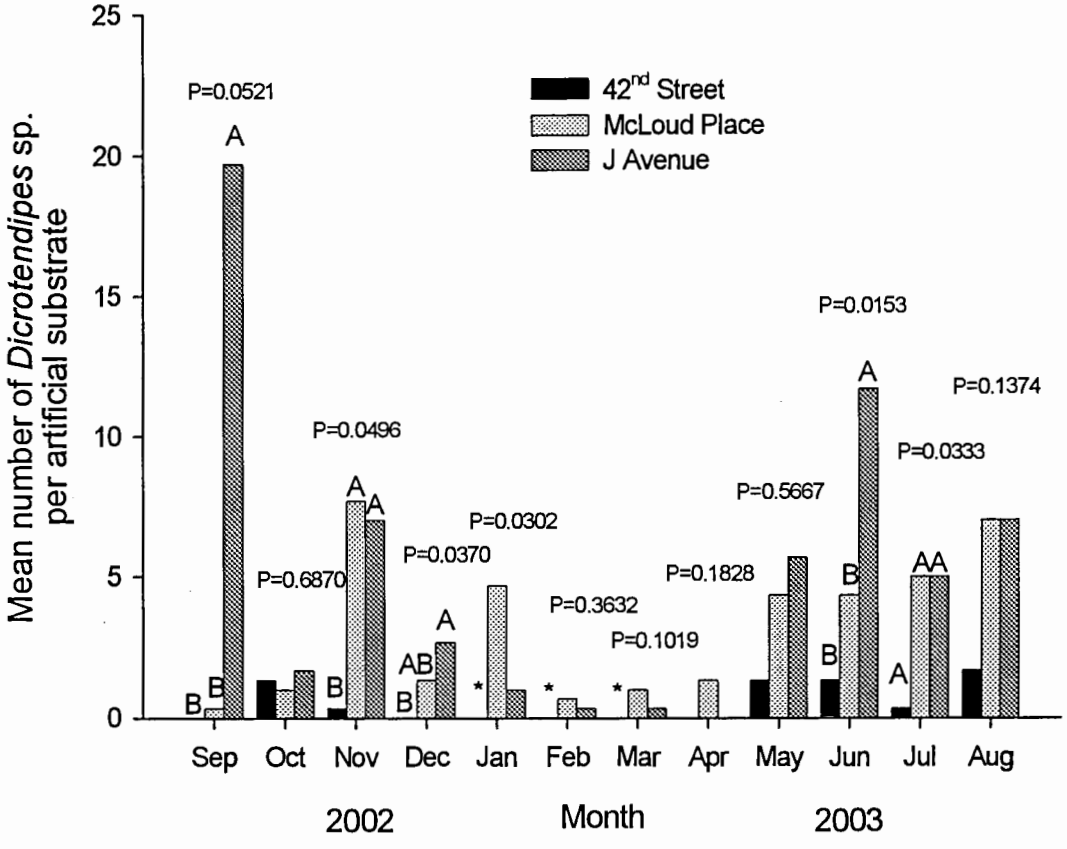

Fig 4.7. Mean number of *Dicrotendipes* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

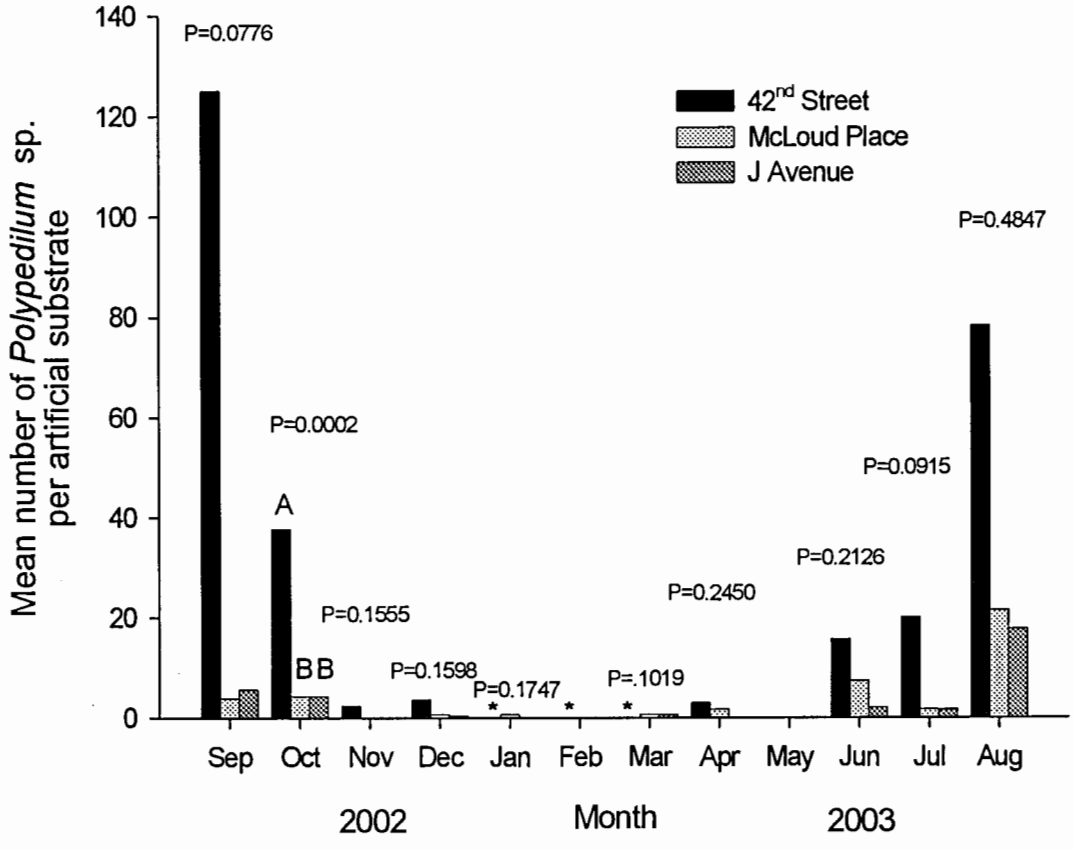

Fig 4.8. Mean number of *Polypedilum* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Polypedilum* sp. were not collected at any site during February and May, 2003.

* 42nd Street was not sampled on these dates due to freezing. McLoud Place and J Avenue were compared with a t-test in January and March, 2003.

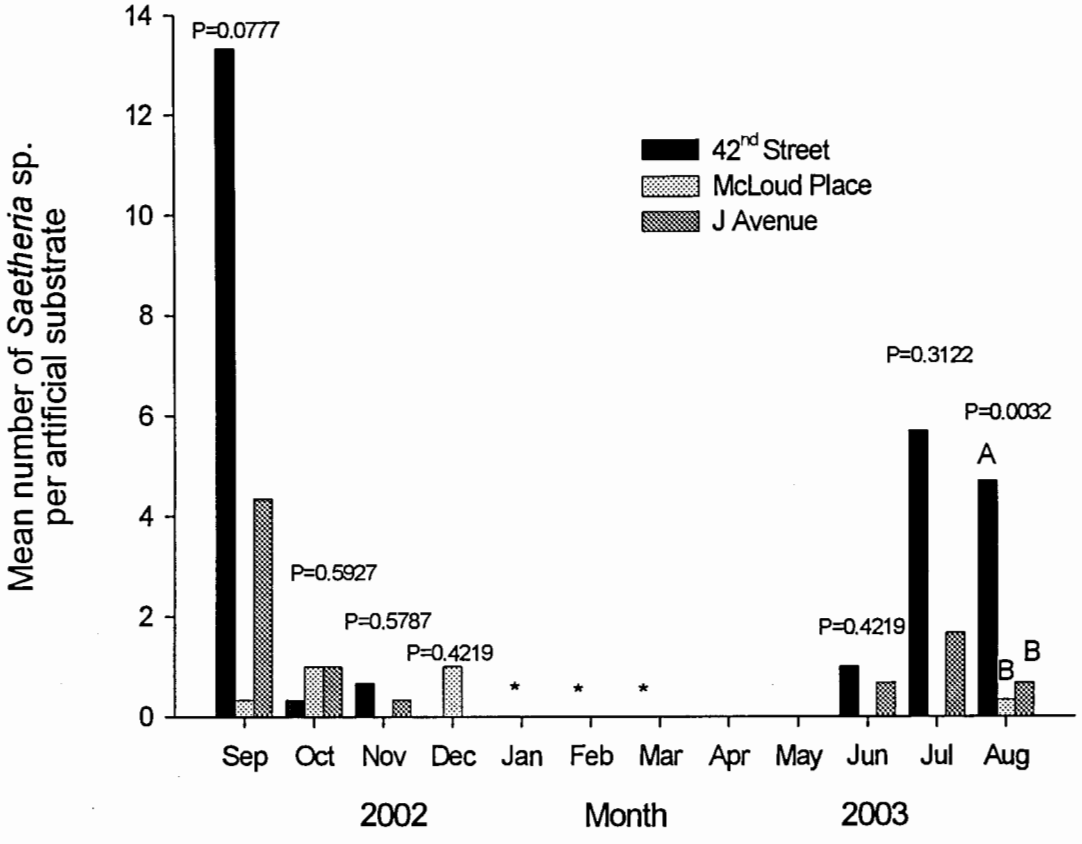

Fig 4.9 . Mean number of *Saetheria* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Saetheria* sp.were not collected at any site from January to May, 2003.

* 42nd Street was not sampled on these dates due to freezing.

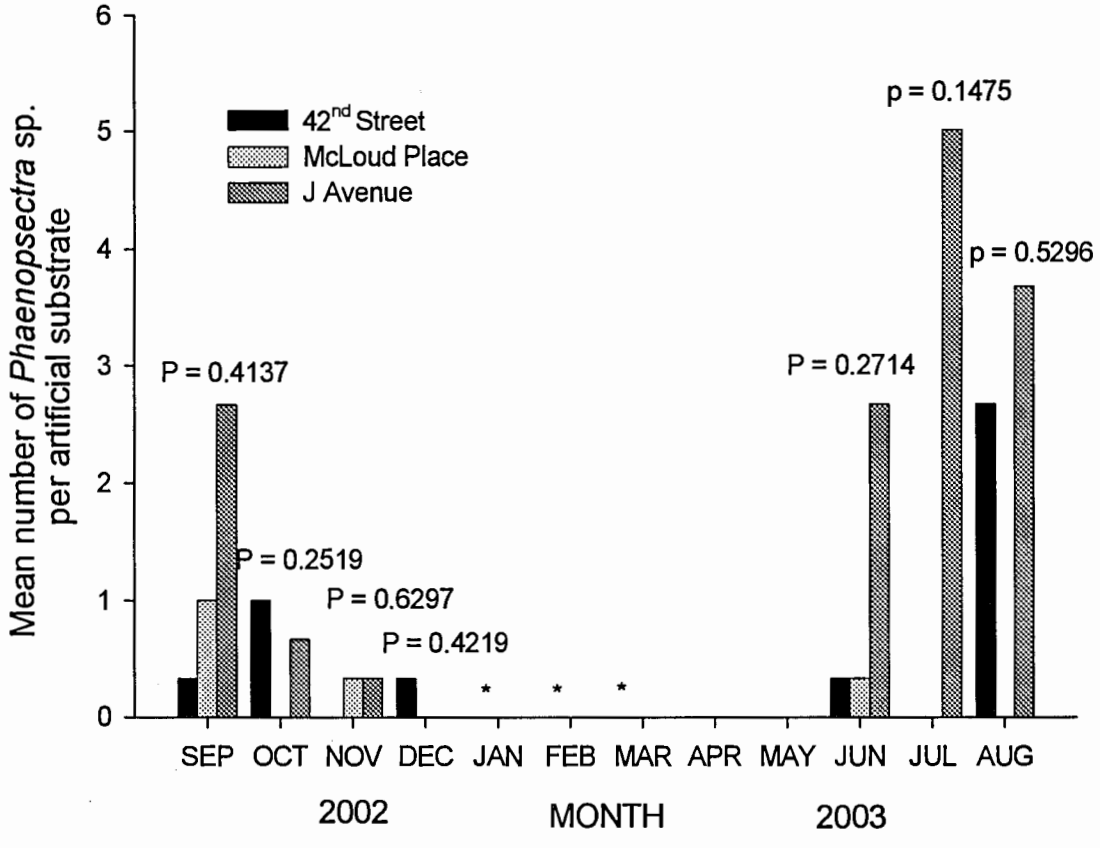

Fig 4.10. Mean number of *Phaenopsectra* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Phaenopsectra* sp.were not collected at any site from January to May, 2003.

* 42nd Street was not sampled on these dates due to freezing.

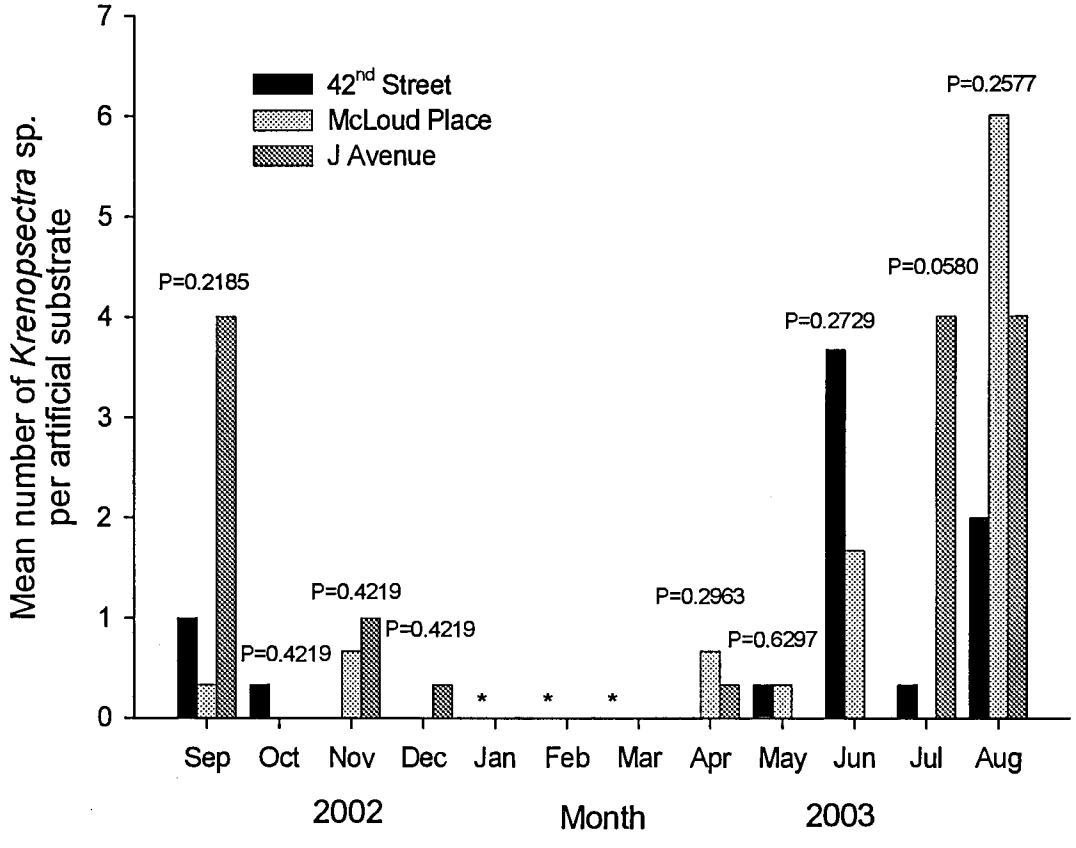

Fig 4.11 . Mean number of *Krenopsectra* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Krenopsectra* sp. was not collected at any site from January to March 2003.

* 42nd Street was not sampled on these dates due to freezing.

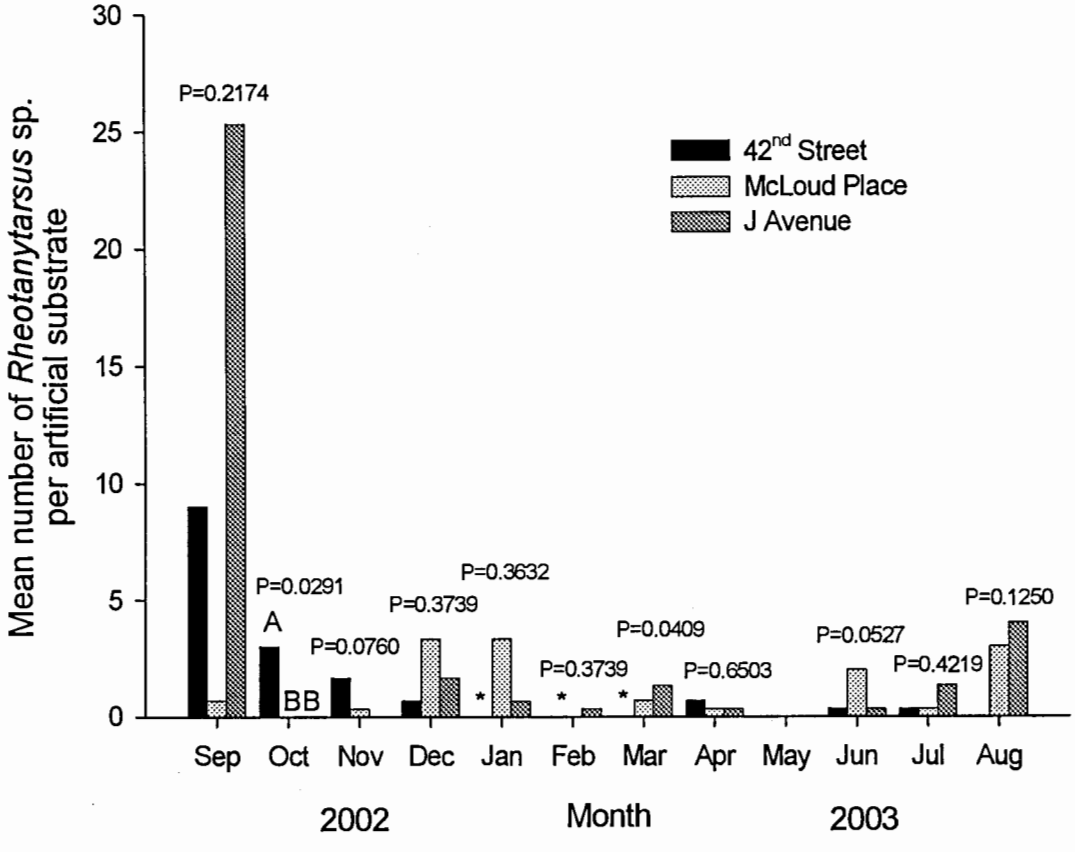

Fig 4.12. Mean number of *Rheotanytarsus* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Rheotanytarsus* sp. was not collected at any site in May, 2003.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

The Psychodidae (Diptera) genera *Telmatoscopus* or *Pericoma* (these genera are indistinguishable as larvae) constituted one "core" taxon. Psychodidae were found in limited numbers at 42nd Street and McLoud Place (Figure 4.13). Psychodidae are amphineustic and therefore, can withstand low dissolved oxygen in areas of high organic enrichment such as wastewater treatment plant trickling filters (Wiederholm, 1984). However, Minshall (1969) collected *Pericoma* sp. in reaches of a relatively unpolluted stream.

All three species of Simuliidae (Diptera) identified were considered "core" taxa. *Simulium vittatum* was the most commonly collected blackfly. This species was collected at all sites on all sampling dates but in the greatest numbers at McLoud Place (Figure 4.14). *Simulium aureum* was found at all sites in limited numbers (Figure 4.15). Both *S. vittatum* and *S. aureum* were found in greatest numbers at J Avenue during August of 2003. *Simulium tuberosum* was collected at J Avenue and more often at McLoud Place (Figure 4.16) but was not found at 42nd Street.

Four dipteran families collected were not considered "core" taxa. One Empididae specimen was collected in January at J Avenue. One Syrphidae was collected during April at J Avenue. Tipulidae were collected in October and June at McLoud Run and during October at 42nd Street. Two Ceratopogonidae (*Culicoides* sp.) were collected; one in August at 42nd Street and another in September at McLoud Place.

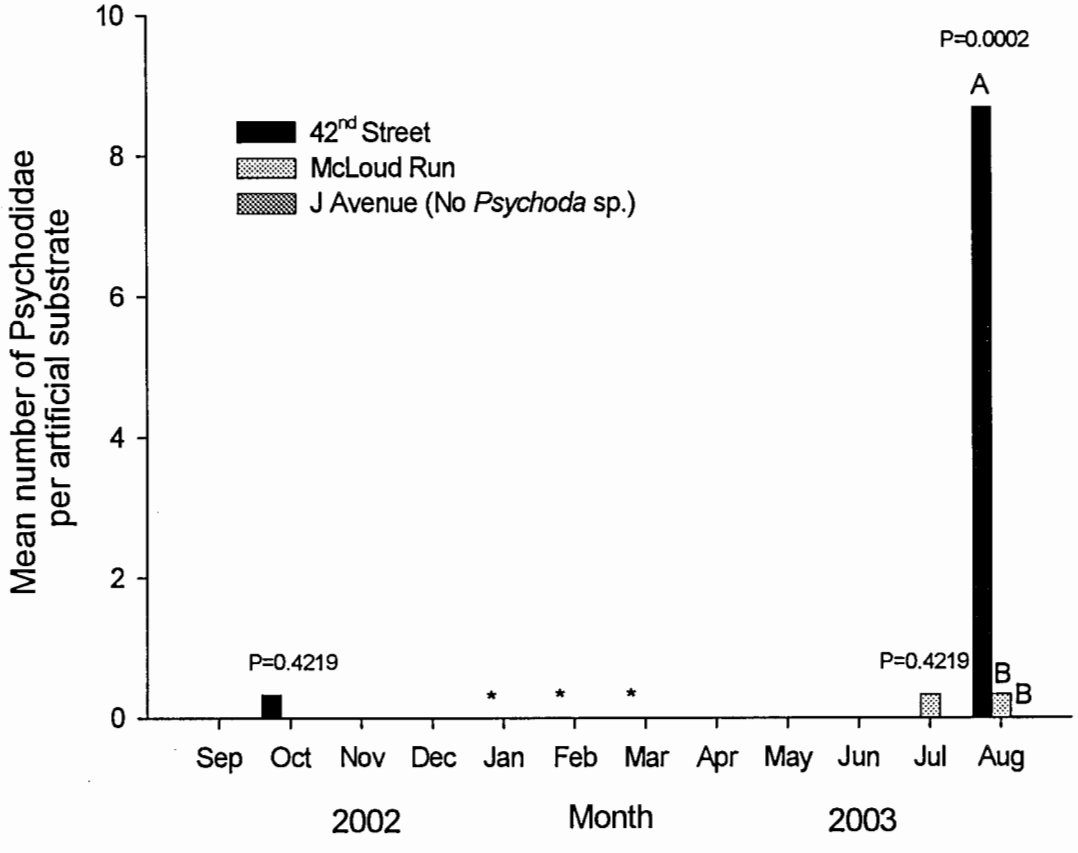

Fig 4.13. Mean number of Psychodidae per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. No Psychodidae were collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing.

