EVALUATION OF THE USE OF THE BIVALVES *Ischadium recurvum* RAFINESQUE, 1820 AND *Corbicula fluminea* MULLER, 1774 AS BIOLOGICAL INDICATORS OF RELATIVE WATER QUALITY IN TERMS OF GROWTH AND UPPER TEMPERATURE TOLERANCE

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Jon Michael Hemming, B. S.
Denton, Texas

December 1997

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Growth of mussels under laboratory conditions was examined under various food regimes in different water types and temperatures. Growth was less than would be useful as an indicator and comparisons with field exposures were of minimal value. The effects of organophosphates on bivalves were examined via toxicity tests, tissue concentration, and by controlling exposure through the use of physical constraints. Upper temperature tolerance of both bivalve species was examined with respect to different acclimation temperatures and organophosphate exposures. Deviations from control exposures occurred at some temperatures. Copper effectively lowered the mean heat coma temperatures of *C. fluminea* at some concentrations, however, chlorine exposures did not alter heat coma temperature.

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INTRODUCTION

Mussels belong to the family Mytilidae which is comprised of 33 genera and more than 250 species. Mytilids present in North American waters are represented by 17 genera and about 57 species which are predominantly estuarine (Knopf, 1981). Their ubiquitous distribution, life history, and economic importance make mussels particularly well suited as bioindicators for eco-physiological and toxicological studies of water quality. For example, mussels are widely distributed and found in a variety of habitats that can range from open areas (burrowed into the sand and gravel) to crevaced areas (including rocks, old burrows, coral, roots of estuarine plants, and oyster clumps). In addition, post-larval mytilids are anchored to the substratum by a series of wire-like, byssus threads laid down by the distal foot (Price, 1983; Lane *et al.*, 1985; Barnes, 1991) and so, are continuously exposed to toxicants in their immediate habitat.

One such bivalve species *Brachidontes variabilis* has recently been studied in an attempt to remedy the lack of life history information available for this group of molluscs. Morton (1988) examined a resident mussel

population in a mangrove at Ting Kok on the northern shore of Tolo Harbour, New Territories of Hong Kong. *B. variabilis* breeds only once per year in the summer. Recruitment occurs from June to September. September gives rise to small individuals with an average length of four mm. The mussels continue to grow until January when new individuals reach a mean length between 8.5 and 11.5 mm (or 15 mm in some years). After winter arrives, growth is diminished until summer, while gonads mature in the new recruits. The second year growth only occurs in the summer, then ceases until December. In second year individuals, substantial growth occurs from December to March. In the third year little growth occurs in the spring as they reach sexually maturity and spawn. During the following winter, mortality ensues after the mussels have reached a final length of 15-20 mm (Morton, 1988).

Many events precede the recruitment phase. Mytilids, like most bivalves, are dioecious with gonads encompassing the intestinal loops. The gonads are usually so close to each other in a paired condition that they are difficult to differentiate. There is no copulation in these bivalves as indicated by the simple gonoducts. The lamellibranchs empty gametes into the nephridium through the short gonoduct. The eggs and sperm exit via the nephridiopores. The gametes are

swept out with the exhalant siphon from the parabranchial cavity into which they are shed. Fertilization often occurs in the surrounding waters and is facilitated by the clumping organization of mussel beds. Once the gametes fuse, they develop into a free-swimming trochophore which changes into a veliger larva (Barnes, 1991).

The veliger, typical of marine bivalves, is symmetrical but eventually becomes enclosed within two characteristic valves. Veligers have potential for dispersal over long distances depending on the water currents. Metamorphosis is characterized by the shedding of the velum. Settling follows, which may delay metamorphosis, and often involves considerable testing of the substratum (Barnes, 1991). Young post-larval mussels secrete long drifting threads which are simple monofilaments distinct in form and function from the final byssal threads. These threads facilitate prolonged suspension in the water column after metamorphosis until a preferred substratum is encountered. Post-larvae or plantigrades drift periodically, temporarily settling on substrata such as bryozoans, hydroids and filiform algae. They eventually permanently settle on existing mussel beds or occasionally begin new mussel beds (Lane et al., 1985).

Bivalves of the subclass Lamellibranchia are filter feeders that posses a greatly enlarged, specialized gill

which supplies maximum surface exposure area for feeding. The combination of relatively large body surface area and highly permeable tissues precludes the need for respiratory pigments in many bivalves (Barnes, 1991). Bivalves are often isosmotic in marine environments and only slightly hyperosmotic in fresh waters. These adaptations minimize water loss. However, the relatively large area of permeable tissues utilized for respiration and feeding facilitates rapid uptake and storage of lipophilic pesticides and metals.