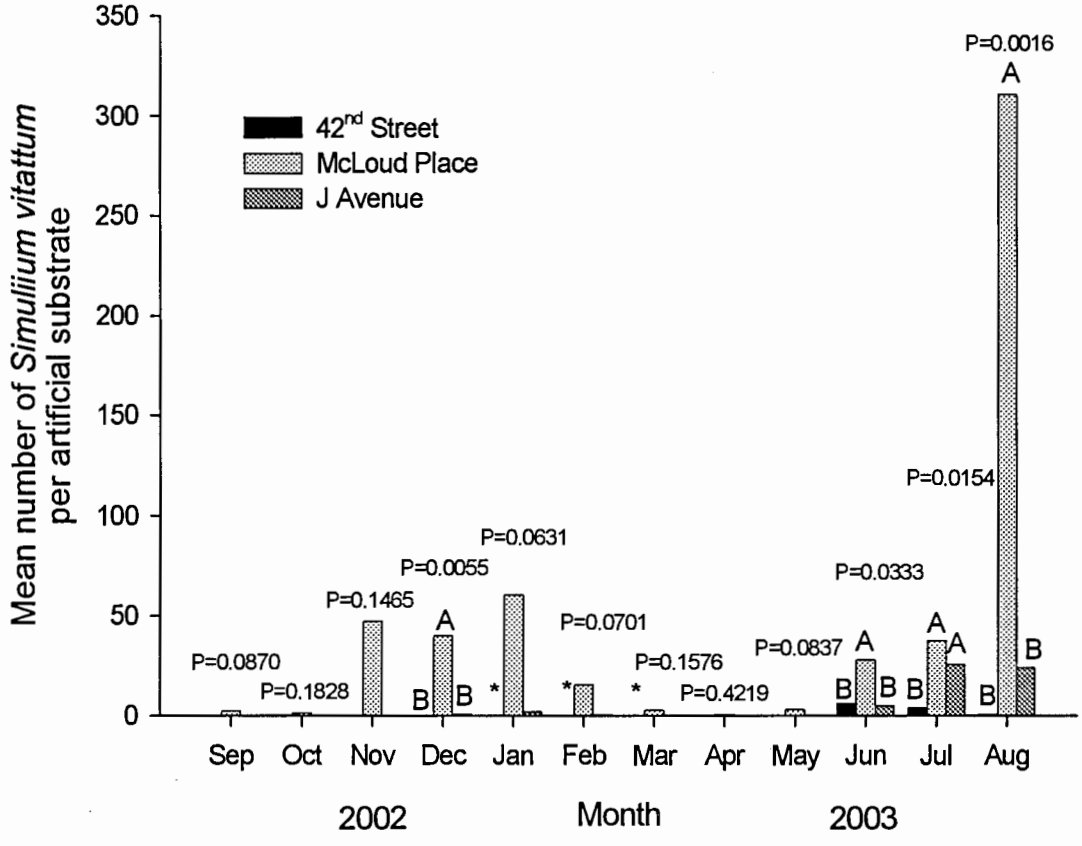

Fig 4.14. Mean number of *Simulium vittatum* per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

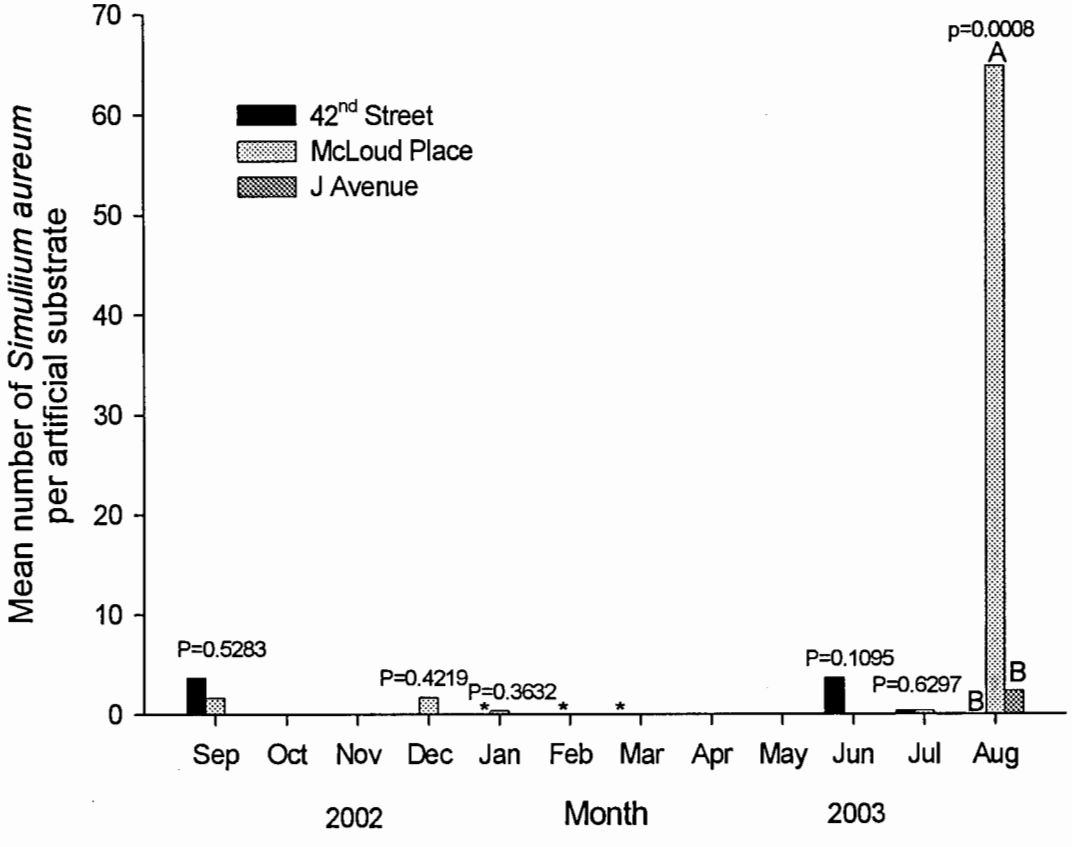

Fig 4.15. Mean number of *Simulium aureum* per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from a one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Simulium aureum* were not collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing. McLoud Place and J Avenue were compared with a t-test in January 2002.

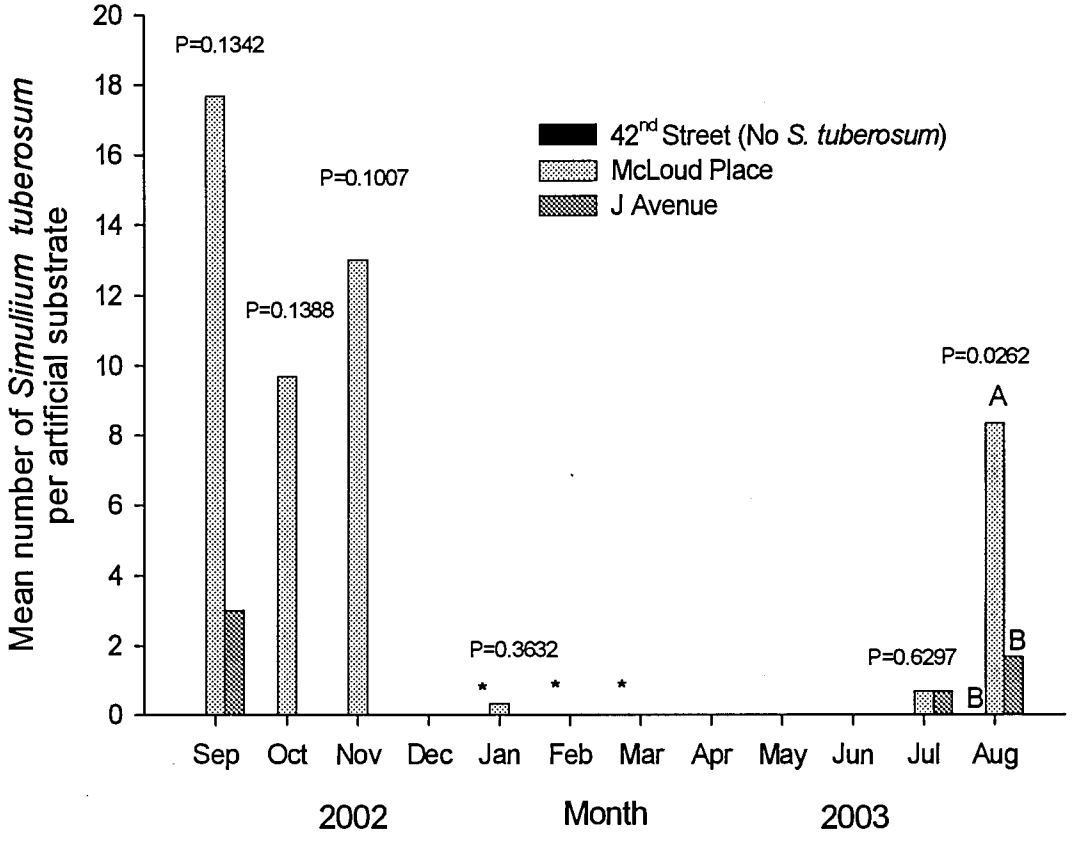

Fig 4.16. Mean number of *Simulium tuberosum* per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from a one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Simulium tuberosum* was not collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing. McLoud Place and J Avenue were compared with a t-test in January, 2003.

Stenelmis sandersoni (Coleoptera: Elmidae) was the only species of beetle collected regularly, but was not considered a "core" taxon. No adults of this species were collected and all other species of Coleoptera were limited to the collection of one or two individuals from each of the families Melyridae, Dytiscidae and Hydrophilidae.

Oligochaetes (Annelida) were the most abundant "core" taxon collected in this study. However, taxonomic resolution is poor. Oligochaetes (aquatic earthworms) are notoriously hard to identify, requiring slide-mounting or serial dissections for lower level identification. Due to poor preservation in ethanol and often large numbers, adequate identification to lower taxonomic levels is nearly impossible and always impractical. Also, the macerating effect of sand can destroy posterior segmentation and separate individuals in the process of asexual budding, both of which are useful in taxonomic identification. Unfortunately, lack of taxonomic knowledge of oligochaetes (with the exception of *Limnodrilus* species and *Tubifex tubifex*) reduces their utility in water quality studies (Goodnight and Whitley, 1960; Aston, 1973). For this study all Oligochaetes were considered one "core" taxon. However, a number of random individuals were slide mounted for further identification. Of the slide mounted specimens all belonged to Tubificidae (likely *Bothrioneurum* sp. or *Limnodrilus* sp.) or Naididae (likely *Stylaria* sp.). Oligochaetes were found at all sites on all sampling dates (Figure 4.17).

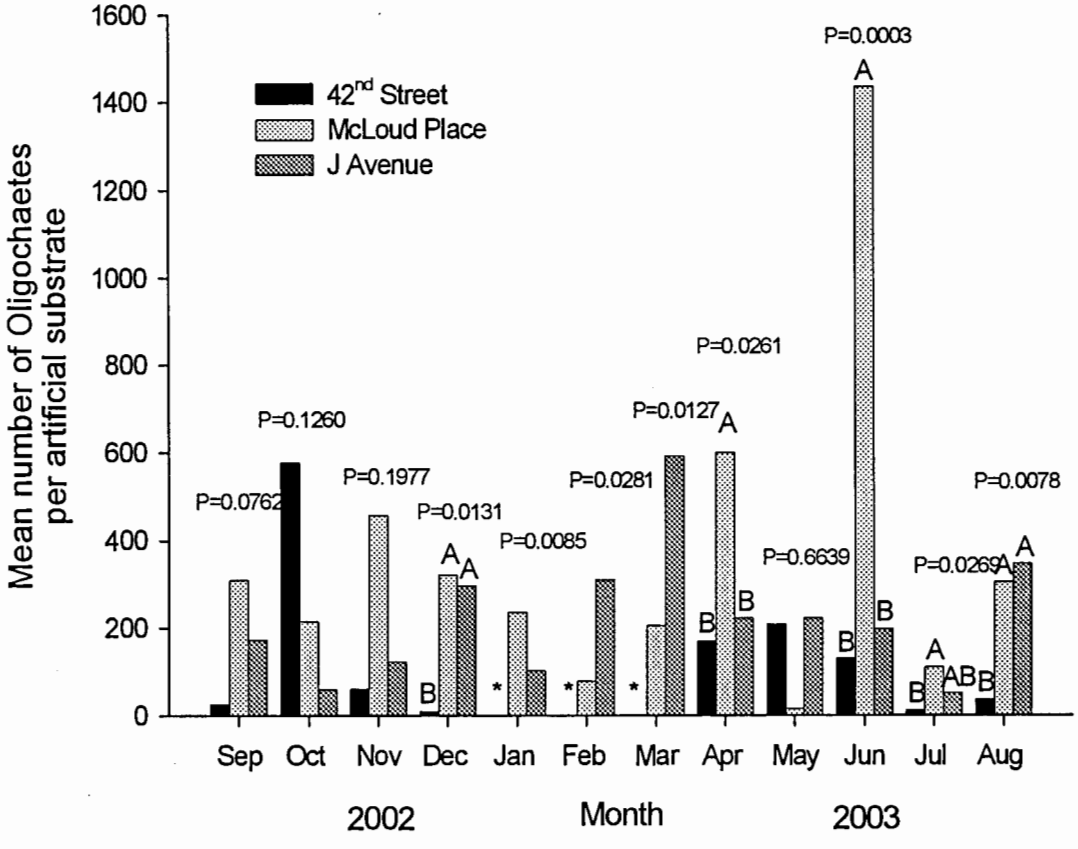

Fig 4.17. Mean number of Oligochaeta per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

Leeches (Annelida: Hirudinea) were considered one "core" taxon. Leeches were collected at 42nd Street and rarely at J Avenue. Numbers at 42nd street were highest during June, July and August (Figure 4.18). Most specimens belonged to the family Erpobdellidae. Although keys exist to further identify leeches, preservation in ethanol makes identification difficult. Some erpobdellids are known to be scavengers and/or predators on gastropods and small oligochaetes (Pennak, 1989). This species' peak abundance occurred with peak *Physella* sp. abundance (see below) suggesting they may have been be part of the erpobdellid diet. Two specimens of Glossiphoniidae were also collected at 42nd street in August.

Bivalve molluscs were represented only by one species of Sphaeriidae (*Pisidium* sp.). All of these specimens were small and could not be identified to the species level. *Pisidium* was only found at 42nd Street in low numbers and was not considered a "core" taxon.

Two families of Gastropoda (Mollusca) were present in the macroinvertebrate samples, Planorbidae (*Helisoma* sp. and *Promenetus* sp.) and Physidae (*Physella* sp.). A third family, Ancylidae (*Ferrissia* sp.) was found on rocks at J Avenue, but not in any of the artificial substrates. *Helisoma* sp. and *Promenetus* sp. were collected rarely at 42nd Street and were not considered "core" taxa. Although these species were collected only rarely in the artificial substrates, they were commonly encountered at 42nd Street along with *Physella* sp., beneath the substrate support frames. Although *Physella* was also occasionally collected at McLoud Place and J Avenue, it was most common at 42nd

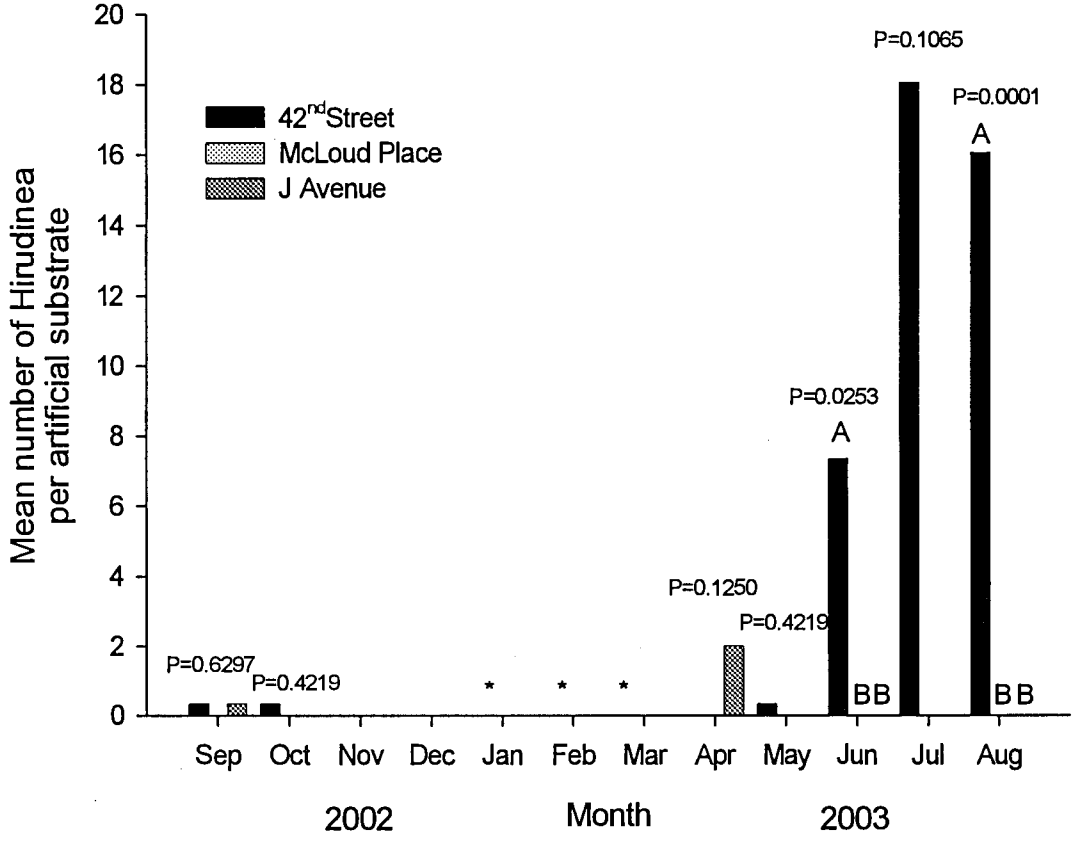

Fig 4.18. Mean number of Hirudinea per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. Hirudinea were not collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing.

Street, with greatest abundance in the summer months (Figure 4.19). This genus is known to prefer areas of high organic enrichment and to withstand dissolved oxygen concentrations of <2 ppm (Pennak 1989).

. Two species of Amphipoda (Crustacea) were collected; neither were "core" taxa. *Gammarus fasciatus* was collected in June at McLoud Place and in April and September at J Avenue. *Hyalella azteca* was collected in December at McLoud Place and in November at J Avenue. Neither species was found at 42nd Street.

Hydracarina (Arachnida) were represented by one genus, *Sperchon*; a "core" taxon. This mite was collected year round at McLoud Place and in July and August at J Avenue (Figure 4.20). It was not found at 42nd Street. These mites were abundant in some samples sorted for chlordane analysis and may be under-represented due to morphological similarity to sand and organic matter.

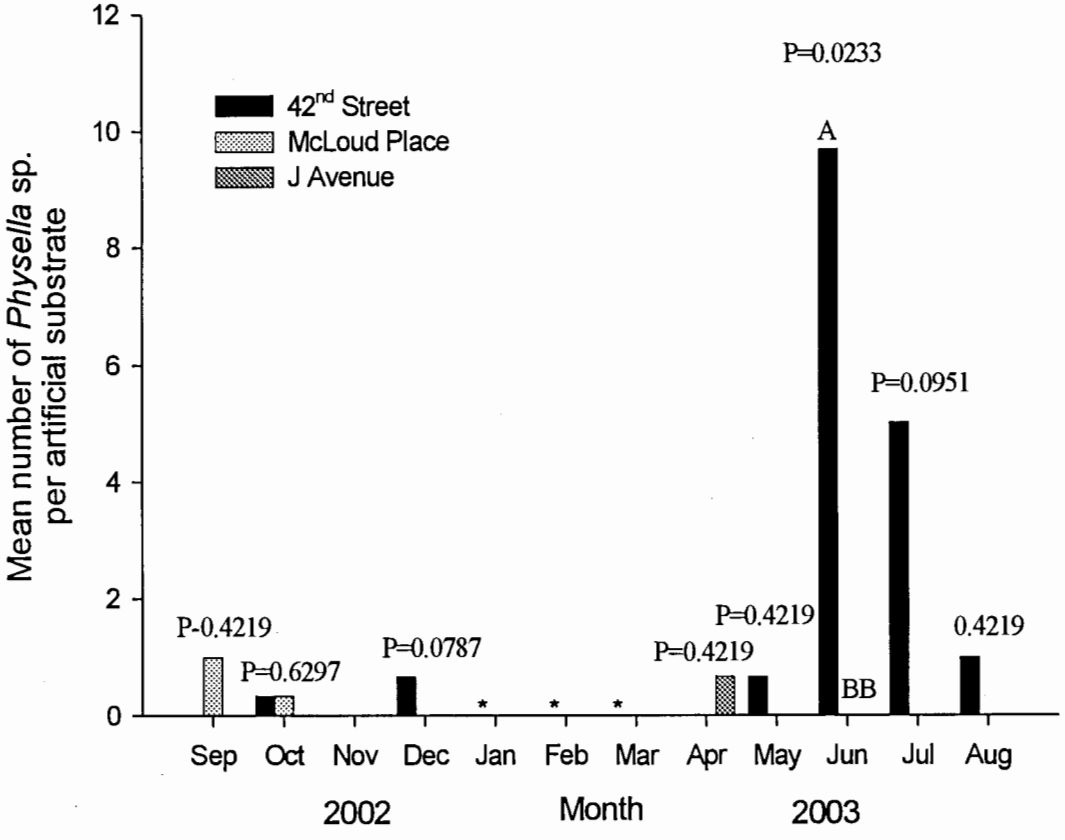

Fig 4.19. Mean number of *Physella* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Physella* sp. was not collected on dates with no P value.

* 42nd Street was not sampled on these dates due to freezing.

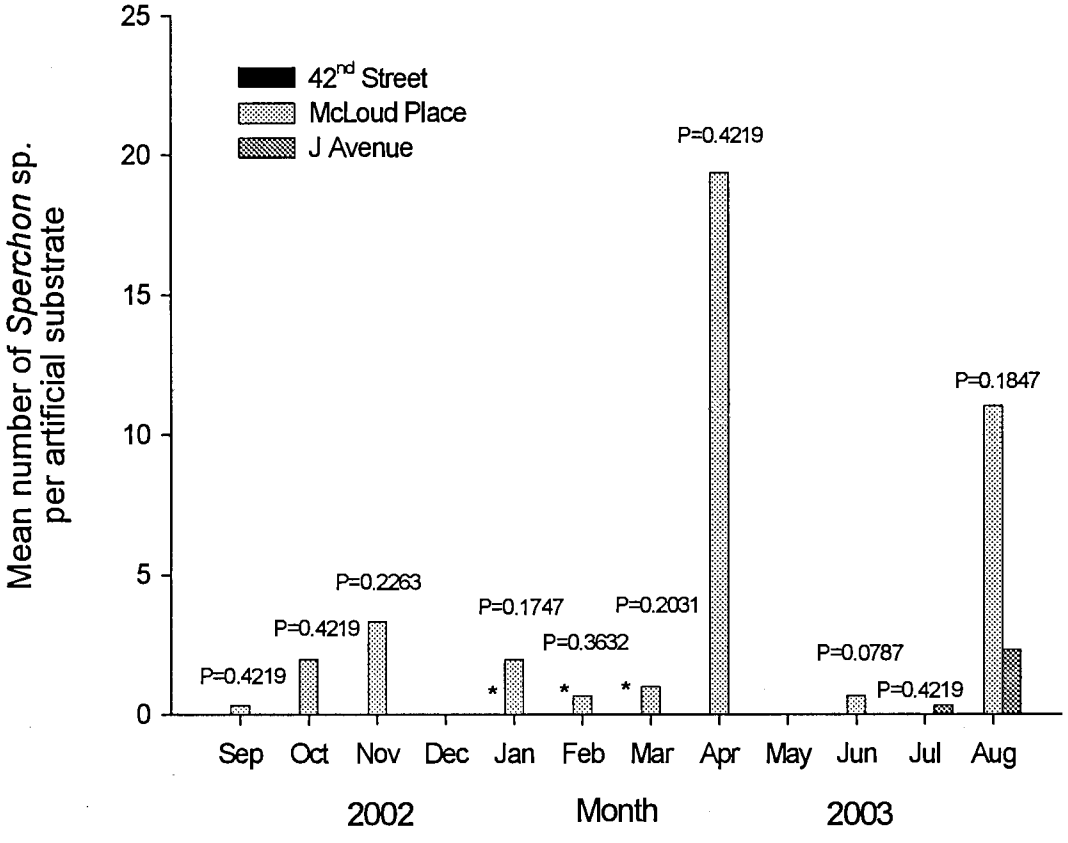

Fig 4.20. Mean number of *Sperchon* sp. per artificial substrate at three sites in McLoud Run (Iowa, USA). P values are from one-way ANOVA for each date among sites. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range Test for the separation of means. *Sperchon* sp. was not collected on dates with no P value and never at 42nd Street.

* 42nd Street was not sampled on these dates due to freezing so McLoud Place and J Avenue were compared with a t-test.

Functional Feeding Groups

In this study each taxon was assigned to a functional feeding group (FFG) using the classification of Merritt and Cummins (1996). If more precise information existed (e.g., to genus or species), it was used. However, species level identification was often lacking and primary modes of feeding often change during development. When specific knowledge was absent the general FFG of the next highest taxonomic level was assigned. When the organism fit more than one FFG it was assigned to the most important or dominant FFG during its aquatic life cycle.

Collector/gatherers were the most abundant FFG at all sites, on most sampling dates, compromising greater than 50% of the benthos on most dates (Figures 4.21-4.23). This group feeds on detritus and other loose material deposited on the substrate. Chironomidae and Oligochaeta compromised most of the collector/gatherers in this study. Shredders feed on coarse particulate organic matter (>1mm diameter) such as dead leaves. Shredders were relatively abundant at 42nd Street but were poorly represented at McLoud Place and J Avenue. Most of the shredders in this study were Chironomidae, with larger shredders (e.g., Plecoptera) notably absent. Collector/filterers utilize fine particulate organic matter (≤1mm diameter) and were relatively abundant at McLoud Place and J Avenue, but were uncommon at 42nd Street. In McLoud Run, Hydropsychidae and Simuliidae comprised most of the collector/filterers. Tanypodinae comprised the majority of predators collected at all sites. Piercer/herbivores were exclusively represented by Hydroptilidae and were seldom collected during this study. Scrapers, mostly Baetidae and

Gastropoda, were relatively abundant at 42nd Street and were occasionally collected in small numbers at J Avenue and McLoud Place.

At 42nd Street (Figure 4.21), collector/gatherers were the most abundant macroinvertebrates sampled except in September 2002 and during July and August 2003 when shredders were more abundant. Predators and shredders were most abundant during summer and fall months. Scrapers collected at 42nd Street were mostly Gastropods. Collector/filterers and piercer/herbivores were poorly represented at 42nd Street.

At McLoud Place (Figure 4.22), collector/gatherers were the predominant FFG on all sampling dates. Collector/filterers were present in relatively large numbers in March, April and May 2003 and in smaller numbers throughout the rest of the year. Relatively small numbers of predators were found in September 2002 and in April and August, 2003. Other FFG's were poorly represented throughout the year.

Collector/gatherers were also the most abundant FFG at J Avenue on all sampling dates except in July when collector/filterers were more abundant (Figure 4.23). From November 2002-May 2003 collector/gatherers compromised nearly 100% of all macroinvertebrates sampled. In September 2002 and during June, July and August 2003 collector/filterers were relatively well represented. Other FFG's were seldom collected at J Avenue.

It is important to note that Chironomidae were typically considered collector/gatherers and compromised a large percentage of collector/gatherer numbers.

However, it is generally accepted that chironomid feeding behavior is poorly understood in many species and chironomids often change feeding behavior during their life cycle. Also, similar morpho-behavioral mechanisms of food acquisition in different species (within a genus) may result in the ingestion of markedly differing food items (Merritt and Cummins, 1996). The role that Chironomidae play in organic matter processing is important, and further research is needed to accurately utilize chironomids in FFG surveys.

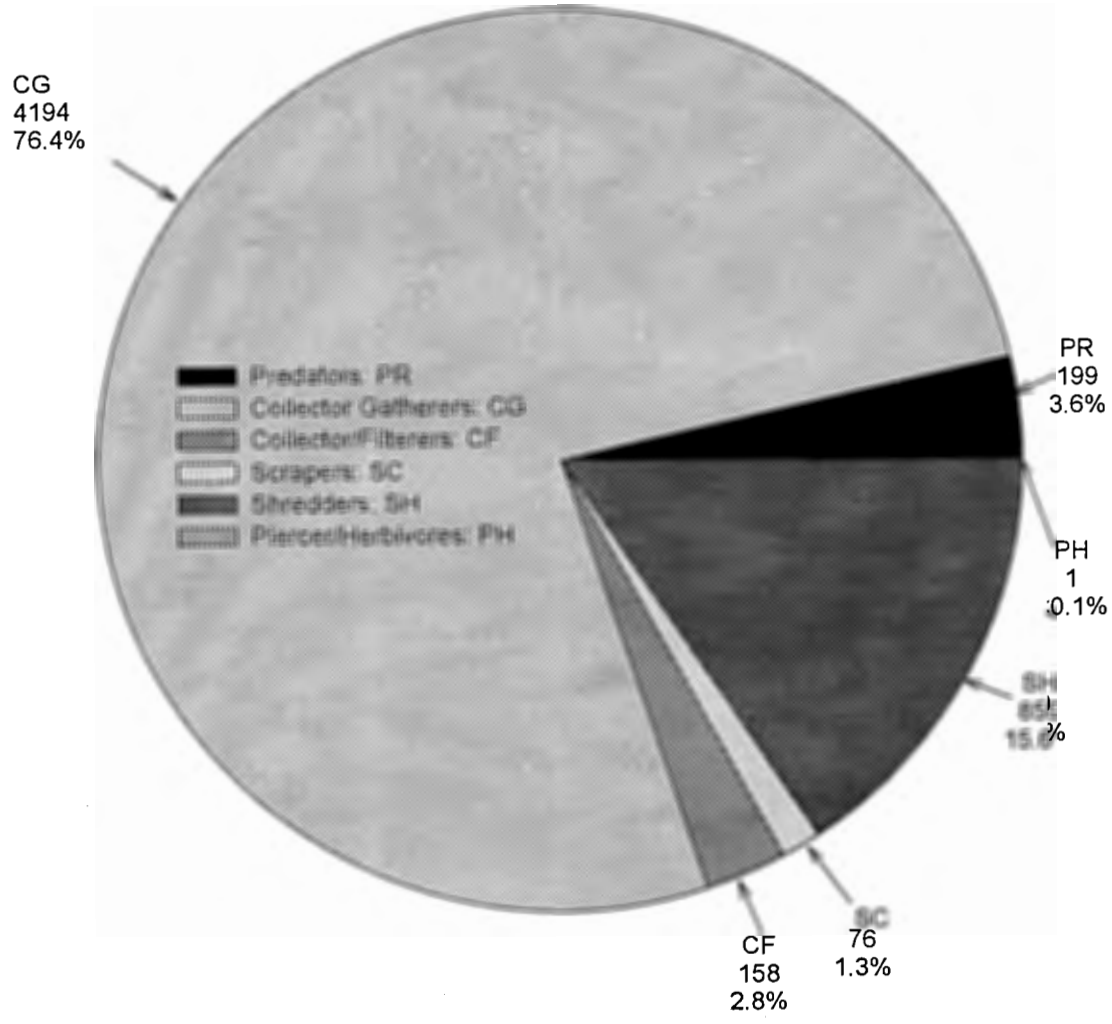

Fig 4.21. Functional feeding groups (FFG) at 42nd Street. Data are combined from all sampling periods (FFG symbol, number collected, percent of total; total n = 5,487).

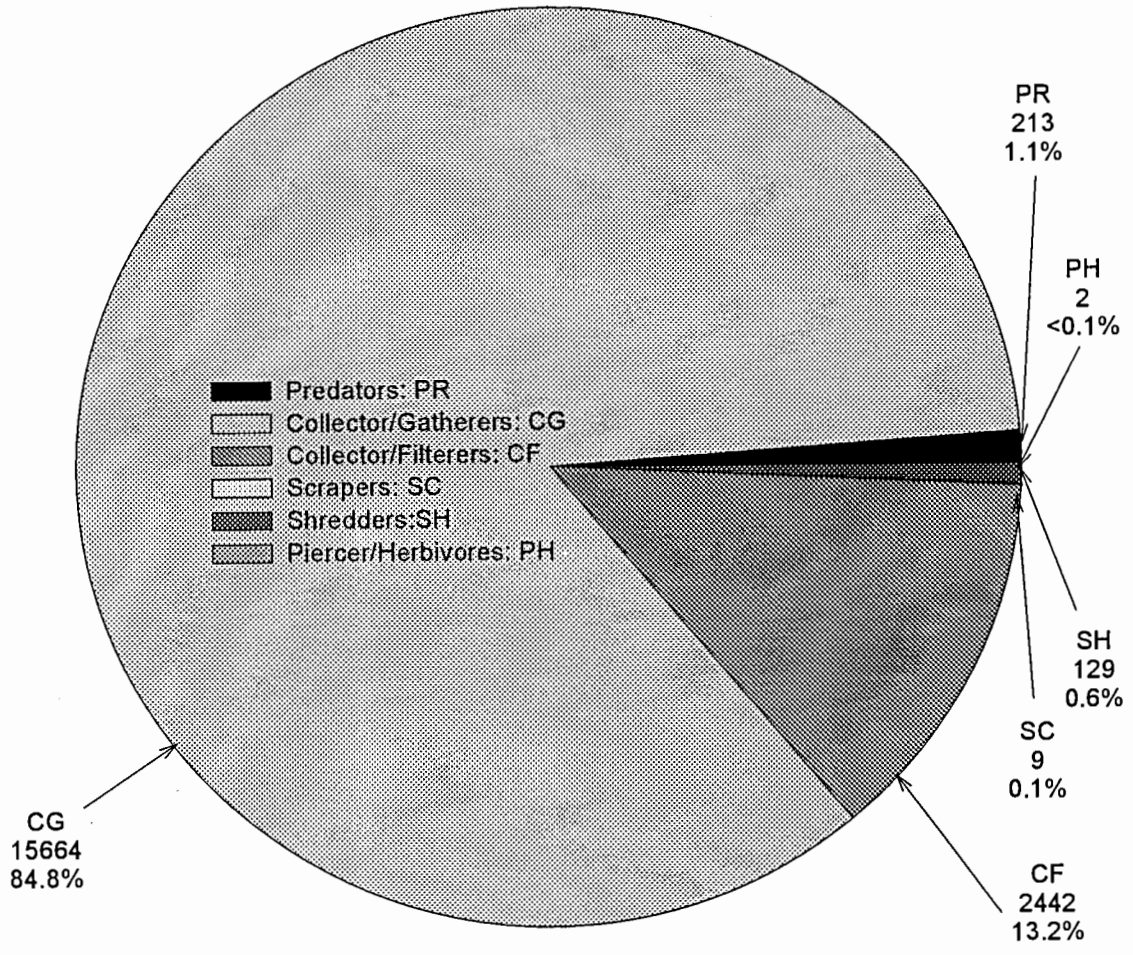

Fig 4.22. Functional feeding groups (FFG) at McLoud Place. Data are combined from all sampling periods (FFG symbol, number from FFG collected, percent of total; n=18,456).

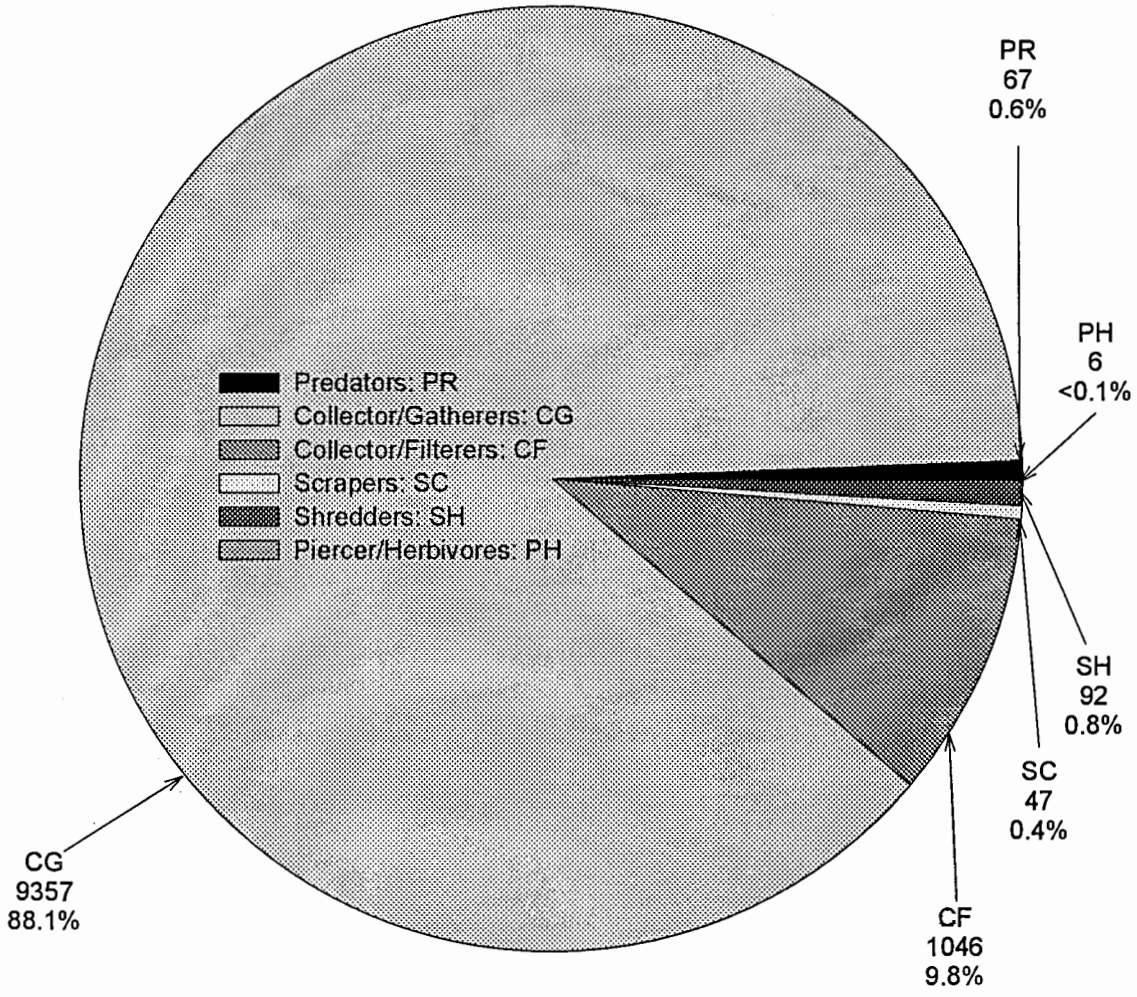

Fig 4.23. Functional feeding groups at (FFG) J Avenue. Data are combined from all sampling periods (FFG symbol, number from FFG collected, percent of total n=10,615).

Hilsenhoff Biotic Index

The Hilsenhoff Biotic Index (HBI) was developed as a means to categorize streams according to amount of organic enrichment using macroinvertebrate community composition. Each insect species (or lowest possible taxon) is given a score (from 1 "good" to 10 "poor"). This score is then multiplied by the number of that species (or taxa) collected. This is repeated for all taxa and then all of these numbers are summed. This number is then divided by the total number of insects collected to give the index value (Hilsenhoff 1987). In this study HBI scores were used from both Hilsenhoff (1998) and from the U.S. Environmental Protection Agency's Rapid Bioassessment Protocols (Barbour, *et al.* 1999). Another version (Modified HBI: Hilsenhoff 1998) uses no more than ten individuals of any taxon to calculate the Modified Hilsenhoff biotic index. Abundant species skew the original HBI and standard deviation between dates is increased. By utilizing a maximum of ten individuals the modified HBI provides a closer approximation of a site's "true" index value.

It is important to note that: (1) the HBI and modified HBI were not intended to compare between sites from the same river and should not be used to compare within a site over time. Hilsenhoff (1977) suggests that diversity and species richness indices are more useful for comparing similar sites on the same stream or the same site from year to year than the HBI or Modified HBI; (2) correction for natural stream variation (substrate quality, streamflow, etc.) and seasonal differences (i.e. species presence/absence) do not exist; (3) important insects such as Cheumatopsyche, Simuliidae and Chironomidae are

difficult or impossible to identify to species and great variations in tolerance may exist within genera; (4) summer collection is discouraged (lower dissolved oxygen may change community composition and can result in "poorer" index values); and (5) Hilsenhoff (1977) suggests using samples with approximately 100 individuals.

In this study both the HBI and the modified HBI were calculated despite inconsistencies with Hilsenhoff's (1977) original guidelines (i.e. sampling procedure, summer sampling). In addition, these indices were designed to classify streams where organic matter is the primary anthropogenic disturbance, and it is not clear how the indices are affected when coupled with other disturbances. The macroinvertebrate sampling called for year-round sampling so these data were used in calculating the HBI. However, the HBI and modified HBI values in summer months were not substantially different than other sampling dates and were therefore included in this analysis. Hilsenhoff was concerned that the HBI from summer dates would be compared between seasons, affecting interpretation. In this study these concerns appear to be unjustified; the cold-water nature of McLoud Run may have negated this effect to some extent. In this study insect numbers were often lower than 100 per sample (14 of 28 samples had >100 insects sampled), however artificial substrates appeared to sample proportional abundance with relative accuracy regardless of total abundance, thereby minimizing the importance of large sample sizes.

Tables 4.2- 4.4 show all HBI and modified HBI from 42nd Street, McLoud Place and J Avenue. Generally, the HBI and the modified HBI were similar on most sampling

dates within a site, but the mean modified HBI values were slightly higher than the mean HBI within each site. The mean HBI and mean modified HBI within-site values were similar at all sites and between 5.5-6.1, which indicates moderate organic enrichment.

Table 4.2. Hilsenhoff biotic index (HBI) and Modified Hilsenhoff biotic index values from McLoud Run sampling 2002-2003 at 42nd Street site. Macroinvertebrate numbers were pooled from 3 replicates for calculation of biotic index.

	HBI		Modified HBI	
Month	Total Macroinvertebrates*	BI value	Total Macroinvertebrates**	BI value
Sep	721	5.71	77	5.30
Oct	195	4.60	48	5.98
Nov	41	5.41	30	5.63
Dec	48	6.02	27	5.81
Jan	Site Frozen/No Sample			
Feb	Site Frozen/No Sample			
Mar	Site Frozen/No Sample			
Apr	18	5.89	†	†
May	11	6.36	†	†
Jun	122	4.07	54	6.39
Jul	104	5.80	45	5.80
Aug	318	6.28	61	6.57
		Mean= 5.57		Mean=5.97
		Range 4.07-6.36		Range 5.30-6.57

* Total number of macroinvertebrates used for HBI analysis. This number includes only taxa for which index values are established.

** Total number of macroinvertebrates used for modified HBI analysis. The Modified HBI utilizes only 10 members of taxa where total numbers are greater than ten.

† No taxa contained 10 or more individuals/ Modified HBI was same as HBI.

Table 4.3. Hilsenhoff biotic index (HBI) and Modified Hilsenhoff biotic index values from McLoud Run sampling 2002-2003 at McLoud Place site. Macroinvertebrate numbers were pooled from 3 replicates for calculation of biotic index.

	HBI		Modified HBI	
Month	Total Macroinvertebrates*	BI value	Total Macroinvertebrates**	BI value
Sep	421	2.73	90	5.28
Oct	157	5.25	62	5.44
Nov	414	5.78	65	6.08
Dec	207	6.76	50	6.36
Jan	425	4.20	61	6.34
Feb	109	6.39	29	6.24
Mar	89	2.66	38	6.21
Apr	44	6.09	25	6.16
May	45	6.73	31	6.87
Jun	340	6.19	61	6.31
Jul	296	6.35	56	6.07
Aug	2264	6.94	121	5.88
		Mean=5.51		Mean=6.10
		Range 2.66-6.94		Range 5.28-6.87

* Total number of macroinvertebrates used for HBI analysis (sum of means for each taxa). This number includes only taxa for which index values are established.

** Total number of macroinvertebrates used for modified HBI analysis (sum of means for each taxa). The Modified HBI utilizes only 10 members of taxa where total numbers are greater than ten.

Table 4.4. Hilsenhoff biotic index and Modified Hilsenhoff biotic index values from McLoud Run sampling 2002-2003 at J Avenue site. Macroinvertebrate numbers were pooled from 3 replicates for calculation of biotic index.

	HBI		Modified HBI	
Month	Total Macroinvertebrates*	BI value	Total Macroinvertebrates**	BI value
Sep	303	6.09	109	5.76
Oct	35	6.00	32	6.00
Nov	74	6.28	42	5.98
Dec	66	3.06	37	6.03
Jan	43	6.19	31	5.29
Feb	39	6.07	18	6.17
Mar	33	6.00	19	6.32
Apr	14	5.57	14	5.57
May	22	7.36	15	7.27
Jun	194	6.05	71	6.25
Jul	552	5.64	102	6.13
Aug	1479	5.56	113	5.84
		Mean=5.82		Mean=6.05
		Range 3.06-7.36		Range 5.29-7.27

* Total number of macroinvertebrates used for HBI analysis (sum of means for each taxa). This number includes only taxa for which index values are established.