The simplicity illustrated in osmoregulation is mirrored by the bilateral nervous system of bivalves. The nervous system contains three pairs of ganglia and two pairs of long nerve cords. Cerebropleural ganglia are found on either side of the esophagus which are connected dorsal of the esophagus. Each of these ganglia give rise to a pair of nerve cords directed posteriorly. The top two nerve cords terminate in visceral ganglia on the posterior adductor muscle. The second or lower pair of cords extend posteriorly and ventrally into the foot connecting to the pedal ganglia. The foot and anterior adductor muscle are under the control of the pedal and cerebral ganglia. The visceral ganglia control the posterior adductor mussels and the siphons. The cerebral ganglia also coordinate movements of the foot and

valves. The margin of the mantle houses the majority of bivalve sensory organs (Barnes, 1991).

Kramer (1989) noted that the concentration of pollutants in molluscs can serve as an indicator for the level of pollution in the environment. Bivalves accumulate toxins making them suitable for the characterization of specific ecosystems. An equilibrium concentration can be obtained after only several weeks of exposure (Kramer *et al.*, 1989). Stirling and Okumus (1994) stated that it is well known that high concentrations of contaminants can play an important role as stressors to mussels. Mussels can respond to stress by valve closure or inhibition of byssal thread production, respiration and filtration rate, and as a consequence poor growth (Stirling and Okumus, 1994).

Some species of mussels are economically important as a fishery (Camacho *et al.*, 1991; Williams *et al.*, 1993). Other species such as the blue mussel *Mytilus edulis* Linnaeus, 1758 (Minchin and Duggan, 1989) are known for their detrimental effects on shellfish culture. The negative impact of the recent introduction of zebra mussels *Dreissena polymorpha* Phallas, 1771 (Mollusca: Dreissenidae) to North America is well documented (Gillis and Mackie, 1994).

Mussel studies have repeatedly illustrated a strong direct relationship between temperature and growth. A study

performed on the Mediterranean mussel (*Mytilus galloprovincialus*) demonstrated that growth and development were greatest from 20-25°C (His and Dinet, 1989). Blue mussels achieve optimal growth at 20°C (Brenko and Calabrese, 1969) and *Mytilus californianus* Conrad, 1837 was reportedly most successful in growth from 18-20°C (Widdows, 1991). Widdows (1991) also recorded the influence of salinity, temperature, dissolved oxygen, and toxicant variations on the growth and development of *Mytilus*. He reported that, although salinity had little effect over a large range, temperature dependent growth is relative to latitude.

Toxicant exposures can also influence mussel growth. For example, Lowe (1967) reported decreases in shell deposition in oysters exposed to various pesticides at concentrations as low as 0.1 mg/L. Toxicant studies utilizing DDT, toxaphene, and parathion produced physiological inhibitions such as reduced growth in oysters (Lowe, 1970). Growth inhibition was also observed in oysters during an exposure to Arochlor 1254 at a concentration of 5 mg/L (Lowe, 1972).

The tensil strengths of byssus threads were also used to demonstrate the sublethal effects of 4-Nonylphenyl on the blue mussel (Granmo *et al.*, 1989). Blue mussels exposed to

4-Nonylphenyl suffered a weakening in byssus thread strength, and in higher concentrations a lack of byssus thread production was observed.

Some relatively new commercial insecticides have become an environmental concern. Their widespread use in agriculture and domestic lawn and garden applications have led to the contamination of aquatic environments via runoff and waste water treatment plant discharge. Two commercially available organophosphorus pesticides are diazinon [0,0-diethyl 0-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate] and chlorpyrifos [0,0-diethyl 0-(3,5,6-trichloro-2 pyridinyl) phosphorothioate]. Diazinon is sold under several trade names including Spectracide, Sarolex, and Diazitol among others. The annual production of diazinon is almost 4 million kg in the United States and is used by professional pest control operators (40%), in private homes (40%), in agriculture (10%), and on turf (10%). It is also used on over 75 food crops to control soil and leaf eating pests (Robertson, 1989).