** Total number of macroinvertebrates used for modified HBI analysis (sum of means for each taxa). The Modified HBI utilizes only 10 members of taxa where total numbers are greater than ten.

Chlordane Analysis

Initially chlordane analyses were to be performed on samples from each site and each date. However, insufficient quantities of insects from each site prevented individual analyses. Therefore, samples from all sites and dates were then pooled. No chlordane was found in the pooled sample. However the detection limit (50 μg/l) may be higher than physiologically relevant levels because 48-96 h LC50's for six macroinvertebrate species range from 15.0 to 50.0 μg/L. Of those species the stonefly *Pteronarcys californica* (Plecoptera: Pteronarcidae) was the most sensitive (Mayer and Ellersiek, 1986).

Periphyton Sampling

Sedimentation hindered periphyton sampling at 42nd Street and J Avenue throughout this study. At 42nd Street samples were not collected in December, 2002 and June, July or August 2003 because of sedimentation or in January, February and March 2003 due to freezing of this portion of the stream. McLoud Place had higher flow over the substrates throughout the year and, therefore, did not have sedimentation problems. Throughout the study this site had lush, green-brown algal growth covering the substrates. Samples could not be collected at J Avenue in September and October, 2002 and February, March, April, May and June 2003 due sedimentation. Flow was higher in July, 2003 and allowed increased algal growth . A high water event prevented collection of periphyton samples at all sites in July 2003.

McLoud Place had significantly ($p \leq 0.05$) higher periphyton biomass on all dates sampled except for August 2003 (Figure 4.24). This site had little shading riparian vegetation and therefore was exposed to sunlight for most of the day. This likely caused the increased periphyton growth at McLoud Place. Periphyton growth was noticeably less at 42nd Street and J Avenue probably due to shading and lower current velocities which allowed deposition of sand and silt on the periphyton substrates. On most dates that periphyton was analyzed at 42nd Street and J Avenue the growth of periphyton appeared as a thin brown biofilm.

McLoud Place also had the highest chlorophyll *a* values on all dates (Fig 4.25). Again, this was likely due to shade and sedimentation at 42nd Street and J Avenue. Chlorophyll *a* values at McLoud Place fluctuated in an anomalous manner throughout the year. Highest values were achieved in November and January and great variation was present throughout the year. Decreased water temperatures in November and January may have slowed grazing insect metabolism and allowed algal growth to exceed grazing.

Autotrophic Index values were high at all sites and dates (Fig 4.26). This is indicative of high organic enrichment. The APHA, *et al.* (1989) state that normal values range from 50-200 and that values above 200 indicate possible organic enrichment. In this study AI values often exceeded 200. The April sample from 42nd Street exceeded 17,000; however it should be noted that there were large amounts of decomposing leaf packs present. The saprophytic microorganisms from this decomposition, coupled with inherently low chlorophyll *a* values at this site, likely caused this ratio to be biased.

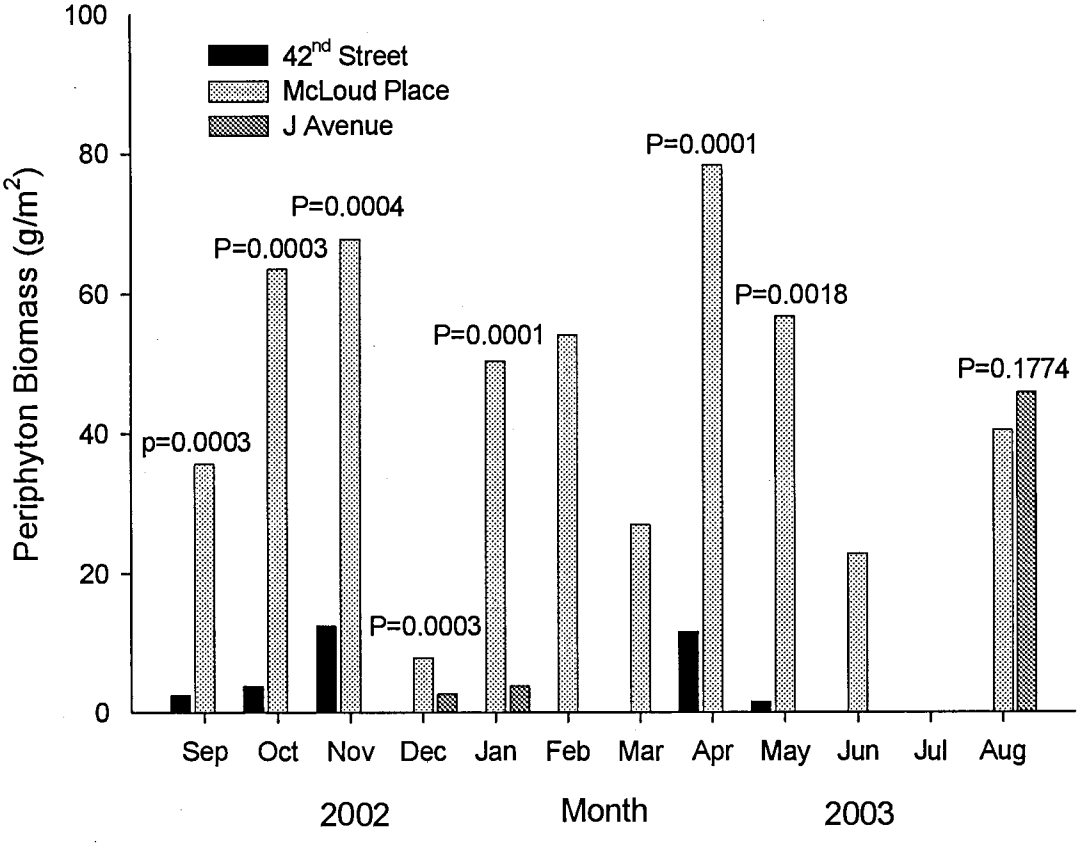

Fig 4.24. Mean periphyton biomass at three sites in McLoud Run (Iowa, USA). P values are from a t-test on each date between sites. Samples were only processed from McLoud Place during February, March and June. No samples were processed in July, 2003.

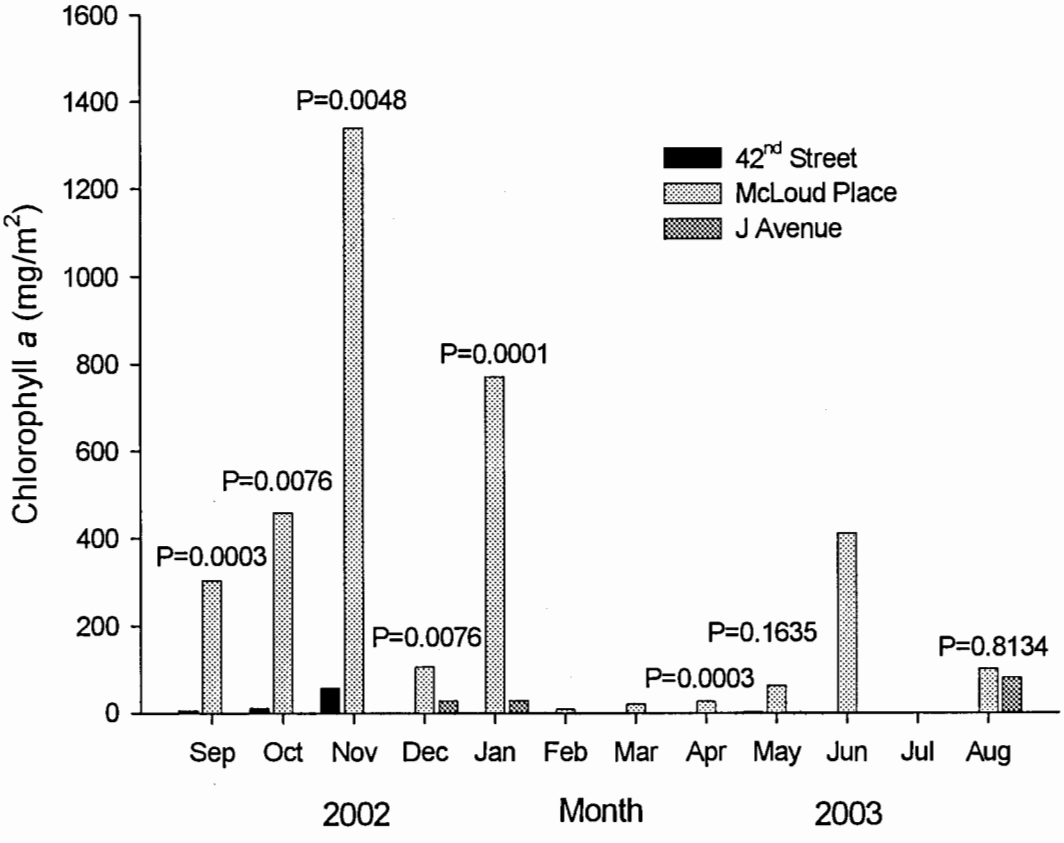

Fig 4.25. Mean chlorophyll *a* at three sites in McLoud Run (Iowa, USA). P values are from a t-test on each date between sites. Samples were only processed from McLoud Place during February, March and June. No samples were processed in July, 2003.

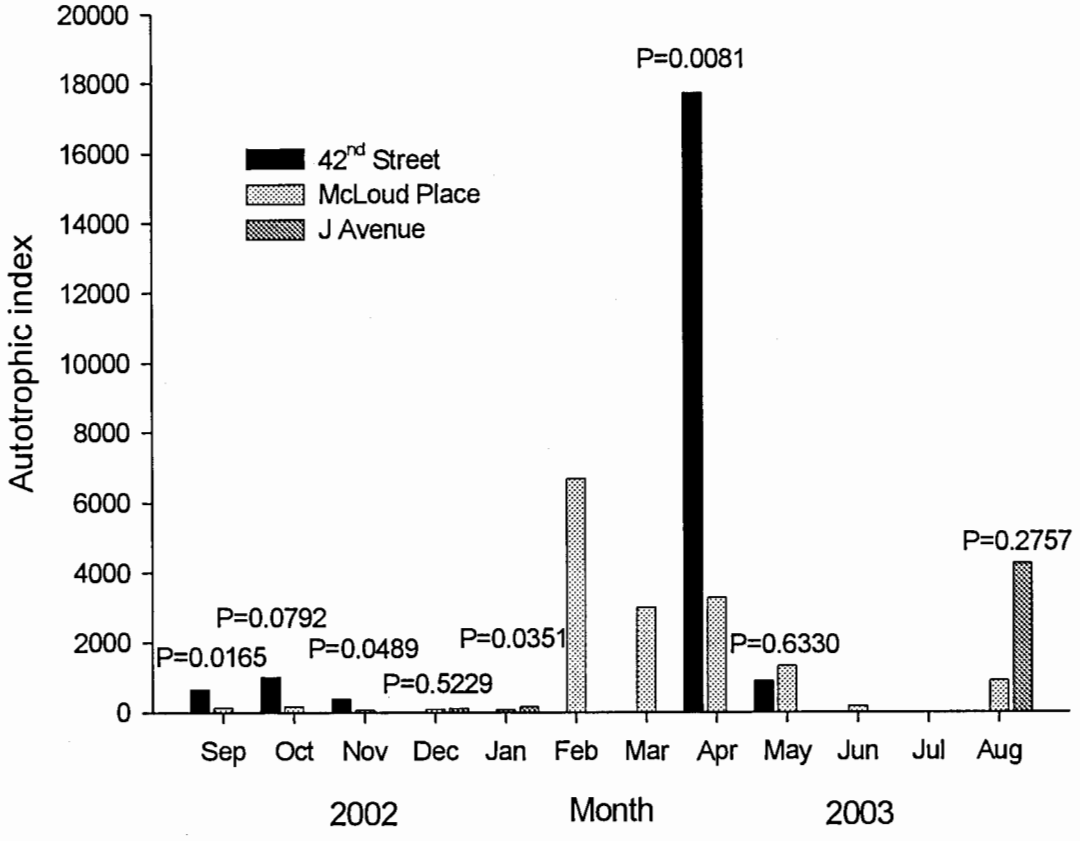

Fig 4.26. Mean Autotrophic Index at three sites in McLoud Run (Iowa, USA). P values are from a t-test on each date between sites. Samples were only processed from McLoud Place during February, March and June. No samples were processed in July, 2003.

Discussion

The macroinvertebrate data indicate that McLoud Run has low numbers of "clean water" fauna typical of unpolluted trout streams. The so called "EPT taxa" (Ephemeroptera, Plecoptera and Trichoptera) are indicative of unpolluted, healthy streams. Baetidae, the only commonly collected Ephemeroptera family in McLoud Run, are considered tolerant mayflies. Pedersen and Perkins (1986) suggest that Baetidae (as opposed to other Ephemeroptera) are more ammenable to disruption of local habitat.

The order Plecoptera, another "clean water" taxon was not collected in this study. Plecoptera (or stoneflies) are commonly collected in most Northeast Iowa rivers. Wiederholm (1984) noted that Plecoptera were the last to reappear after periods of organic enrichment. Trichoptera were collected at all sites and on most sampling dates in relatively high numbers. However, most Trichoptera collected belonged to the family Hydropsychidae, a filter-feeding family found in higher numbers in areas of increased organic enrichment (Wiederholm, 1984; Pontasch and Cairns, 1991). Generally, pollution intolerant species were uncommon in McLoud Run, while oligochaetes and tolerant Diptera fauna were present in large numbers.

This pattern of decreased numbers of "EPT taxa" and large numbers of Diptera and Oligochaetes is consistent with organic pollution. In classic cases of organic enrichment (such as below inadequately treated sewage treatment outflows); macroinvertebrate communities are dominated by Diptera, especially Chironomidae and

Simuliidae, as well as tubificid oligochaetes (Wiederholm 1984). Increased biochemical oxygen demand and decreased dissolved oxygen from increased organic load may eliminate more sensitive macroinvertebrate taxa (Hynes 1960). High organic enrichment may also explain the high biomass and AI values found at McLoud Run. In areas of high organic enrichment AI values are high due to increased heterotrophs (bacteria, saprophytic fungi, etc.) feeding on the carbon source. McLoud Run does not receive any apparent point-source inputs (i.e. wastewater treatment, manufacturing effluent, etc.) of organic pollution. However, urban runoff likely contributes a high organic burden. Sources such as lawn clippings, petroleum products and pet waste can contribute to increased organic loads. Analyses of macroinvertebrate data with the HBI and modified HBI suggest only moderate organic impact.

Organic pollution is one of many components of urban runoff that may be contributing to the degradation of McLoud Run. Urban storm runoff contains many substances toxic to aquatic life such as salt from roads, polycyclic aromatic hydrocarbons from incomplete combustion of gas, coal or wood and heavy metals such as lead, zinc, copper, cadmium and chromium. Ide (1967) noted that after DDT application to a stream, chironomid populations developed early and larger insects (especially Trichoptera) did not recover until four or more years had passed. Minshall (1969) also noted a similar phenomena after a flood. A study by Benke, *et al.* (1981) found that taxa with large body size (i.e. Plecoptera, Megaloptera) were seldom found in urban streams. The authors

suggested this was because of their inability to recover and recolonize quickly from stresses.

Insecticides and herbicides may also enter McLoud Run via storm runoff. This may be especially important in urban areas where these chemicals are applied to lawns. Insecticides such as organophosphates may hydrolyze quickly but may still enter urban streams. Persistent non-polar compounds such as chlordane may be flushed from areas of application and then bind to the organic fraction of stream sediments. When chlordane is found in solution it is typically found in the ng/L range. However, concentrations are usually higher (0.03-580 ppb) in suspended solids and sediments where it may be accumulated by benthic invertebrates (ATSDR, 1989). Unfortunately, the few studies that investigated chlordane toxicity reported chlordane concentrations in the ambient water only. No field studies have been found that looked at the significance of chlordane body burden in aquatic macroinvertebrates. This paucity of data renders interpretation of data from this study impossible. Because the detection limits for chlordane in this analysis were higher than physiologically relevant levels, the impact of chlordane on this stream is likely obscured.

Urban runoff may also cause large amounts of sediment to be deposited into McLoud Run, especially from construction areas. Throughout this study silt and sediment tended to fill interstices and cover the artificial substrates, especially at 42nd Street and J Avenue. This effectively eliminates microhabitats necessary for species-rich macroinvertebrate communities and obscures the effects of chemical contamination

(Arthington, 1982). A study by Blyth (1980) found siltation (due to damming) created a habitat for small oligochaetes and midge larvae and excluded many previously common "clean water" stream insects. This is consistent with the community composition found in this study.

High water flow in McLoud Run during storm events is another factor potentially affecting the macroinvertebrate communities in McLoud Run. Debris indicating high water could be found as high as 2.5 m above 42nd Street's low flow level, even after mild rain showers. Impervious surfaces such as parking lots and roads, coupled with storm sewers allow sudden, punctuated surges of water. These surges can scour macroinvertebrates and algae from the substrate, and as they subside, deposit fine sediments in riffle interstices rapidly. Also, these surges may rapidly increase temperatures. Many "normal" trout stream macroinvertebrates require constant cold temperatures. Temperature spikes may eliminate these cold stenotherms and allow other more tolerant species to become established.

Conclusions

McLoud Run is subject to a number of perturbations common to many urban streams. Urban runoff carries many toxic compounds directly to streams. Although chlordane was found in McLoud Run's trout, it was not found in the macroinvertebrates. However, the detection limits used in this study may have been higher than ecologically

relevant levels. Decreased EPT taxa and results of the HBI and Autotrophic Index suggest moderate organic enrichment in McLoud Run.

During the course of this study debris indicating high water could be found 2.5 m above 42nd Street's low-flow level, even after relatively insignificant rainfall events. Frequent inundation rapidly alters the hydrology of streams, changes the temperature drastically, scours the substrates and deposits sediment. The effects of flooding were not investigated, but they also likely affect community composition in McLoud Run drastically.

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EPILOGUE

Populations of aquatic macroinvertebrates in Northeast Iowa are potentially exposed to organophosphorus (OP) and carbamate insecticides throughout much of the spring and summer. Insecticide drift and runoff may reach surface waters where intensive agriculture is in close proximity to streams and wetlands. Carbamate and OP insecticides are the most commonly used in Northeast Iowa and both are acetylcholinesterase (AChE) inhibitors. These insecticides can be difficult to detect with chemical analysis and methods to analyze some of their degradation products do not exist.

AChE activity from field populations of aquatic macroinvertebrates could be used as a biomarker of exposure to OP and carbamate insecticides. In order to demonstrate inhibition in the field, background AChE activity fluctuations need to be clearly understood. Previous studies have shown that *Isonychia bicolor* (Ephemeroptera: Isonychiidae) AChE activities from Northeast Iowa streams have a great deal of variability both among sites and over time (Andersen, 2002). The studies described in this thesis expressed AChE activity both in μmoles acetylthiocholine (AThCh) hydrolyzed/min/gram tissue (as used in Andersen, 2002) as well as nmoles AThCh hydrolyzed/min/mg protein to potentially minimize variability. However, using the Bradford Assay in conjunction with the Ellman assay did not always clarify results. However, the assay is easy to use, inexpensive and sensitive. For these reasons it is suggested that future studies utilize these methods. Throughout this thesis AChE activity per g tissue was included to allow

comparison to previous studies. In future studies it may also be included, but treated as less accurate than the activity per mg protein.

Research presented in Chapter One monitored AChE activity in *I. bicolor* field populations. During 2002, weekly monitoring on the Volga River indicated a significant ($p \leq 0.05$) increase over time in both activity per mg protein and per g tissue at VRNB , but found no significant ($p > 0.05$) changes at VRTB. At VRSP, no significant ($p > 0.05$) changes in AChE activity per g tissue were found, but AChE activity per mg protein increased significantly ($p \leq 0.05$) between 5/14/02 and 6/3/02, then decreased ($p \leq 0.05$) significantly by 6/18/02. This decrease coincided with a flood event that occurred between 6/3/02 and 6/5/02.

Sampling at ten sites in May, 2002 indicated CRED, CRJV and UIKV had among the highest activities in both per g tissue and per mg protein. In addition, UICR and UIDE were notably lower than the upstream UIKV site. During July, 2002 the Upper Iowa River sites were lower than most other sites, while LCCP had the highest activity (21 μmoles AThCh/min/g tissue) of any site reported in this thesis. During September, 2002 upstream Upper Iowa River sites (UIKV and UICR) were significantly ($p \leq 0.05$) higher than UIDE in activity per mg protien. A similar decrease was reflected in the activity per g tissue; however, these activities were not significantly ($p > 0.05$) different.

The 2003 weekly sampling on the Volga and Upper Iowa Rivers indicated a significant ($p \leq 0.05$) decrease in activities at downstream sites following a major rainfall event between July 2 and July 14 that may have carried pesticide runoff into these streams.

No significant ($p>0.05$) differences were found among the ten sites during 2003 sampling in activity per g tissue or per mg protein. Similarly, monthly sampling of the Cedar River found no significant ($p>0.05$) differences in 2002 or 2003. Overall, the significant ($p\leq0.05$) decreases in AChE activities on the Volga River (2002) and Volga and Upper Iowa Rivers (2003) following rainfall events in July, suggest an association between decreased AChE activities and possible insecticide runoff events.

The studies described in Chapter Two investigated the effects of larval body size and photoperiod on *I. bicolor* AChE activity. During June and August, 2002, *I. bicolor* were sampled from the Cedar River, Cedar Falls, Iowa, USA. These insects were divided into three size classes: small, medium, and large, and analyzed for AChE activity. No significant ($p>0.05$) differences were found among size classes in either June or August.

During July, 2002, 540 *I. bicolor* were sampled from the Cedar River in Cedar Falls. 60 insects were transferred to each of 9 stream microcosms and acclimated to one of three photoperiod regimes (18:6, 12:12, and 6:18 L:D). AChE activity was monitored weekly for three weeks. No significant ($p>0.05$) differences were found, suggesting photoperiod is not an important factor affecting AChE activity in *I. bicolor*. Results of these studies suggest that any size *I. bicolor* larvae can be used for AChE analysis, and that photoperiod is not an important factor influencing AChE activity in future microcosm or field studies with *I. bicolor*.

If biomarkers are to be used to estimate environmental insecticide concentrations or simple exposure in the field, laboratory dose-response studies are necessary. Such

experiments associate AChE inhibition with dose. During research reported in Chapter Three, stream microcosms were used to expose late instar *I. bicolor* to the OP insecticide terbufos for 24 h at nominal concentrations of 0.0, 2.5, 5.0, 10.0 and 20.0 µg/L and then were purged with 200 ml/min of clean water. Streams were sampled prior to dosing, after 24 h exposure, 24 h after purging and 8 d after purging.