Chlorpyrifos is another widely utilized active ingredient in commercial pesticides. Chlorpyrifos is also known as dursban which can be found in many fire ant treatments, mole cricket baits and general insecticides. This chemical is somewhat persistent in the environment with

a half-life of 24 days (95% confidence 20-29 days) based on a sea water-sediment persistence test (Schimmel *et al.*, 1983). Chlorpyrifos degrades via biological and chemical interaction and photolysis. However, the intermediate compounds resulting from the breakdown of chlorpyrifos are often more toxic than the original active ingredient itself (Barcelo, 1993). Unfortunately, the assays used to determine the presence of these pesticides are often expensive.

Organophosphorus pesticides act at nerve endings primarily by phosphorylation of the acetylcholinesterase enzyme (AChE; Gyson and Margot, 1958). Inhibition of AChE retards normal control of nerve impulse transmissions from nerve fibers to muscle, gland cells, other nerve cells in autonomic ganglia and in the brain itself. Symptoms and signs of poisoning are not evident, however, until some critical portions of the tissue enzyme mass are inactivated by phosphorylation. The loss of enzyme function allows for accumulation of the impulse transmitter acetylcholine (ACh) at cholinergic neuroeffector junctions (muscarine effects), at skeletal nerve muscle junctions and autonomic ganglia (nicotinic effects), and in the brain. High concentrations of ACh cause muscle contraction in smooth muscles and secretion in gland cells at the cholinergic nerve junctions.

The resulting excess of acetylcholine at skeletal muscular junctions can be excitatory or may weaken or paralyze the cell by depolarizing the end-plate. High ACh concentrations in the brain may result in sensory and behavioral disturbances, as well as lack of coordination and depression of motor function. The ultimate cause of death from organophosphorus poisoning are depression of respiration and pulmonary edema. Regeneration of new unaffected AChE in critical tissues is the recovery process (Morgan, 1989; Eckert et al., 1988).

Organophosphates are efficiently absorbed via inhalation, ingestion and diffusion through permeable membranes including the epidermis. Breakdown of the pesticide within an organism occurs predominately through hydrolysis. However, if the breakdown is slow, as is the case for some organophosphorus pesticides, the toxicants may be stored in body fat tissues (Morgan, 1989; Eckert et al., 1988).

Conversions occur in many organophosphorus pesticides readily from -thions (P=S) to -oxons (P=O). In the environment the conversions occur in the presence of oxygen and light, but in the body the change is facilitated mainly by the action of liver microsomes. Unfortunately, the -oxons are much more toxic than the precursor -thions, but the -

oxons break down faster. These compounds are much more dangerous than the parent compounds causing enzyme inhibition 14,000 times more toxic (Soliman et al., 1982; Singmaster, 1990). Hydrolyzing the ester linkage of -oxons and -thions produces alkyl groups and a leaving group. Their relative toxicity is low when compared to their precursors (Morgan, 1989).

Phosphorylated acetylcholinesterase enzymes can be dephosphorylated (or reactivated) by the oxime antidote pralidoxime within the first day or two of initial organophosphate-acetylcholinesterase binding. However, the enzyme-phosphoryl bond is strengthened over time by the loss of one alkyl group forming the phosphoryl adduct. Pralidoxime cannot reactivate the enzyme after this has occurred (Morgan, 1989).

Members of the family Mytilidae have been used in toxicity (Beaumont and Budd, 1984), temperature, salinity (His and Dinet, 1989), and dissolved oxygen studies (Riisgard and Randlov, 1981). Many mytilids species are indigenous to the bays and bayous of the Florida panhandle where they occur in relative abundance. Included in the benthiofauna are brachidontids (Personal Communication Charles D. Asaro, UWF, Dept. Biol.). *Ischadium recurvum*, the brachidontid called the hooked mussel, has been reported

from Cape Cod to the West Indies (Knopf, 1981) including the relatively clean waters of the Santa Rosa Sound and highly polluted estuarine sites, e.g. Bayou Chico, in northwest Florida (Hand *et al.*, 1994). *Ischadium recurvum* was previously known as *Mytilus recurvus* (1928), *Mytlilus hamatus* (1945), and until 1980 *Brachidontes recurvus* Rafinesque, 1820.