Following 24 h exposure, AChE activity (per g tissue) in the 20 μg/L treatment was significantly ($p \leq 0.05$) different from the control, but AChE activity (per mg protein) in the 20 μg/L treatment was not significantly ($p > 0.05$) different from the control. Following 24 h purging, AChE activities (per g tissue and per mg protein) were significantly ($p \leq 0.05$) lower in the 20 μg/l treatment. By the eighth day after purging, all organisms in the 20 μg/L treatment were dead, and among the remaining treatments there were no significant ($p > 0.05$) differences in AChE activities (per g tissue or per mg protein). An analysis of AChE activities within a treatment over time indicated that in the 10 μg/L treatment activity per g tissue was significantly higher ($p \leq 0.05$) on day 9 than on day 2 , but the increase was not significant ($p > 0.05$) for activity per mg protein.

Overall research reported in Chapter Three, indicated that terbufos concentrations near 20 µg/L significantly inhibit *I. bicolor* AChE activity and may lead to mortality following 24 h exposure. In addition, *I. bicolor* exposed to 10 µg/L or less rebounded to pre-exposure AChE activity levels within 9 days.

The research reported in Chapter Four was undertaken to quantify possible impacts on the aquatic macroinvertebrate communities in McLoud Run, an urban,

coldwater stream in Cedar Rapids, Iowa, USA. Aquatic macroinvertebrate communities were allowed to colonize rock-filled artificial substrates at three sites for one month before sampling. Sampling occurred monthly from August, 2002 until August, 2003. The colonizers from three artificial substrates at each site were preserved in alcohol on each date. Also, monthly invertebrate samples were collected for chlordane analysis. Chlordane is a persistent organochlorine pesticide previously found in trout from McLoud Run. Additionally periphyton samples were collected monthly for determination of chlorophyll *a*, biomass and the Autotrophic Index.

Invertebrate sampling indicated low numbers of "clean water" taxa such as Ephemeroptera, Trichoptera and Plecoptera (absent) as well as high numbers of "dirty water" taxa such as Chironomidae and Oligochaeta. In addition, collector/gatherers were predominant at all sites and mean Hilsenhoff Biotic Index values ranged between 5.5 and 6.1. These data suggest moderate organic enrichment at all sites.

Samples for chlordane analysis were pooled due to small macroinvertebrate biomass. However, chlordane levels were below detection limits (50 μg/l). Periphyton sampling was often hindered by sedimentation and spates. One site (McLoud Place) had significantly ($p \leq 0.05$) higher biomass and chlorophyll *a* on most dates compared to the remaining sites (J Avenue and 42nd Street). Autotrophic Index values were relatively high (i.e. a high ratio of heterotrophs to autotrophs) at all sites, again suggesting organic enrichment.

Future AChE Research

This study, as well as the study by Andersen (2002), have helped to illustrate some important details of AChE activity in *I. bicolor* populations. If this biomarker is to become a useful and accurate tool in insecticide exposure assessment a number of issues need to be addressed. The foremost problem with interpretation of field and laboratory data is from fluctuating AChE activity. If activity levels under "normal" circumstances remained static, interpretation would be quite easy. However, this is not the case. This and previous studies were the first steps of interpreting background AChE activity and its variability. Future studies need to look more closely at other sources of this variability.

Although this study found no differences among photoperiod regimes, it may be possible that AChE activity varies at certain times of the year due to photoperiodic or temperature changes. Lutz (1974) stresses the importance of photoperiodic regulation of growth and development (i.e. synchronous larval development, emergence, mating and oviposition) in aquatic insects, especially near the vernal and autumnal equinox. Important physiological changes at these times of year may be accompanied by changes in AChE activity.

AChE activity may also change daily with diel temperature or photoperiod changes. A study by Bauer (1976) found that the AChE activity of the locust *Schistocerca gregaria* rapidly peaked following morning temperature increases, but little change followed temperature decreases at night. Numerous, but less dramatic changes occurred following various diel temperature or photoperiod changes. While the details of this are

beyond the scope of this discussion, it is important to note that diel fluctuations can cause important shifts in AChE activity in insects. In our study, field sampling occurred throughout the day. If AChE activity in *I. bicolor* changes throughout the day, as suggested by Bauer (1976), great variability may be seen, despite little change in mean daily activity. A study with design similar to Bauer's (1976) may illustrate important diel changes in *I. bicolor* AChE activity.

In order to better understand the role seasonal photoperiod and temperature changes play in AChE activity, the voltinism of *I. bicolor* in Iowa streams needs to be ascertained. Sweeney (1978) found that *I. bicolor* were bivoltine in Pennsylvania streams. If the same voltinism pattern occurs in Iowa, *I. bicolor* were likely sampled from both generations in a given year. If AChE activity patterns differ between "winter" and "summer" generations, this may complicate interpretation of results. Using Sweeney's (1978) methods, voltinism patterns could be established with relative ease. Also, Sweeney (1978) could sex late instar nymphs just prior to emergence at certain times of the year. It is possible that differences exist in AChE activity between sexes, creating variability when head capsules of both sexes are pooled. A study to determine AChE activity of both sexes might demonstrate another potential source of variability.

Sweeney (1978) cited a method for rearing *I. bicolor* from eggs in the laboratory. Since *I. bicolor* are relatively easy to maintain in a laboratory, this method may reduce confounding results from use of field-collected test organisms in microcosm studies as well as in future assay optimization applications. Not only would this eliminate the

potential for insecticide exposure, it would utilize organisms of approximately the same age with a relatively homogeneous genetic makeup. While developing a method to produce large numbers of insects may take considerable time, the benefits of such homogeneity may outweigh the difficulties associated with rearing and maintenance.

Carbamate and OP insecticides have been used for many years, and these compounds may enter streams regularly. Future studies may need to establish if *I. bicolor* in Northeast Iowa have become resistant to commonly used insecticides. The details of insect resistance to OP's and carbamates are beyond the scope of this discussion. However, if *I. bicolor* populations are resistant, that may cloud the interpretation of AChE inhibition studies. A number of mechanisms for resistance to OP and carbamate insecticides have been suggested.

Charpentier, *et al.* (2000) suggest three mechanisms of resistance to OP and carbamate insecticides: (1) increased hydrolysis of acetylcholine (ACh) by AChE in resistant strains, (2) increased production of AChE , and (3) changed conformation of active (or other) binding sites in resistant strains. If a population of *I. bicolor* was repeatedly exposed to cholinesterase inhibitors and became resistant by producing an AChE enzyme with increased activity rates and/or by increased enzyme production, high activity in field populations might represent chronic exposure. However, with both of these resistance mechanisms exposure to cholinesterase inhibitors would result in decreased activity following exposure, despite higher baseline AChE activity. Numerous studies have shown increased AChE activity in resistant insect populations (e.g., Voss,

1980; Fournier and Mutero, 1994; Parker and Callaghan, 1997; Charpentier, *et al.* 2000; Lee, *et al.* 2000). If resistance is conferred by changed conformation of the enzyme's active site (i.e. insensitivity to inhibitors) exposure to OP or carbamate insecticides would cause little decrease in AChE activity following exposure. With this type of resistance, baseline activities could be high or low depending on the physiological state of the organism. However, since they are not readily inhibited by OP's or carbamates, activities would remain more stable than if resistance was by other means (i.e., increased activity rates or increased enzyme production).

A study by Bourget, *et al.* (1997) illustrated another potential mechanism of resistance to OP and carbamate insecticide. Choline acetyltransferase, the enzyme responsible for acetylcholine (ACh) production, may decrease toxicity of OP's and carbamates by producing lower amounts of ACh. By reducing the amount of ACh produced when AChE is inhibited, accumulation of ACh in the synapse is diminished. By exposing mosquitoes (heterozygous with resistant and sensitive AChE molecules) to the carbamate insecticide propoxur, Bourget, *et al.* (1997) found that as dose increased, sensitive AChE molecules were inhibited. Acetylcholine breakdown from insensitive molecules continued, but at levels near the minimum for survival. As doses increased, propoxur began to inhibit choline acetyltransferase, decreasing the release of ACh and reducing the continuous neural stimulation associated with OP and carbamate toxicity. Such resistance allows greatly depressed AChE activity, but reduces mortality. However, with further increasing propoxur dosages, choline acetyltransferase became completely

inhibited, resulting in mortality due to lack of ACh production. If *I. bicolor* were resistant by such a mechanism, decreased AChE activity may not result in increased mortality. It would be useful to know if the common insecticides terbufos or chlorpyrifos produce similar changes in *I. bicolor* biochemistry. Bourget, *et. al* (1997) also suggest that resistance may result from increased detoxification of AChE inhibitors by compounds such as glutathione S-transferase or cytochrome P_{450}. However, establishing cause/effect relationships of increased levels of detoxifying compounds with OP or carbamate exposure may be difficult since they often detoxify numerous compounds.

Voss (1980) believed that increased carboxylesterase activity may act as a form of resistance, by sequestering OP's before binding to AChE. It may be useful to establish the activity of other cholinesterases or "pseudocholinesterases" such as butyrylcholinesterase (BChE), and proprionylcholinesterase (PChE) as well as nonspecific esterases to determine their roles in ACh hydrolysis, as well as their OP and carbamate binding potential. Considerable diversity exists in aquatic invertebrate cholinesterase activity in terms of properties such as substrate specificity, sensitivity to inhibitors, as well as importance of true AChE, BChE, and PChE in ACh hydrolysis (Habig and DiGuilio 1991). Understanding the role of the pseudocholinesterases may clarify some causes of variability in AChE activity.

A number of lab methods exist that may enable greater understanding of AChE inhibition. This study utilized a protein assay to establish AChE activity in relation to total protein content. Ideally, AChE activity would be expressed as amount of acetyl

thiocholine iodide hydrolyzed by a quantity of AChE (instead of the amount hydrolyzed by the amount of total protein), resulting in greater precision. However, it is difficult to accurately establish the amount of AChE present. A study by Charpentier, *et al.* (2000) titrated AChE *in vitro* with an OP to establish the amount of enzyme present. This method would only be useful in studies where insects were not exposed to AChE inhibitors. If the insects were previously exposed, AChE amounts would likely be underestimated. However, it may be possible to establish amounts of inhibited AChE. Hunt and Hooper (1993) established a number of techniques to reactivate AChE that had been exposed to certain inhibitors. By using the oxime 2-PAM the investigators were able to reactivate inhibited AChE if it had not become aged (aging is a condition where the inhibitor is permanently bound to AChE). While this method was only effective in removing OP's, many oximes exist with varying effectiveness on different OP and carbamate compounds (Wilson, *et al.* 1992). A combination of oxime treatment and insecticide titration may be effective in determining precise amounts of AChE in tissue homogenates as well as the amount of inhibited enzyme present. Most information about these methods exist in literature dealing with resistant strains of target organisms, and have seldom been utilized in analysis of non-target organisms (with the exception of Hunt and Hooper, 1993). While time consuming, these methods would likely aid interpretation of AChE activity assay results.

It may also be useful to incorporate a commercially prepared cholinesterase control into the protocol used in this research that utilized a check standard prepared from *I.*

bicolor homogenate as a form of in-house control. However, a commercially prepared control, such as purified electric eel AChE, may be useful for inter-laboratory comparisons as suggested by Marden, *et al.* (1994).

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APPENDIX A. Site descriptions and map of field sites from *Isonychia bicolor* sampling during 2002 and 2003.

Volga River

North Branch - first bridge on W25 S off Hwy 93 (**VRNB**) (42^0 49.08 N, 91^0 52.78 W)

This riffle (~40 x 10 m) was upstream from a bridge and composed of cobble (6-13 cm) 75% embedded in pebble (1-2 cm). Substrate was covered by filamentous algae. Deciduous trees and grasses provided ~10% canopy cover to this shallow (0.05-0.1 m) riffle.

Twin Bridges Park - W25 just S of Hwy 93 (**VRTB**) (42^0 49.08 N, 91^0 52.76 W)

Substrate composed of cobble (13-25 cm) 25% embedded in pebble (0.2-1 cm). The riffle area was ~50 x 25 m and bordered by recreational area with deciduous trees providing ~5% canopy cover. Stream depth ranged from 0.15-0.3 m.

Volga River State Park - N of Fayette on HWY 150 near campground (**VRSP**)

(42^0 51.85 N, 91^0 45.42 W) Substrate composed of cobble (13-25 cm) was unembedded in pebble (2-6 cm) throughout the riffle area (~40 x 10 m). Deciduous trees and grasses made up the riparian vegetation (0% canopy cover). Approximate stream depth was between 0.25-0.5 m.

Upper Iowa River

Kendallville Park - off Hwy 139-access from campground (**UIKV**) (43^0 26.50 N, 92^0 02.24 W) This riffle (~50 x 25 m) was composed of cobble (6-13 cm) 50% embedded in pebble (0.1-1 cm). Deciduous trees in recreational areas on both sides of the river provided ~25% canopy cover. Stream depth ranged from 0.25 to 0.5 m.

Chimney Rock Park - Bluffton Road NW of Bluffton (**UICR**) (43^0 24.94 N, 91^0 56.16 W) This riffle consisted of separate channels composed of pebble (2-6 cm) 25% embedded in smaller pebble (1-2 cm) for most of the area (~75 x 15 m). Deciduous trees provided ~5% canopy cover. Stream depth ranged from 0.25 to 0.5 m.

Decorah - under bridge downtown (**UIDE**) (43^0 18.20 N, 91^0 47.78 W) This larger riffle (~70 x 25 m) was composed of cobble (13-25 cm) 50% embedded in smaller cobble (6-13 cm). Stream depth (0.25-1 m) and velocity were higher than at most other Upper Iowa sites. River banks were covered with grasses and deciduous trees provided ~5% canopy cover.

APPENDIX A. (Continued) Site descriptions and map of field sites from *Isonychia bicolor* sampling during 2002 and 2003.

Little Cedar River

Chickasaw Park - B57 S of Bassett (**LCCP**) (43^0 02.09 N, 92^0 30.27 W)

This riffle (~90 x 25 m) was composed of cobble (13-25 cm) and boulders (> 25 cm) unembedded in pebble (2-6 cm). Deciduous trees made up most of the riparian vegetation and provided ~10% canopy cover. Large boulders in and around the riffle limited sampling to specific areas. Stream depth is less than 0.75 m.

Cedar River

Evansdale - Downstream from the Hwy 20 bridge, 0.5 mile S of Evansdale. (**CRED**)

(42^0 27.54 N, 92^0 17.63 W) The river is bordered by deciduous trees providing no canopy cover. Springs from the river bank flow into the river near the sampling site. Substrate was composed of cobble (13-25 cm) 50% embedded in pebble (1-2 cm). Depth (~.5 to 1 m) and flow are similar to that of the Cedar River in Cedar Falls.

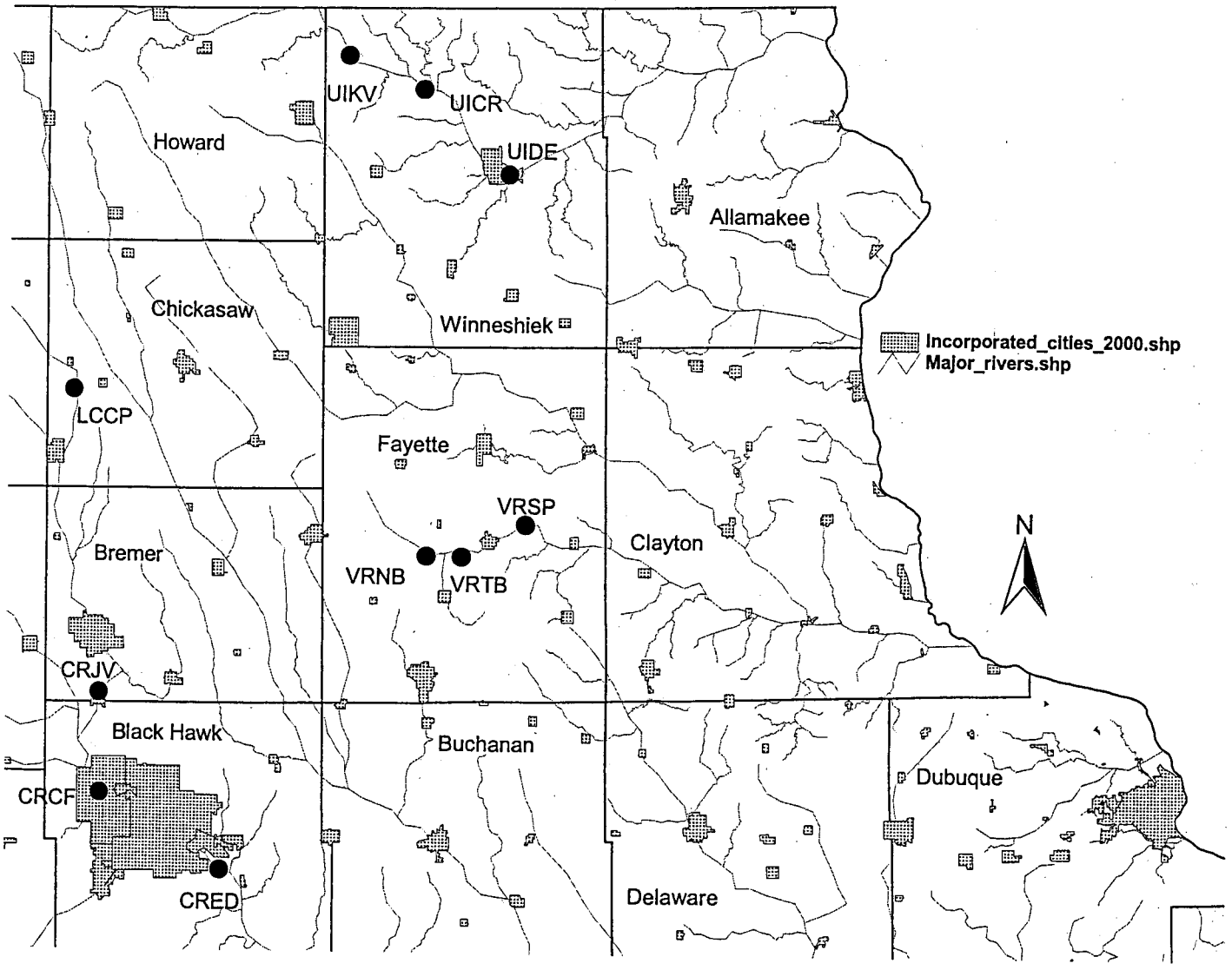
Janesville City Park - W end of town (**CRJV**) (42^0 38.93 N, 92^0 27.91 W)

This riffle site was ~75 x 50 m and bordered by recreational area on one side. Substrate was composed of cobble (13-25 cm) 25% embedded in pebble (0.2-1 cm). Deciduous trees provided ~10% canopy cover. Stream depth was ~0.5-1.0 m.

Cedar Falls - under Hwy 57 Bridge (**CRCF**) (42^0 32.22 N, 92^0 26.33 W)

No canopy cover was available at this riffle (~100 x 50 m). Substrate was composed of cobble (13-25 cm) 50% embedded in pebble (2-6 cm). Depth (~0.5-1 m) and flow were greater than at most other sites.

APPENDIX A. (Continued) Map of sampling sites from *Isonychia bicolor* acetylcholinesterase studies.



APPENDIX B. *Isonychia bicolor* densities from 2002 and 2003 "10 site" sampling.

Hess sample densities (number/0.093 m^2) during May, July and September, 2002. Data are mean number of individuals (SD) for n = 3.

Riffle Site	May	July	September
CRCF	247.33 (281.74)	15.67 (12.01)	5.0 (3.61)
CRED	131.33 (168.53)	17.67 (9.07)	3.0 (1.73)
CRJV	197.33 (189.12)	43.02 (46.11)	0.0 (0.0)
LCCP	0.0 (0.0)	7.67 (2.89)	33.33 (16.50)
UICR	0.67 (1.15)	142.67 (118.81)	38.00 (28.58)
UIDE	2.33 (2.52)	14.33 (16.17)	7.67 (7.23)
UIKV	8.67 (9.02)	59.00 (43.86)	7.02 (11.33)
VRNB	1.33 (1.15)	32.00 (29.82)	4.33 (3.79)
VRSP	0.33 (0.58)	26.67 (23.07)	7.33 (6.51)
VRTB	1.67 (2.89)	9.00 (4.36)	24.67 (23.69)

Hess sample densities (number/0.093 m^2) during May, July and September, 2003. Data are mean number of individuals (SD) for n = 3.

Riffle Site	May	July	September
CRCF	2.89 (6.33)	7.57 (8.33)	17.62 (15.67)
CRED	1.00 (2.00)	20.88 (36.00)	2.52 (4.33)
CRJV	0.0 (0.0)	16.7 (22.33)	10.54 (11.00)
LCCP	0.0 (0.0)	2.31 (2.33)	20.55 (33.67)
UICR	0.0 (0.0)	2.52 (8.33)	69.84 (58.00)
UIDE	2.52 (3.67)	221.40 (233.00)	27.22 (46.00)
UIKV	0.0 (0.0)	0.58 (0.67)	15.62 (20.00)
VRNB	0.58 (0.33)	1.73 (4.00)	6.56 (6.00)
VRSP	0.0 (0.0)	12.02 (18.50)	38.31(26.00)
VRTB	0.58 (0.33)	0.58 (2.33)	1.41 (12.00)

APPENDIX C. Physical/chemical properties from *Isonychia bicolor* sampling during 2002 and 2003.

Site	Date	Time	Temp (°C)	DO (mg/L)	pH	Hard. (mg/L $CaCO_3$)	Alk. (mg/L $CaCO_3$)	Cond. (mS/cm)
CRCF	12/19/01	10:00 a.m.	2.8	NA	8.12	210	164	0.350
CRCF	4/8/02	12:00 p.m.	9.5	NA	11.6	184	96	0.282
CRCF	5/13/02	1:00 p.m.	12.5	NA	8.46	254	155	0.402
VRNB	5/14/02	12:30 p.m.	15.3	NA	8.74	253	144	0.428
VRTB	5/14/02	11:30 a.m.	13.5	NA	8.50	289	157	0.412
VRSP	5/14/02	9:30 a.m.	11.3	NA	8.53	331	159	0.408
UIKV	5/15/02	10:00 a.m.	12.6	NA	7.74	295	169	0.378
UICR	5/15/02	11:00 a.m.	13.2	NA	7.78	280	192	0.392
UIDE	5/15/02	12:00 p.m.	15.1	NA	7.81	266	198	0.420
LCCP	5/16/02	10:00 a.m.	15.2	NA	7.74	236	149	0.396
VRSP	5/20/02	10:30 a.m.	13.2	NA	7.68	259	169	0.416
VRTB	5/20/02	12:00 p.m.	14.5	NA	7.7	252	159	0.404
VRNB	5/20/02	1:30 p.m.	14.8	NA	7.73	233	163	0.410
VRSP	5/28/02	10:30 a.m.	20.6	NA	7.76	240	170	0.458
VRTB	5/28/02	11:30 a.m.	21.5	NA	7.77	216	167	0.436
VRNB	5/28/02	12:30 p.m.	21.9	NA	7.80	205	143	0.434
CRED	5/29/02	11:00 a.m.	21.5	NA	7.84	202	160	0.440
CRJV	5/29/02	12:00 p.m.	21.8	NA	7.86	185	114	0.364
VRSP	6/3/02	10:45 a.m.	15.0	9.32	7.91	255	167	0.446
VRTB	6/3/02	11:30 a.m.	14.7	9.42	7.89	250	154	0.432
VRNB	6/3/02	12:00 p.m.	15.0	10.30	7.85	253	155	0.420
VRTB	6/11/02	10:00 a.m.	19.9	9.70	7.87	252	155	0.486
VRSP	6/11/02	10:45 a.m.	20.2	8.00	7.87	283	172	0.508
VRNB	6/11/02	12:30 p.m.	20.0	9.70	7.88	257	155	0.498
VRSP	6/18/02	10:20 a.m.	18.3	14.30	7.90	271	165	0.472
VRTB	6/18/02	12:00 p.m.	19.2	13.75	7.90	255	150	0.464
VRNB	6/18/02	12:30 p.m.	17.0	12.95	7.80	250	156	0.457

Appendix continued...

APPENDIX C (continued). Physical/chemical properties from *Isonychia bicolor* sampling during 2002 and 2003.

Site	Date	Time	Temp (°C)	DO (mg/L)	pH	Hard. (mg/L $CaCO_3$)	Alk. (mg/L $CaCO_3$)	Cond. (mS/cm)
CRCF	6/20/02	11:30 a.m.	20.5	8.90	7.91	247	137	0.402
VRSP	6/24/02	10:15 a.m.	23.8	10.16	7.94	254	162	0.506
VRTB	6/24/02	11:15 a.m.	24.5	12.90	7.95	249	170	0.500
VRNB	6/24/02	12:15 p.m.	24.9	11.89	7.95	246	149	0.498
UIDE	7/8/02	10:30 a.m.	24.5	10.36	8.16	262	201	0.528
UICR	7/8/02	11:30 a.m.	24.5	10.36	8.16	262	201	0.528
UIKV	7/8/02	12:30 p.m.	23.7	8.94	8.17	262	210	0.508
VRSP	7/10/02	9:30 a.m.	23.3	6.71	8.18	267	175	0.502
VRTB	7/10/02	10:30 a.m.	22.2	7.58	8.22	224	176	0.504
VRNB	7/10/02	11:15 a.m.	21.9	8.02	8.18	235	172	0.446
LCCP	7/11/02	10:00 a.m.	19.0	8.73	8.19	232	163	0.384
CRJV	7/11/02	12:00 p.m.	22.9	9.15	8.19	214	149	0.424
CRCF	7/11/02	1:00 p.m.	22.2	10.20	8.20	231	153	0.442
CRED	7/11/02	2:30 p.m.	22.2	10.16	8.20	219	142	0.436
CRCF	7/23/02	10:00 a.m.	25.9	10.03	8.09	199	139	0.444
CRCF	8/6/02	12:30 p.m.	24.5	10.43	8.23	239	151	0.480
LCCP	9/12/02	9:15 a.m.	16.1	8.10	8.04	222	157	0.370
CRJV	9/12/02	11:00 a.m.	20.1	6.87	8.02	220	166	0.432
CRCF	9/12/02	1:00 p.m.	22.1	10.27	8.04	265	166	0.460
CRED	9/12/02	2:00 p.m.	23.6	16.40	8.05	265	155	0.504
VRSP	9/26/02	10:00 a.m.	14.4	11.10	8.26	223	180	0.400
VRTB	9/26/02	11:15 a.m.	15.3	11.83	8.28	243	173	0.392
VRNB	9/26/02	12:30 p.m.	15.9	10.70	8.30	244	182	0.413
UIKV	9/29/02	11:00 a.m.	12.6	10.30	8.20	303	212	0.402
UIDE	9/29/02	12:45 p.m.	15.6	12.25	8.24	319	216	0.438
UICR	9/29/02	1:00 p.m.	14.2	10.04	8.28	288	239	0.430

Appendix continued...

APPENDIX C (continued). Physical/chemical properties from *Isonychia bicolor* sampling during 2002 and 2003.

Site	Date	Time	Temp (°C)	DO (mg/L)	pH	Hard. (mg/L $CaCO_3$)	Alk. (mg/L $CaCO_3$)	Cond. (mS/cm)
VRSP	4/23/03	12:00 p.m.	15.2	13.40	8.03	252	170	0.402
VRNB	4/23/03	12:30 p.m.	15.3	13.95	8.12	261	143	0.393
VRTB	4/23/03	1:00 p.m.	11.0	14.26	8.09	272	149	0.384
UIKV	5/29/03	10:00 a.m.	16.3	11.09	7.68	233	150	0.414
UICR	5/29/03	11:00 a.m.	17.2	10.94	7.76	278	190	0.430
UIDE	5/29/03	12:00 p.m.	19.4	11.20	7.71	263	224	0.454
VRSP	5/29/03	2:00 p.m.	21.2	10.30	7.76	248	139	0.476
VRTB	5/29/03	3:30 p.m.	22.4	12.70	7.76	236	151	0.487
VRNB	5/29/03	4:00 p.m.	22.4	12.70	7.74	240	155	0.477
LCCP	5/30/03	11:30 a.m.	18.1	8.96	7.81	251	150	0.440
CRJV	5/30/03	12:30 p.m.	20.3	13.30	7.79	273	163	0.486
CRED	5/30/03	2:00 p.m.	21.0	10.32	7.80	297	160	0.532
CRCF	5/30/03	3:00 p.m.	20.6	10.76	7.78	304	167	0.512
UIKV	6/6/03	10:45 a.m.	15.1	10.45	7.76	265	182	0.410
UICR	6/6/03	12:30 p.m.	15.7	12.10	7.79	270	182	0.481
UIDE	6/6/03	2:30 p.m.	15.8	10.20	7.76	263	179	0.412
VRSP	6/6/03	3:00 p.m.	16.5	9.20	7.79	266	145	0.414
VRNB	6/6/03	4:40 p.m.	16.1	8.50	7.79	265	130	0.386
VRTB	6/6/03	5:00 p.m.	16.0	10.90	7.80	259	133	0.414
UIKV	6/13/03	10:00 a.m.	19.4	9.79	7.67	280	160	0.474
UICR	6/13/03	11:00 a.m.	20.6	10.79	7.67	299	156	0.488
UIDE	6/13/03	2:00 p.m.	22.0	8.00	7.69	294	170	0.500
VRSP	6/13/03	3:00 p.m.	22.7	9.88	7.65	269	167	0.536
VRTB	6/13/03	4:00 p.m.	25.3	10.79	7.73	309	147	0.570
VRNB	6/13/03	4:30 p.m.	25.6	10.83	7.84	315	153	0.550
UIKV	6/19/03	10:00 a.m.	19.5	8.53	7.65	259	160	0.472
UICR	6/19/03	11:30 a.m.	21.2	9.77	7.69	289	186	0.500
UIDE	6/19/03	12:00 p.m.	22.4	10.80	7.67	370	188	0.512

Appendix continued...

APPENDIX C (continued). Physical/chemical properties from *Isonychia bicolor* sampling during 2002 and 2003.

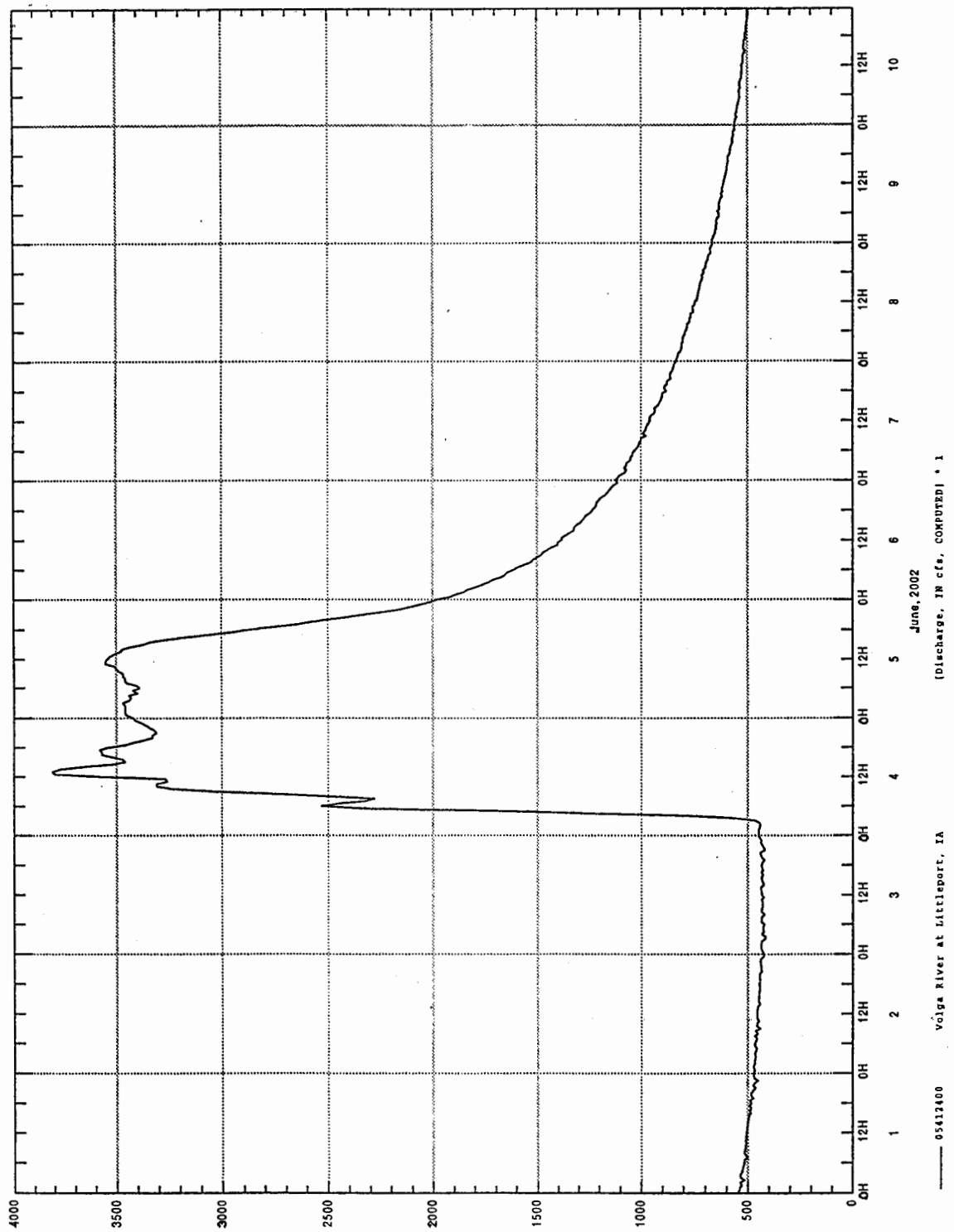
Site	Date	Time	Temp (°C)	DO (mg/L)	pH	Hard. (mg/L $CaCO_3$)	Alk. (mg/L $CaCO_3$)	Cond. (mS/cm)
VRSP	6/19/03	2:00 p.m.	25.5	10.40	7.67	271	147	0.556
VRTB	6/19/03	4:00 p.m.	26.2	10.20	7.71	NA	152	0.510
VRNB	6/19/03	4:30 p.m.	27.0	10.00	7.72	NA	151	0.509
UIKV	6/27/03	10:00 a.m.	17.7	8.86	7.68	267	144	0.422
UICR	6/27/03	10:45 a.m.	18.8	10.06	7.71	296	145	0.446
UIDE	6/27/03	12:00 p.m.	20.1	10.06	7.73	268	242	0.494
VRSP	6/27/03	1:30 p.m.	20.7	10.06	7.67	335	144	0.494
VRTB	6/27/03	2:30 p.m.	22.6	9.07	7.72	212	188	0.558
VRNB	6/27/03	3:00 p.m.	23.1	9.10	7.74	210	193	0.446
UIKV	7/2/03	12:00 p.m.	21.6	9.10	7.67	224	163	0.500
UICR	7/2/03	1:00 p.m.	23.1	9.75	7.66	267	171	0.516
UIDE	7/2/03	1:30 p.m.	23.2	9.65	7.66	230	165	0.503
VRSP	7/2/03	2:30 p.m.	26.5	11.20	7.65	256	194	0.582
VRNB	7/2/03	3:30 p.m.	28.9	10.61	7.70	251	192	0.591
VRTB	7/2/03	4:00 p.m.	28.9	10.59	7.69	255	190	0.575
UIKV	7/14/03	11:30 a.m.	21.2	8.40	7.70	260	172	0.512
UICR	7/14/03	12:30 p.m.	21.3	9.53	7.72	256	175	0.516
UIDE	7/14/03	1:30 p.m.	22.0	10.77	7.70	246	177	0.519
VRSP	7/14/03	2:30 p.m.	24.7	7.80	7.69	267	183	0.535
VRTB	7/14/03	3:00 p.m.	24.1	9.12	7.71	277	175	0.521
VRNB	7/14/03	4:00 p.m.	24.1	7.90	7.70	271	190	0.540
LCCP	7/22/03	10:00 a.m.	20.9	9.16	7.73	262	170	0.490
CRJV	7/22/03	11:00 a.m.	23.3	8.91	7.75	267	189	0.570
CRCF	7/22/03	12:00 p.m.	22.5	8.78	7.60	258	203	0.780
CRED	7/22/04	2:30 p.m.	20.9	8.08	7.74	282	171	0.534

Appendix continued...

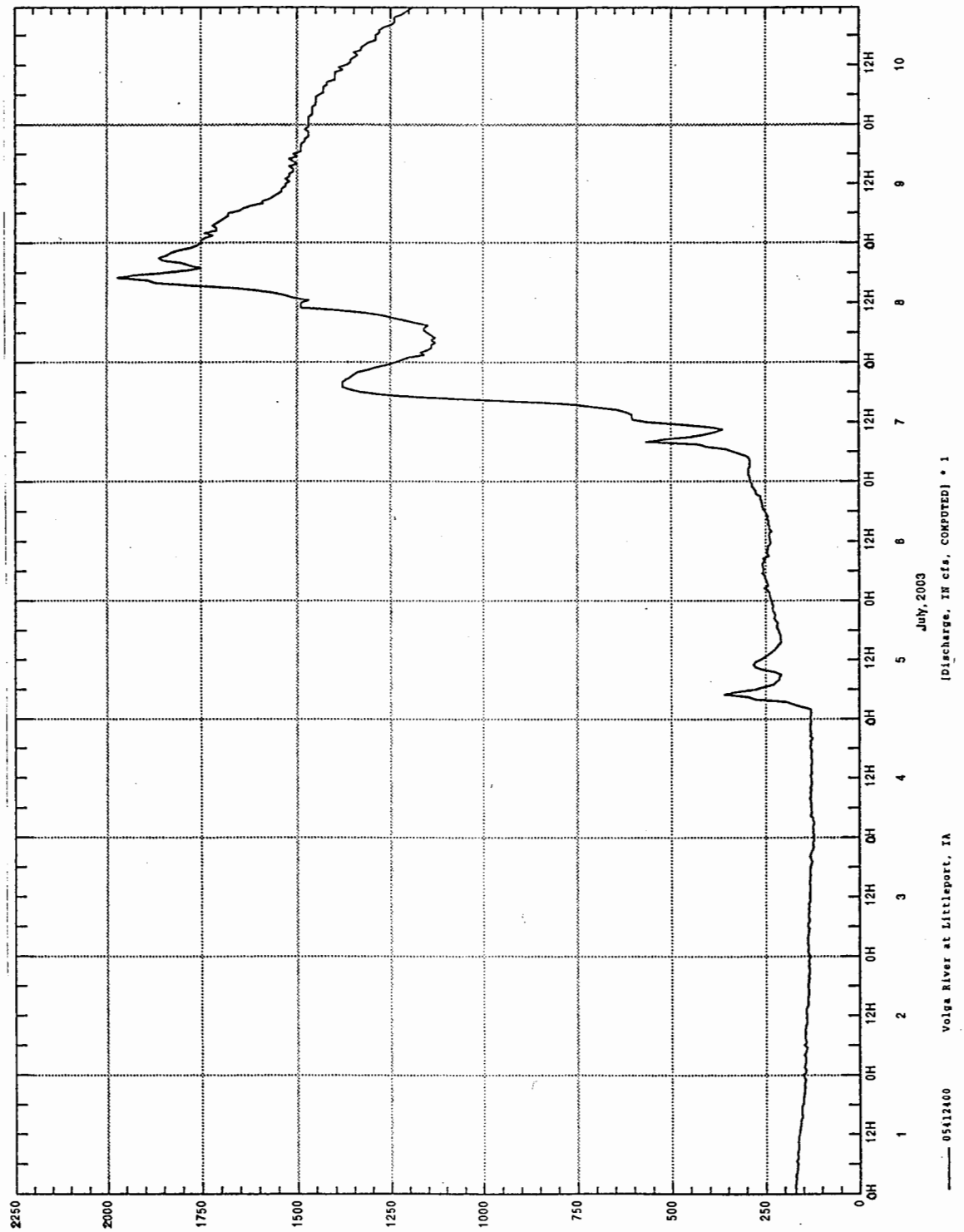
APPENDIX C (continued). Physical/chemical properties from *Isonychia bicolor* sampling during 2002 and 2003.

Site	Date	Time	Temp (°C)	DO (mg/L)	pH	Hard. (mg/L $CaCO_3$)	Alk. (mg/L $CaCO_3$)	Cond. (mS/cm)
LCCP	9/16/03	10:00 a.m.	15.9	10.05	7.63	312	181	0.374
CRJV	9/16/03	12:00 p.m.	19.1	10.10	7.54	270	182	0.420
CRCF	9/16/03	2:00 p.m.	21.0	9.87	7.56	258	156	0.430
CRED	9/26/03	2:30 p.m.	22.5	10.50	7.62	244	165	0.424
UIKV	9/26/03	9:30 a.m.	11.1	11.60	7.69	270	205	0.364
UICR	9/26/03	10:30 a.m.	11.7	6.70	7.75	260	209	0.382
UIDE	9/26/03	1:00 p.m.	12.8	8.60	7.73	264	205	0.378
VRSP	9/26/03	1:30 p.m.	13.1	9.02	7.71	230	170	0.380
VRTB	9/26/03	2:30 p.m.	13.0	10.15	7.73	222	152	0.350
VRNB	9/26/03	3:30 p.m.	13.0	10.23	7.74	220	145	0.320

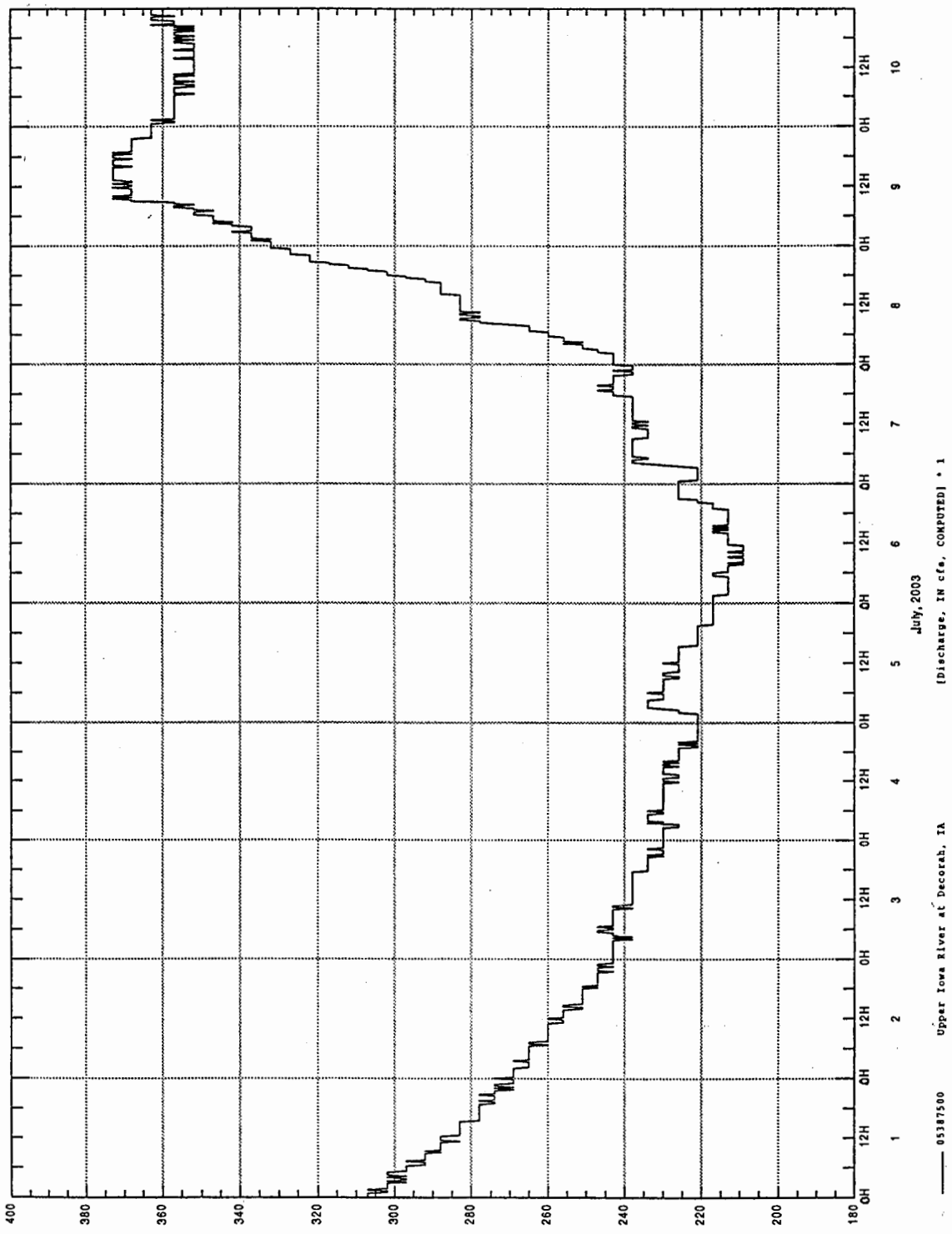
APPENDIX D. Streamflow data from the Volga River (2002 and 2003) and the Upper Iowa River (2003) during intensive sampling.