The hooked mussel is the most common subtidal mussel in the brackish waters of Chesapeake Bay (Allen, 1960). At times, they are so abundant that they become a serious fouling agent on oyster beds (Chanley, 1970). Chanley noted that oyster bed fouling led to extensive research on the hooked mussel. The influence of low salinity and temperature on this mussel was studied in depth (Chanley, 1958; Allen, 1960; Nagabhushanam, 1965). Unfortunately, despite the importance of this organism as a fouling entity and its wide distribution little is known about its biology with the exceptions previously mentioned (Allen, 1962). More specifically, nothing is known about the ecological/physiological or toxicological responses of mussels such as *I. recurvum*. This, however, is not the case for other mussels (e.g. *Mytilus edulis* Linne, *Mytilus californianus* Conrad, *Modiolus demissus* Dillwyn, and *Modiolus modiolus* Linne) on which considerable data are

available pertaining to numerous biological aspects (Allen, 1962).

Recently, researchers have begun investigating the bioaccumulation ability of the hooked mussel for metals (Reidel et al., 1995). This line of research has been studied in depth for other mussels mentioned above among others (Laughlin, 1988; Hutagalung, 1989; Lakshmanan and Nambisan, 1989; Balogh, 1988).

A mytilid highly similar to the hooked mussel, *Brachidontes variabilis*, is one of the most common high zoned mytilids on a wide variety of local estuarine shores in Hong Kong (Morton, 1988). Morton (1988) remarked that prior to his study nothing was known about *B. variabilis* with respect to the reproduction and life history of the species. *B. variabilis*, is on average smaller than *I. recurvum*, and mature at a shell length of approximately 7.5 mm during their first year. *Mytilus edulis* (a much larger mussel) juveniles have been reported at lengths of 17-26 mm (Mallet and Carver, 1989).

Another bivalve under current investigation for use as a bio-indicator is the freshwater Asiatic clam *Corbicula fluminea* Muller (Doherty, 1990; Graney et al., 1984). *C. fluminea* is in the subclass Lamellibrachia, as is the hooked mussel, and is also widely distributed throughout the United

States (McMahon, 1982) having been introduced into U.S. waters in the early 1900's (Britton and Morton, 1979; Sinclair and Isom, 1963: as cited by Foe and Knight, 1986). *C. fluminea* is hermaphroditic (McMahon, 1986, 1991), able to produce dispersal filaments (McMahon, 1982), and is also known primarily as a highly invasive fouling agent (Doherty, 1990; Graney et al., 1984). The numerous similarities between these two species may allow for comparative study and similar uses as indicators of water quality.

The upper temperature tolerance of some bivalve species has also been examined (Liu and Morton, 1994; Spidle, et al. 1995; Urban, 1994, and Byrne et al., 1988). The research to date has focused primarily upon the limits of temperature tolerance with respect to environmental temperature extremes. Liu and Morton (1994) investigated the affects of dramatic elevations in air and rock temperatures on a sessile mussel community. Spidle et al. (1995) studied the limits of tolerance to temperature and salinity on two *Dreissena* species. Urban (1994) examined the upper temperature tolerance of ten commercially important bivalve species off Peru and Chili with reference to the temperature extremes presented by El Nino. *C. fluminea* was observed to be less tolerant of aerial exposure at higher temperatures (35°C; Byrne et al., 1988). Temperature was said to be one

of the most important environmental factors controlling physiological processes, and temperature tolerance was related to the temperature to which mussels were acclimated (Urban, 1994).

The effect of stressors on temperature tolerance has been investigated primarily on fishes. The effects of salinity on the temperature preference and tolerance of Mayan cichlids was examined for potential range expansion in Florida (Stauffer and Boltz, 1994). A different approach was taken in assessing the effects of acute temperature change on the rate of respiration and toxicant uptake of the rainbow trout, *Salmo gairdneri* (Black et al., 1991).

Classic temperature tolerance experiments have traditionally been performed on fish and quantified using either static or dynamic methods (Bennett and Beitinger, 1997). Temperature tolerance can be estimated using static methods which quantify time of resistance of fish suddenly exposed to either high or low temperatures near their lethal limits. Utilizing acclimation temperatures ranging across an organism's zone of tolerance makes possible the identification of zones of tolerance, resistance and lethality (Fry, 1947; Brett, 1970; as cited by Bennett and Beitinger, 1997). Static methods do not allow partial acclimations during trial exposures and death is usually the

endpoint making this method an indicator of physiological thermal tolerance (Fry, 1947: as cited by Bennett and Beitinger, 1997).

In dynamic trials, temperature tolerance is quantified as the critical thermal maximum (CTMax) or critical thermal minimum (CTMin) by exposing organisms to a constant rate of unidirectional water temperature change until a nonlethal endpoint is reached (e.g., loss of equilibrium). Cowles and Bogert (1944) introduced and defined CTMax and CTMin. The concepts were then modified by Lowe and Vance (1955) to include statistical variation and were later ratified for standardization by Hutchinson (1961). The concept of CTMax was re-defined by Cox (1974) as the arithmetic mean of the thermal points at which locomotive activity begins to become disorganized, leaving the organism without the option of fleeing adverse conditions which will lead to death. CTM's are quantified estimates of thermal tolerance and are ecologically defensible (Hutchinson, 1976; Bennett and Judd, 1992a: as cited by Bennett and Beitinger, 1997) and are often used as biological assays for various stressors (as mentioned above from Beitinger and McCauley, 1990).

Research has shown a strong direct relationship between temperature and growth (His and Dinet, 1989) in that maximum growth occurs at the optimum temperature. Similarly, there

is a relationship between acclimation temperature and temperature tolerance. As acclimation temperatures increase, so will the upper temperature tolerance of the organism within a given range specific to that organism. Both are effected by stresses placed on the organism. The purpose of this study was to gain a better understanding of the effects of temperature and the pesticides diazinon and chlorpyrifos on hooked mussel growth and upper temperature tolerance. Growth and temperature tolerance comparisons of mussels in the laboratory were made with field samples from Bayou Chico, known to have poor water quality, and the relatively uncontaminated Santa Rosa Sound, which receives very little fresh water input (Hand, et al. 1994). These experiments had the potential to reveal information relative to the use of hooked mussel growth or upper temperature tolerance as an indicator of water quality in a field setting. The potential use of mussels as bio-indicators may help to alleviate a portion of the financial burden associated with chemical analysis by reducing random sampling to specifically indicated problem areas or sites sometimes associated with non-point source pollutants.

Secondary testing was conducted to investigate the use of the Asiatic clam (*Corbicula fluminea*) in the same role for freshwater systems. Upper temperature tolerance and

overall sensitivity of the clams was investigated in an attempt to assess its usefulness as a bio-indicator of water quality.

OBJECTIVES

Experimentation on the hooked mussel was designed to measure variation in growth with respect to different temperature constants over a given period. The temperature at which the mussels achieved maximum growth were utilized in pesticide exposures. Concentrations reported as lethal median values for other bivalve molluscs were utilized in determining approximate sublethal exposure concentrations. Diazinon was utilized in a static toxicity test to determine a more accurate sublethal range of exposure. The sublethal concentration range was then to be used in exposures of hooked mussels to determine if growth was influenced by the presence of diazinon at sublethal concentrations. Growth of mussels was monitored in exposures to sublethal diazinon concentrations at the predetermined optimal temperature for growth. The same procedure was also followed for the second pesticide chlorpyrifos. These exposures were then used as a comparison for mussel growth in the field. Growth was recorded during exposures of mature and juvenile mussels at

four field locations.

The above assays were used to test the following hypotheses:

1) Ho: Growth of *Ischadium recurvum* is not significantly different among temperatures of 20, 25, 30, and 35°C

   Ha: Growth of *Ischadium recurvum* is significantly different among temperatures of 20, 25, 30, and 35°C

2) Ho: Growth of *Ischadium recurvum* is not significantly different among four sublethal diazinon concentration and a control.

   Ha: Growth of *Ischadium recurvum* is significantly different among four sublethal diazinon concentrations and a control.

3) Ho: Growth of *Ischadium recurvum* is not significantly different among four sublethal chlorpyrifos concentrations and a control.

   Ha: Growth of *Ischadium recurvum* is significantly different among four sublethal chlorpyrifos concentrations and a control.

4) Ho: Growth of *Ischadium recurvum* is not significantly different among four field locations.

   Ha: Growth of *Ischadium recurvum* is significantly different among four field locations.

5) Ho: Growth of juvenile *Ischadium recurvum* is not significantly different among four field locations.

   Ha: Growth of juvenile *Ischadium recurvum* is significantly different among four field locations.

The results of the initial assays led to subsequent tests pertaining to the bioaccumulation ability of the hooked mussel in the presence of chlorpyrifos.

6) Ho: Chlorpyrifos tissue concentration of *Ischadium recurvum* is not related to chlorpyrifos exposure concentration.

   Ha: Chlorpyrifos tissue concentration of *Ischadium recurvum* is related to chlorpyrifos exposure concentration.

The minimal growth of *I. recurvum* in the laboratory prompted investigation into another laboratory method in which mussels could be utilized as indicators of organophosphates in the environment. Upper temperature tolerances among four acclimation temperatures and different sublethal exposures to organophosphates was examined to test the following hypotheses.