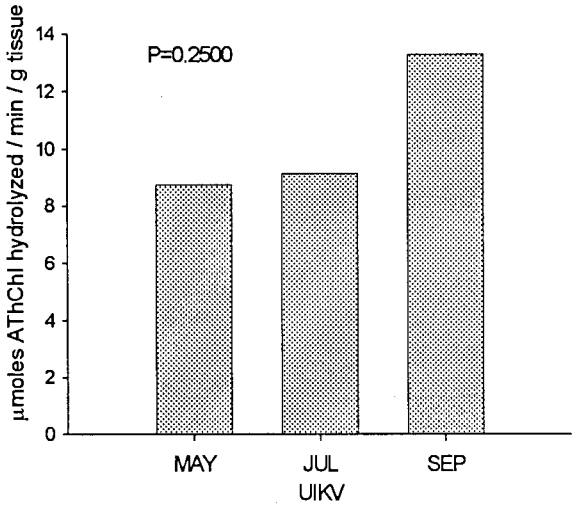
APPENDIX D (continued). Streamflow data from the Volga River (2002 and 2003) and the Upper Iowa River (2003) during intensive sampling.

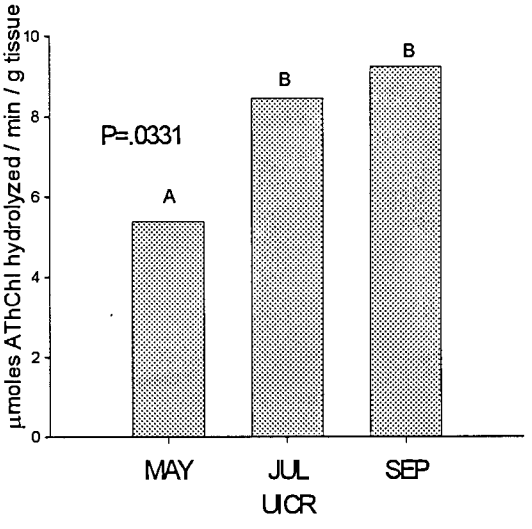


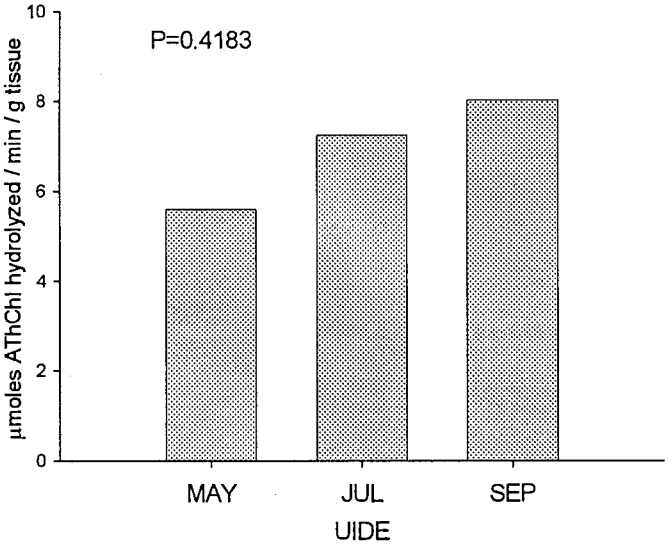
APPENDIX D (continued). Streamflow data from the Volga River (2002 and 2003) and the Upper Iowa River (2003) during intensive sampling.



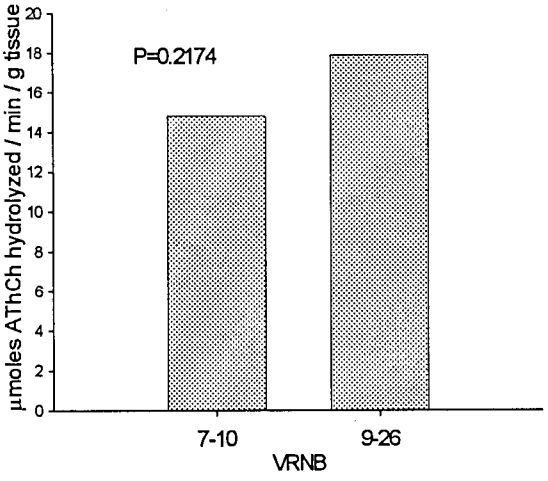
APPENDIX E. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2002. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range. Test.

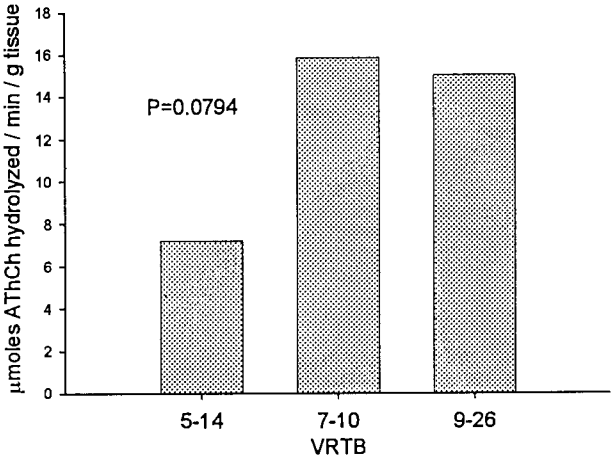


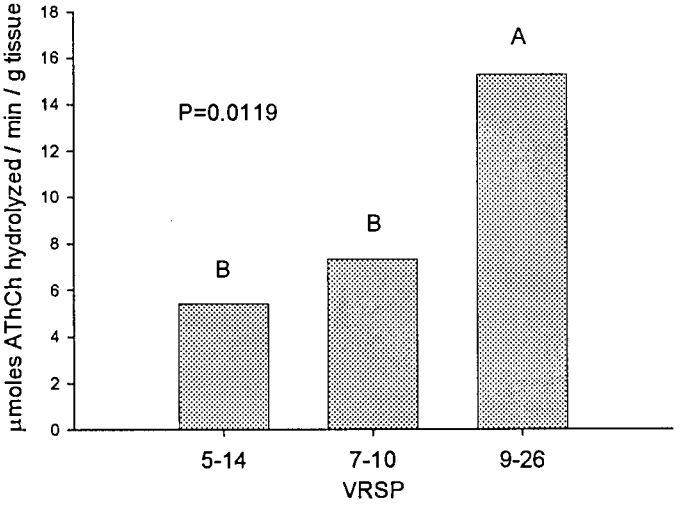




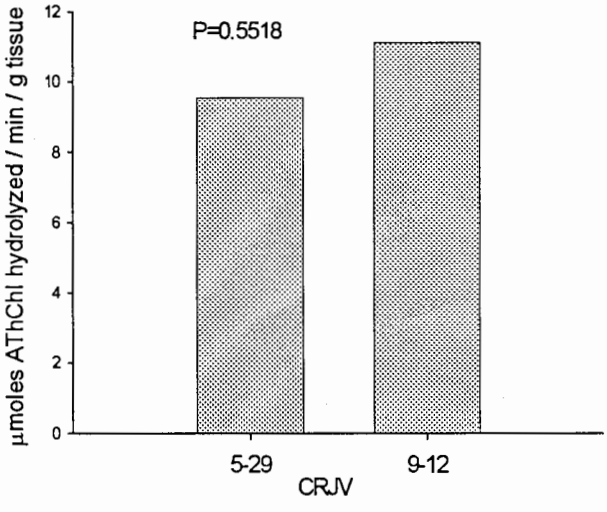
APPENDIX E. (continued). AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2002. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range.

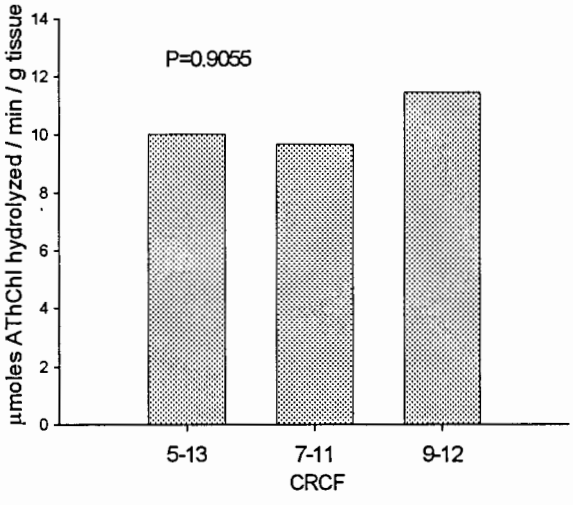


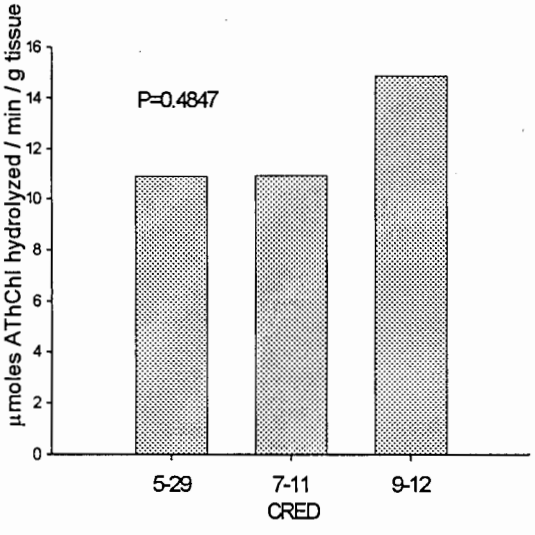


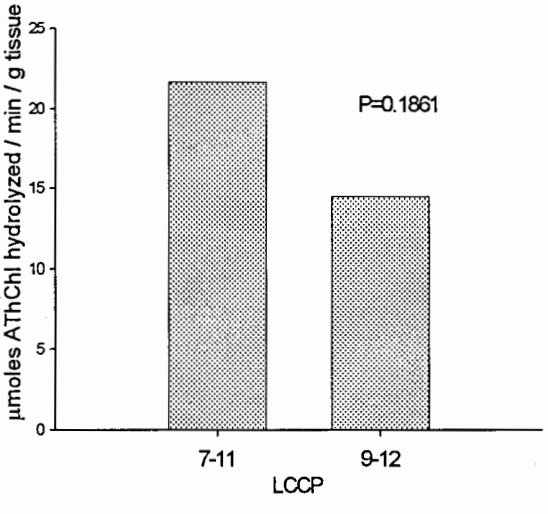


APPENDIX E.(continued). AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2002.

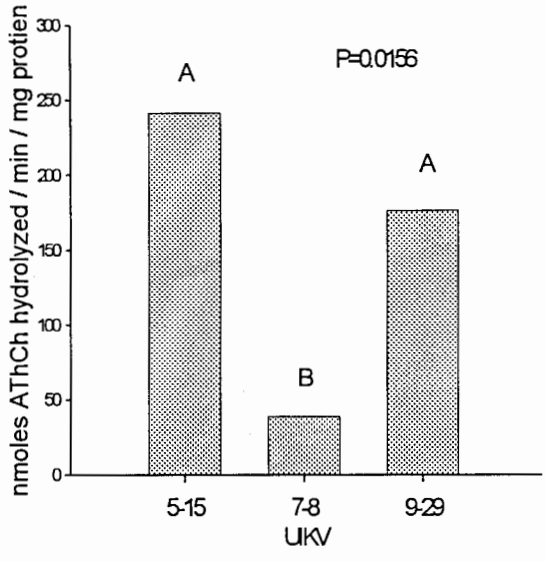


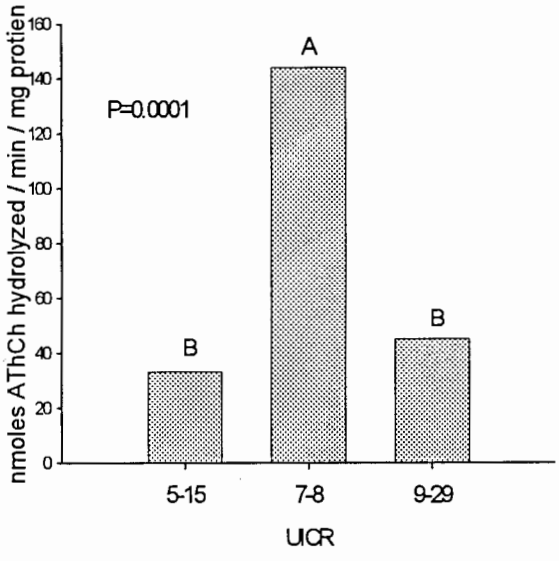


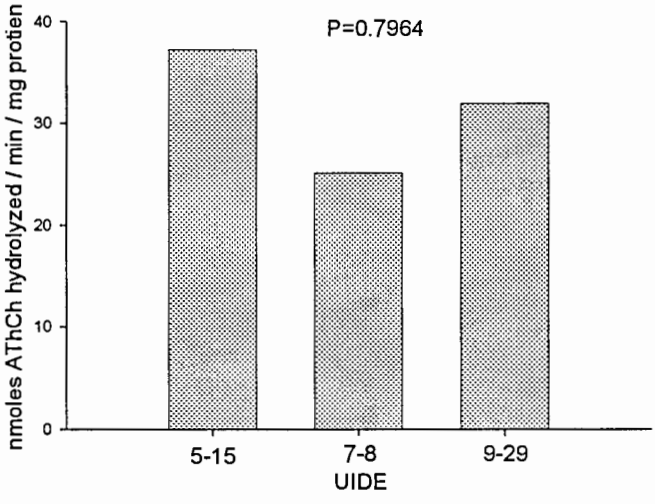




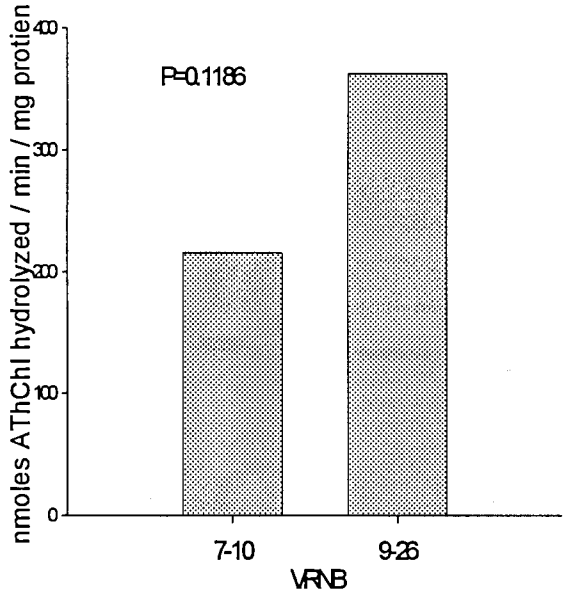
APPENDIX E. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2002. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range.

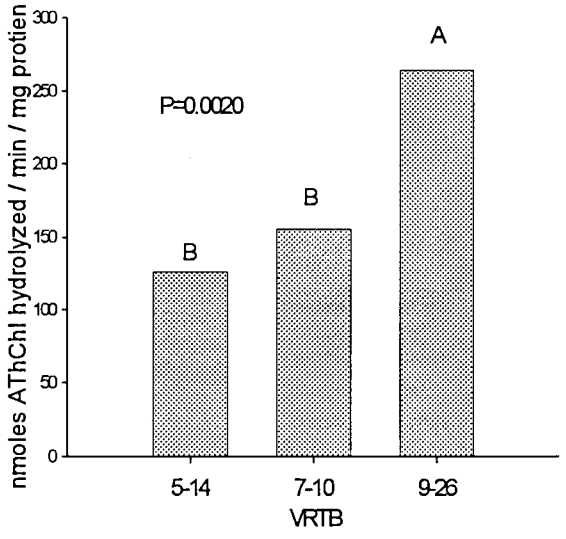


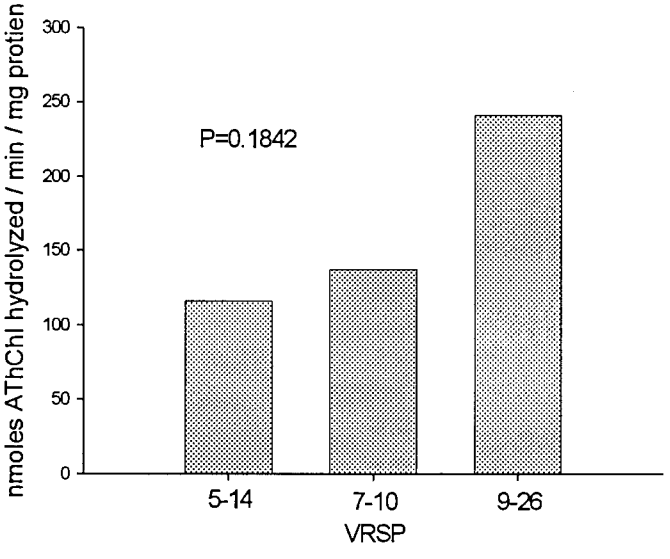




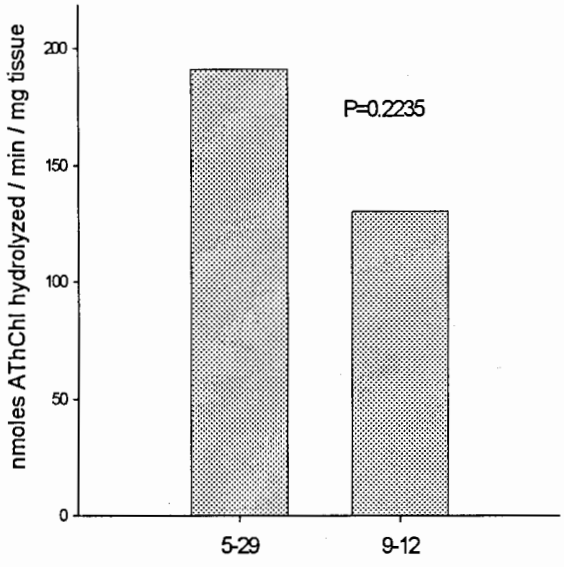
APPENDIX E. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2002. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range.

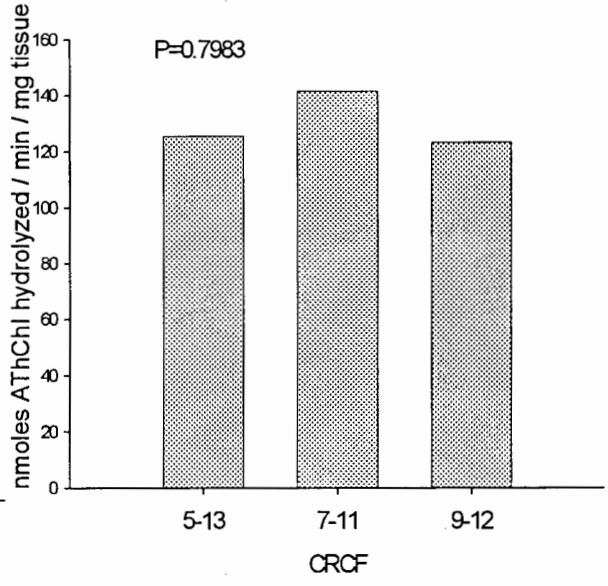


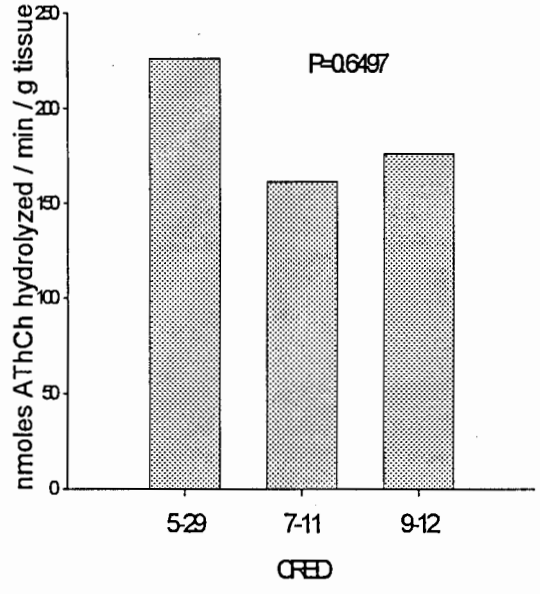


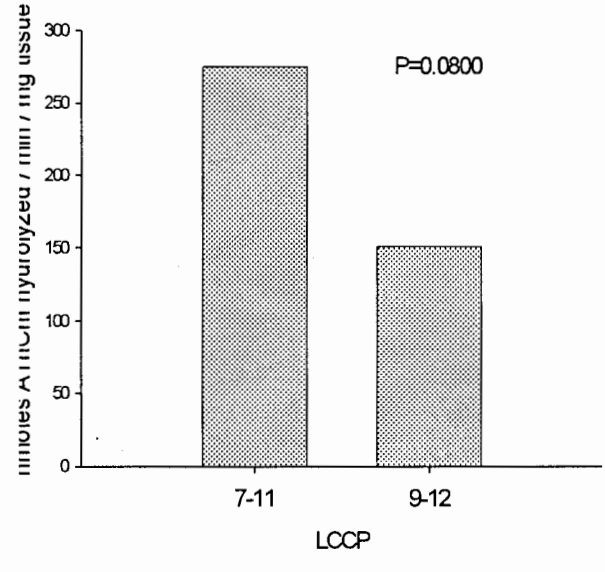


APPENDIX E. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2002. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range.

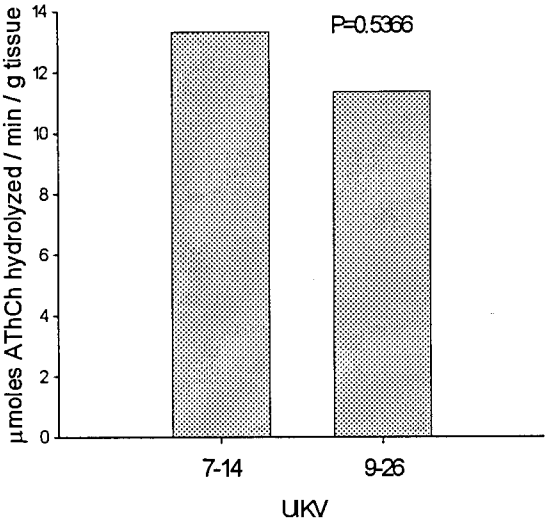


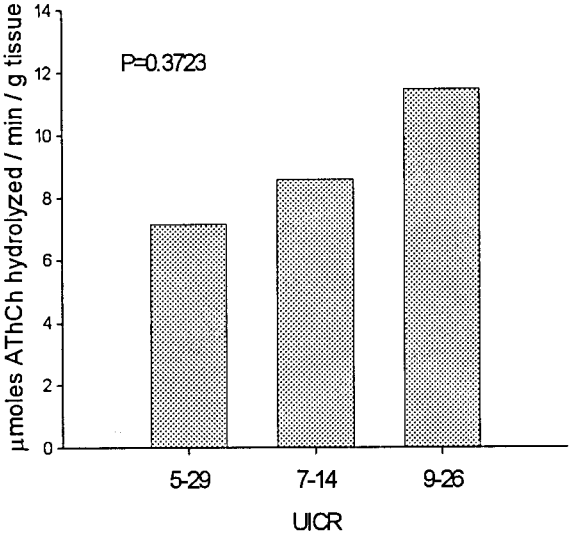


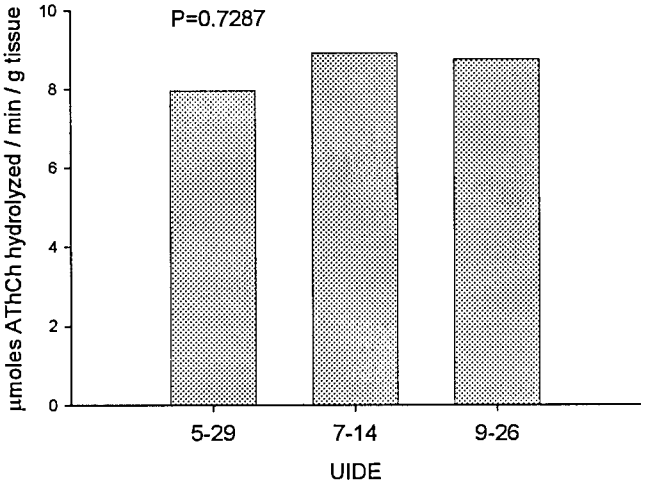




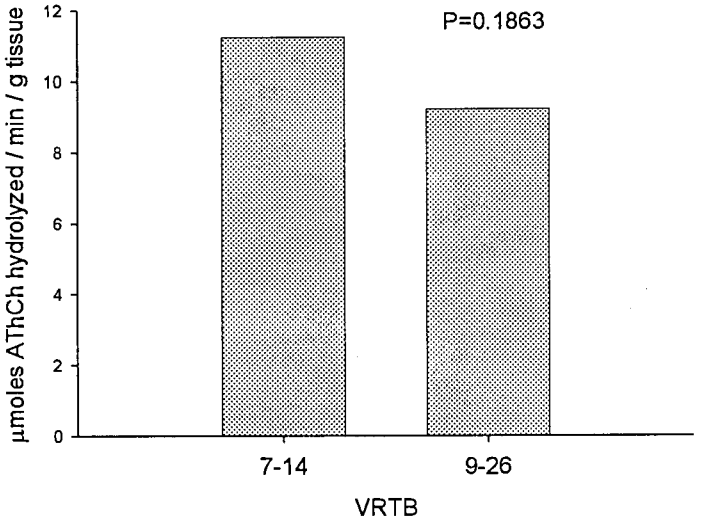
APPENDIX F. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2003.