7) Ho: Upper temperature tolerance of *Ischadium recurvum* is not significantly different among four acclimation temperatures.

   Ha: Upper temperature tolerance of *Ischadium recurvum* is significantly different among four acclimation temperatures.

8) Ho: Upper temperature tolerance of *Ischadium recurvum* is not significantly different among a control and sublethal diazinon concentration exposures.

Ha: Upper temperature tolerance of *Ischadium recurvum* is significantly different among a control and sublethal diazinon concentration exposures.

9) Ho: Upper temperature tolerance of *Ischadium recurvum* is not significantly different among a control and sublethal chlorpyrifos concentration exposures.

Ha: Upper temperature tolerance of *Ischadium recurvum* is significantly different among a control and sublethal chlorpyrifos concentration exposures.

The preceding experiments led to the investigation of bivalve temperature tolerance as an indication of water quality. The organism used was the Asiatic clam *Corbicula fluminea* because of its similarity to *Ischadium recurvum* in morphology and behavior and its availability. Background research to determine the the sensitivity of *C. fluminea* to organophosphates (specifically diazinon) was the first area of study.

10. Ho: The toxicity of diazinon to *Corbicula fluminea* is independent of valve closure.

Ha: The toxicity of diazinon to *Corbicula fluminea* is dependent of valve closure.

After the background information was collected, additional hypotheses were tested to assess the usefulness of the Asiatic clam's upper temperature tolerance as an indicator of water quality.

11. Ho: The upper temperature tolerance of *Corbicula fluminea* is not different among three acclimation temperatures of 10,20, or 30°C.

    Ha: The upper temperature tolerance of *Corbicula fluminea* is different among three acclimation temperatures of 10, 20, or 30°C.

12. Ho: The upper temperature tolerance of *Corbicula fluminea* is not different among four diazinon concentrations and a control at 10, 20, or 30°C.

    Ha: The upper temperature tolerance of *Corbicula fluminea* is different among four diazinon concentrations and a control at 10, 20, or 30°C.

13. Ho: The upper temperature tolerance of *Corbicula fluminea* is not different among six copper concentrations and a control at 10, 20, or 30°C.

    Ha: The upper temperature tolerance of *Corbicula fluminea* is different among six copper concentrations and a control at 10, 20, or 30°C.

14. Ho: The upper temperature tolerance of *Corbicula fluminea* is not different among six chlorine concentrations and a control at 10, 20, or 30°C.

    Ha: The upper temperature tolerance of *Corbicula fluminea* is different among six chlorine concentrations and a control at 10, 20, or 30°C.

MATERIALS AND METHODS

Growth Exposures in Various Media and Food

Mussels (*Ischadium recurvum*) were collected in Pensacola Bay in Pensacola, Florida in the shallow waters adjacent to the L&N Railroad (N 30°25'23", W 87°10'45"). Experiments were carried out in glass aquaria utilizing filtered, raw (unfiltered) or synthetic sea water. The mussels were fed equal volumes of cultured marine algae and/or Roti-Rich invertebrate food (formulated by Florida Aquafarms Inc.; composition, page 24). The mussels were fed equal volumes of a homogeneous mixture of the various foods. Each food and water parameter tested was carried out over the determined temperature range at four intervals. For example, the temperature range of 20-35°C was tested at 20, 25, 30 and 35°C. Twenty-four or thirty mussels were exposed to each of the four temperatures. Mussels were measured (overall length) with a digital caliper to the nearest 0.01 mm and, in initial trials, weighed (wet weight) on a Mettler balance at the beginning and end of the 21-day exposure.

The first trial consisted of the exposure of 120 juvenile hooked mussels. Thirty mussels were divided into three replicates of ten for each temperature. Mussel size

varied from 4.5 to 11.2 mm in overall length. Exposures were carried out in four 10L glass aquaria. The medium was Fritz's synthetic sea salt with micronutrients at a salinity of 35 part per thousand in dechlorinated Denton, Texas tap water. The mussels were fed approximately 100 ml/day *Isochrysus galbana* marine algae. The cultured algae were maintained at a concentration of approximately 1.0 X 10^6 cells/ml as per the Standard Operating Procedure for the maintenance of algae systems from the Environmental Protection Agency's Gulf Ecology Division in Gulf Breeze, Florida. According to that protocol, the algae were cultured in autoclaved, super-filtered sea water with Fritz algal nutrients with the exception of the first trial where synthetic sea water was used. Algal concentrations were confirmed using a hemacytometer.

Mussel exposures were 21-days in duration. Temperatures were held at 20±1.0, 25±1.0, 30±1.0 and 35±1.0°C. The mussels were measured (overall length) and weighed (wet weight) prior to the trial and at the end of each seven day period. After the initial measurement, the mussels were removed from the substratum to make measurements. This removal caused breakage of byssal threads. Wet weight measurements proved variable and inconsistent because of differential water retention within the valves, and so were

a poor indication of growth. Wet weight was not utilized in further trials.

The second temperature trial, consisting of a series three tests, was conducted in flow through ten gallon aquaria. Filtered sea water from the Santa Rosa Sound at naturally varying salinity concentrations (22.4-27.5 parts per thousand) was continuously added to the aquaria at a rate of 1L every hour. Temperatures were held at 20±1.0, 25±1.0, 30±1.0 and 35±1.0°C. To eliminate the need to brake byssal threads for measurements, the mussels were held in place on a plexiglass sheet using Super Glue. Mussel valves were marked with paint pens to improve measurement consistency by ensuring placement of the calliper in the same position for each repeated measure.

Isochrysus galbana marine algae were the food source for the initial exposure under these conditions. *I. galbana* (20 ml) at a concentration ≈1.0 $X10^6$ cells per ml was injected into each aquaria twice daily. Young mussels(n=120) ranging from 22-29 mm in overall length were used. Mussels were measured in overall length on the first, seventh, fourteenth, and twenty-first day of exposure.

The mussels exposed in the second trial under these conditions were fed Roti-Rich invertebrate food. Roti-Rich (Florida Aqua Farms, Inc.) is a commercial food formula

containing quality food grade ingredients including an inactivated yeast base, micro algae, a formula of vitamin mix and specific trace nutrients that have been found to enhance invertebrate growth and reproduction. The formula has been designed for a wide range of filter-feeders including rotifers, crustaceans, mollusks, corals, tube worms, sponges among others. The formula is available to research institutions, zoos, public aquaria and aquaculture facilities.

Each aquarium containing two replicates of fifteen mussels was fed 10 ml of Roti-Rich twice daily. This amount clouded the water thoroughly as per the instructions for the liquid invertebrate food. The water was cleared by the second feeding approximately eight hours later. The invertebrate food was removed from the water by means of bivalve filtration and, to some extent, overflow. The 120 mussels used in the filtered sea water *I. galbana* marine algae trial were also used in this trial because they were further acclimated to the environment, appeared healthy upon visual inspection, and their byssal threads were well established. These conditions suggested that the energy utilized to acclimate and produce byssal threads would act as less of a negative factor for growth than that of newly introduced mussels. The mussels were measured (overall

length) at the initiation and conclusion of this twenty-one day trial. The seven and fourteen day measurements were omitted to eliminate the stress of being removed from the water.

The third and final temperature exposure trial utilized the same 120 mussels as were exposed in the previous trials for the above mentioned reasons. The mussels were measured for overall length at the beginning and twenty-first day of the trial. Mussels were fed fifty ml of *I. galbana* marine algae (at standard concentration) and 10 ml of Roti-Rich invertebrate food two times daily. Feedings occurred approximately eight hours apart.

Raw (unfiltered) sea water from the Santa Rosa Sound was utilized for an exposure of thirty mussels in two replicates of fifteen mussels each. The raw sea water was arranged to flow through two 1.5L shallow glass aquaria at a rate of ~8L per hour. The mussels were immobilized on plexiglass squares with Super Glue and marked as before. The temperature range for this exposure was naturally varying (27.8-29.1°C) and the salinity varied from 21.7-23.4 parts per thousands.

The initial length and growth in terms of the difference between initial and final length for the mussels in each water type, feeding method, and acclimation

temperature were examined. The data were analyzed using the Statistical Analysis System (SAS) for normality (Shapiro-Wilks). The data summary also provided the mean, median, variance, standard deviation and sample size. A variance ratio test was also performed (Zar, 1984). A paired T-Test was performed on each data group to assess the significance of the difference between the initial and final length. Analysis of variance, Kruskal-Wallis chi squared approximation, and the Median 1-way chi squared approximation were also performed on both the initial length and growth among groups.

If the data were normal and homogeneous the parametric ANOVA was used to analyze the data. If the data were non-normal or heterogeneous the Kruskal-Wallis or Median 1-Way methods were used to analyze the data. Both parametric and non-parametric analyses were performed in cases where the data were only slightly non-normal or heterogeneous. Parametric analysis was used to analyze the data in such cases when it showed distinctions among means.