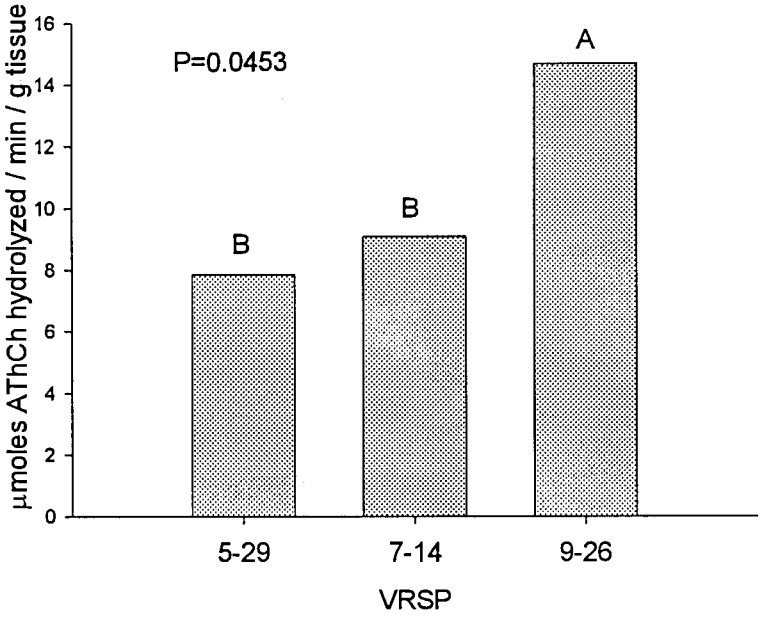




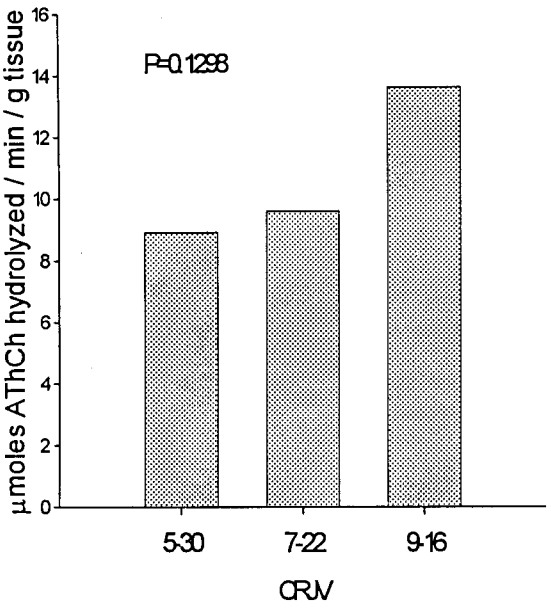


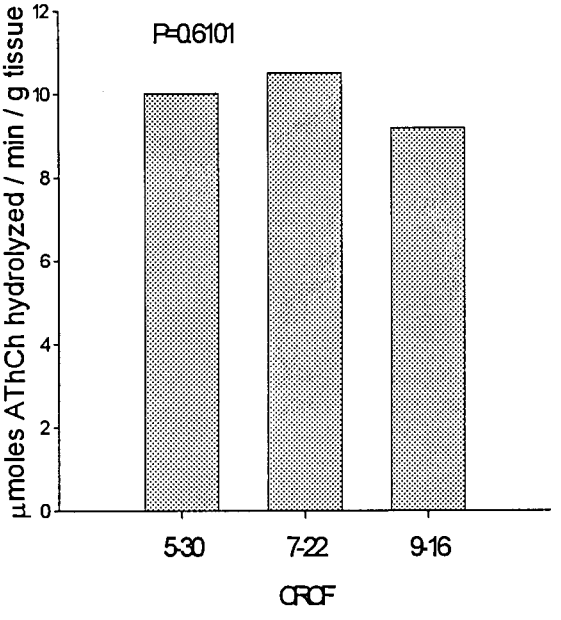
APPENDIX F. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2003. *Isonychia bicolor* were not collected at VRNB during May or July sampling. Bars with the same letter are not significantly different (P>0.05) based on Duncan's Multiple Range.

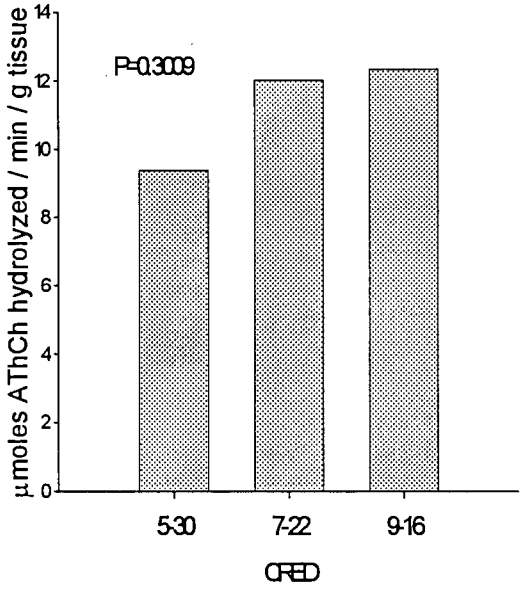


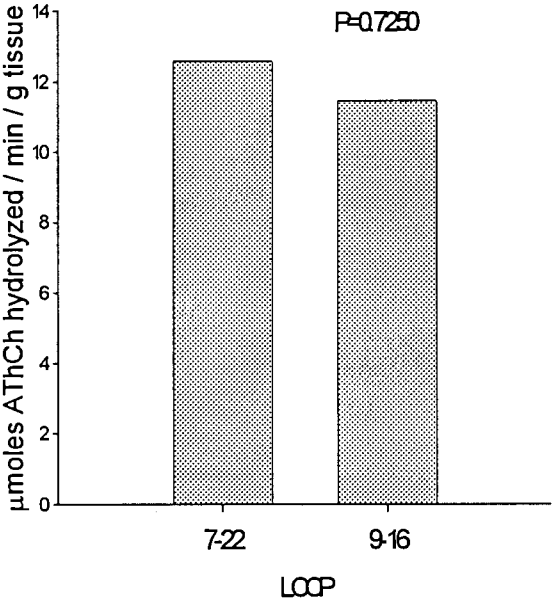


APPENDIX F. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2003.

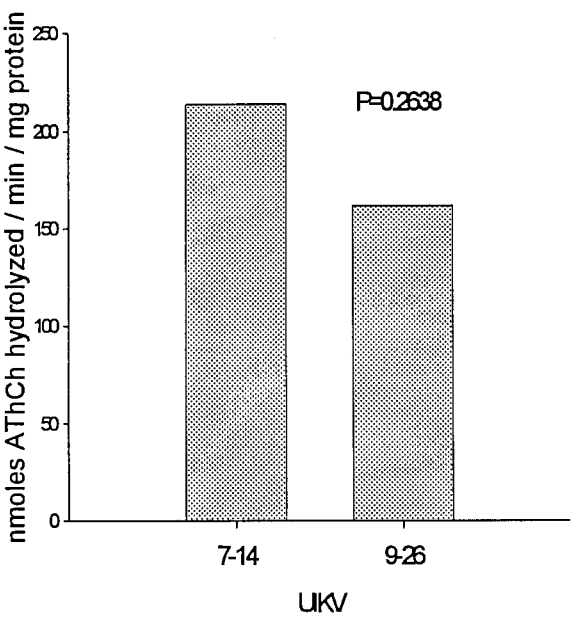


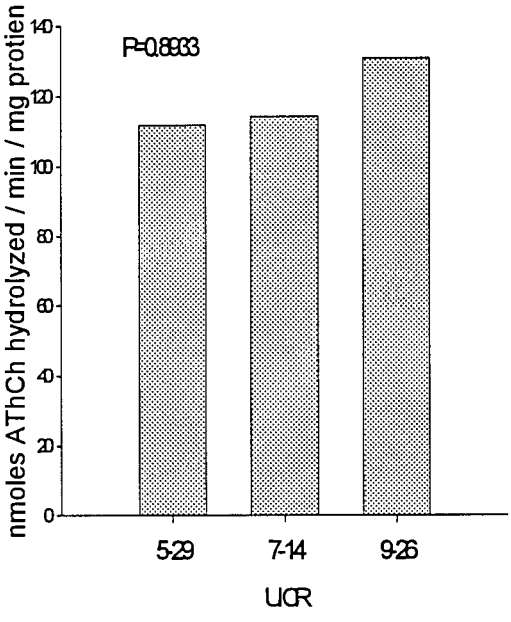


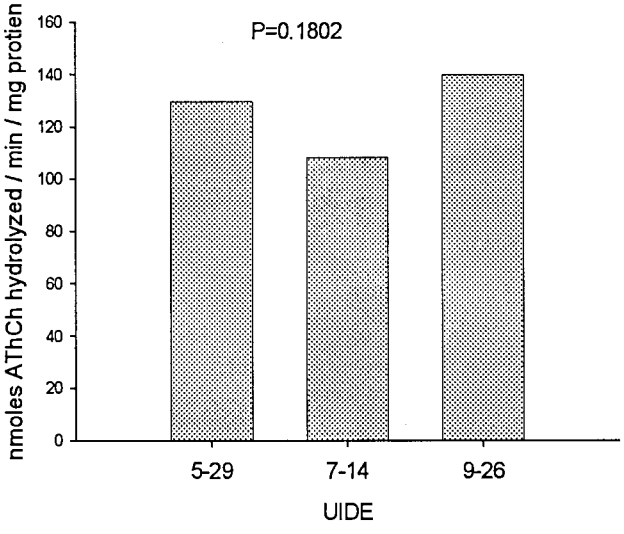




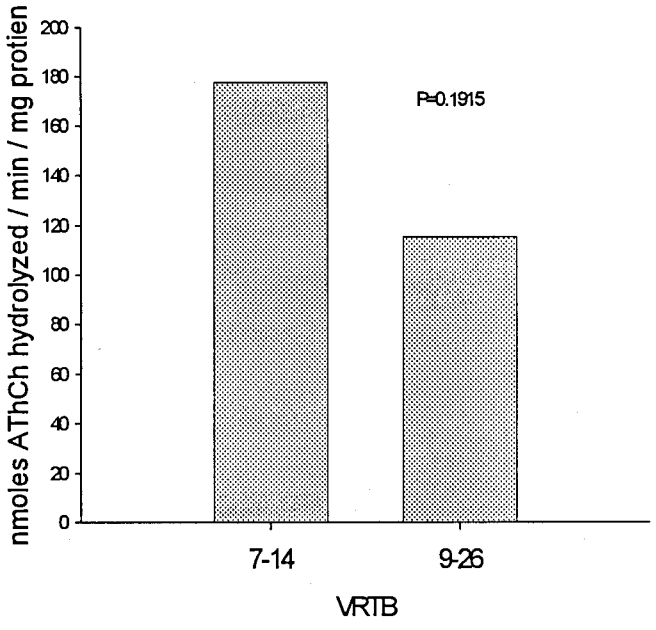
APPENDIX F. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2003.

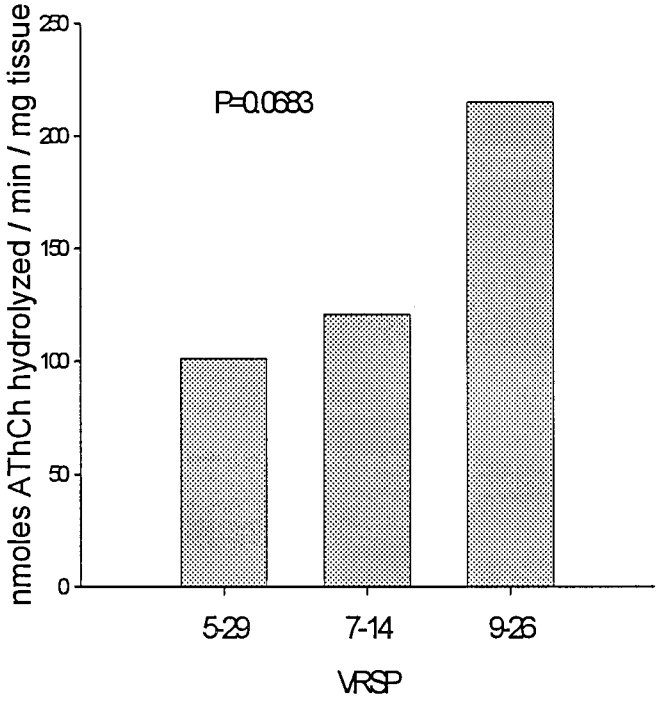




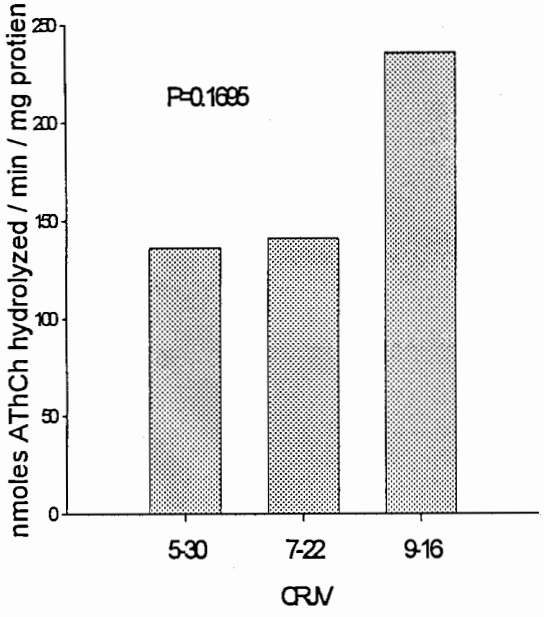


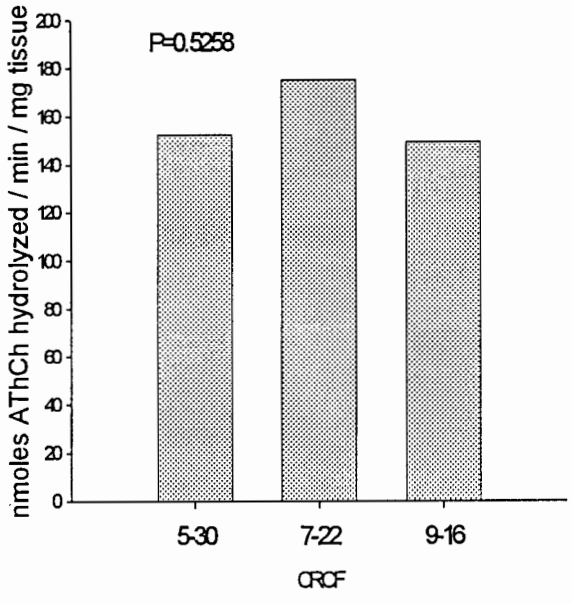
APPENDIX F. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2003.

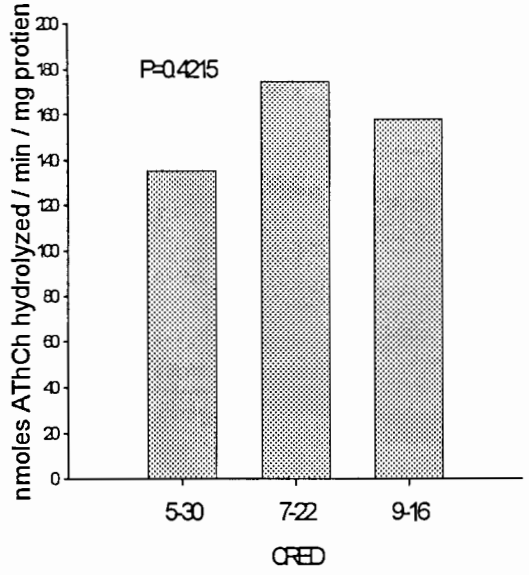


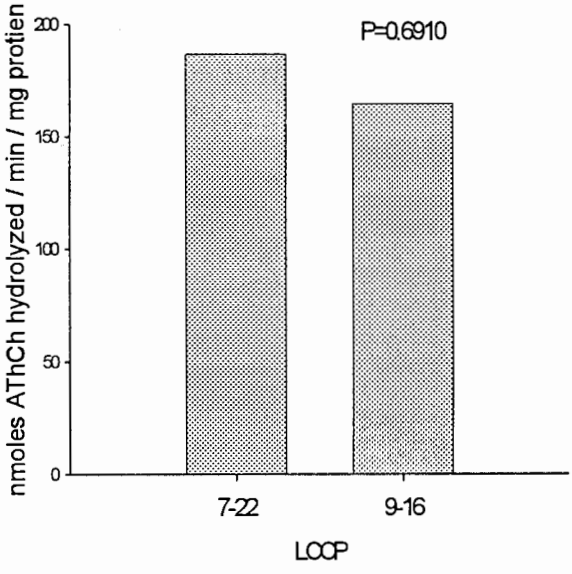


APPENDIX F. AChE activities at 10 sites from May, July and September "10 Sites Sampling" 2003.









APPENDIX G. Physical/Chemical characteristics at three sites on McLoud Run from September 2002 through August 2003.

Date Site	Time	Temperature °C	Dissolved O_2 mg/L	pH	Hardness mg/L $CaCO_3$	Alkalinity mg/L $CaCO_3$	Conductivity mS/cm
8/15/02							
42nd Street	9:00 a.m.	21.1	6.75	8.16	197	128	0.400
McLoud	10:00 a.m.	18.7	16.4	8.17	227	179	0.582
J Avenue	11:15 a.m	16.8	11.14	8.07	367	214	0.570
9/17/02							
42nd Street	10:00 a.m.	17.7	7.42	8.20	159	93	0.396
McLoud	11:00 a.m.	14.7	12.7	8.19	301	217	0.530
J Avenue	11:30 a.m.	14.0	11.9	8.20	277	196	0.490
10/17/02							
42nd Street	11:00 a.m.	9.3	9.15	8.33	249	125	0.364
McLoud	12:00 a.m.	11.2	11.2	8.30	338	239	0.462
J Avenue	1:00 p.m.	10.0	11.9	8.29	330	219	0.486
11/16/02							
42nd Street	11:45 a.m.	4.8	9.28	8.19	314	162	0.812
McLoud	12:30 a.m.	11.0	16.6	8.20	345	205	0.482
J Avenue	1:15 p.m.	8.0	16.22	8.22	320	198	0.458
12/19/02							
42nd Street	9:30 a.m.	3.9	7.72	8.20	225	80	0.396
McLoud	10:00 a.m.	4.2	9.33	8.60	356	208	0.403
J Avenue	10:30 a.m.	4.4	10.31	8.50	300	211	0.530
1/16/03							
42nd Street							
McLoud	11:00 a.m.	5.6	11.87	8.01	309	219	0.422
J Avenue	12:30 p.m.	2.1	14.77	7.99	403	201	0.390
2/18/03							
42nd Street		Frozen					
McLoud	10:30 a.m.	7.2	10.37	8.07	324	217	0.468
J Avenue	11:30 a.m.	4.0	14.04	8.08	409	219	0.506
3/18/03							
42nd Street	11:00 a.m.	7.0	2	8.40	152	88	0.496
McLoud	12:30 a.m.	12.6	13.3	8.03	349	194	0.550
J Avenue	1:00 p.m.	11.9	8.09	8.02	347	214	0.547

APPENDIX G. (Continued) Physical/Chemical characteristics at three sites on McLoud Run from September 2002 through August 2003.

Date Site	Time	Temperature °C	Dissolved O_2 mg/L	pH	Hardness mg/L $CaCO_3$	Alkalinity mg/L $CaCO_3$	Conductivity mS/cm
4/17/03							
42nd Street	11:00 a.m.	9.0	4.35	8.24	259	135	0.690
McLoud	12:00 p.m.	9.7	14.9	8.11	327	209	0.494
J Avenue	1:00 p.m.	9.7	12.56	8.09	339	218	0.530
5/19/03							
42nd Street	10:30 a.m.	14.2	12.7	7.82	271	139	0.611
McLoud	11:30 a.m.	13.9	13.1	7.80	301	203	0.621
J Avenue	12.30 p.m.	14.6	12.8	7.78	322	199	0.634
6/20/03							
42nd Street	10:00 a.m.	16.9	4.98	7.70	181	95	0.446
McLoud	11:30 a.m.	15.4	10.21	7.67	324	211	0.564
J Avenue	12:30 a.m.	15.2	11.42	7.67	306	179	0.570
7/16/03							
42nd Street	11:00 a.m.	21.4	5.56	7.69	166	144	0.374
McLoud	12:00 p.m.	15.9	12.47	7.65	292	236	0.562
J Avenue	1:00 p.m.	15.8	11.51	7.72	232	231	0.568
8/18/03							
42nd Street	9:30 a.m.	20.0	NA	7.62	361	261	0.728
McLoud	1:00 p.m.	17.7	NA	7.63	339	244	0.580
J Avenue	11:30 a.m.	17.3	NA	7.62	343	240	0.594