Growth determined to be useful for the purpose of bio-indication was defined as a minimum increase in overall length of 0.5 mm. The statistical differences shown in the analyses of these data groups were compared to that which was defined as useful for bio-monitoring purposes.

Field Exposure Growth

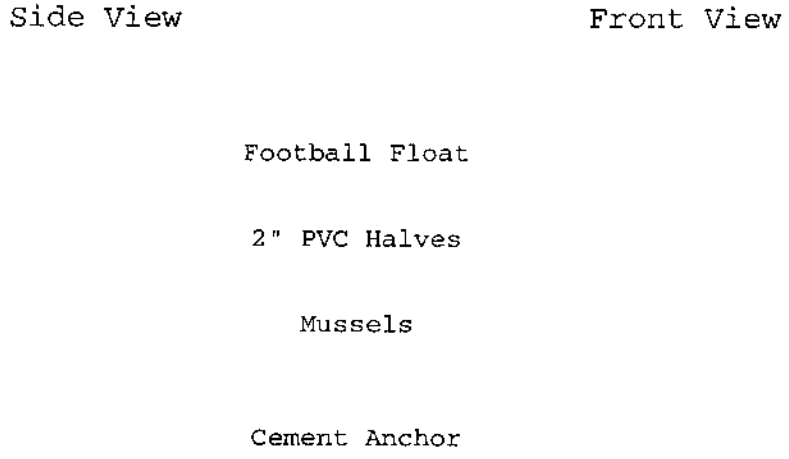
This assay was used to compare laboratory growth with field growth. Hooked mussels were deployed at selected sites in the Pensacola area, specifically the relatively clean Santa Rosa Sound and the contaminated Bayou Chico. One site was chosen in the Santa Rosa Sound (SR) (N 30°20'22", W 87°09'30") adjacent to Sabine Island (next to the Bob Sikes Bridge in Pensacola, Florida) to represent an area of relatively good water quality. Three locations were chosen in Bayou Chico to represent areas of relatively and historically poor water quality. Bayou Chico site 1 (BC 1) (N 30°24'09", W 87°15'57") was located in the western fork of the bayou, Bayou Chico site 2 (BC 2) (N 30°24'51", W 87°15'35") was located in the eastern fork, and Bayou Chico site 3 (BC 3) (N 30°24'16", W 87°15'13") was located centrally in the main bayou body. Temperature, dissolved oxygen and salinity were recorded three times for each site at high and low tide. All measurements were taken during daylight hours.

Mussels were mounted with Super Glue on racks constructed from two inch PVC pipe halves approximately one foot long. PVC pipe halves were used to minimize mussel removal via rough waters and abrasion without hindering filtration capacity. A mesh over the mussels would have

quickly fouled with the copious amounts of filamentous algae present in these waters in the summer months. The racks consisted of four 2" PVC halves defined as shelves (Figure 1). Each shelf housed ten mussels. Three shelves housed adults and one shelf per rack housed ten juvenile mussels. The racks were held together with nylon line. They were weighted down with a cement anchor and buoyed with a styrofoam "football" float. The racks were checked one to two times weekly for algae buildup, stability, and vandalism.

Figure 1

Field Exposure Rack Design



The mussels were measured (overall length) at the beginning and end of the 21-day exposure in each the respective habitats. The field exposures at the contaminated and clean sites occurred simultaneously to eliminate as much variation as possible. The growth of mussels at the field sites were compared to each other and that of the laboratory assays in the terms of statistical differences and usefulness for biological monitoring of relative water quality. Concentrations of the two pesticides, diazinon and chlorpyrifos, were measured during the field exposures with GC analysis (Appendix 1). The relative growth of the field exposures in reference to the pesticide concentration data collected was examined.

The initial length and growth in terms of the difference between initial and final overall length for the mussels in each field area was examined. The data were analyzed in the same manner as the laboratory growth tests for statistical differences and growth relative to the biological monitoring minimum.

I. recurvum LC50 Determination

Diazinon

Utilizing existing LC50 data for bivalve molluscs, multiple range finding tests were performed (by the same

exposure methods as the following LC50 determination). Static toxicity tests were performed following the range finding tests to determine an appropriate sublethal diazinon concentration range for the hooked mussel. Forty mussels were exposed to each diazinon concentration and a control. Mussels were removed from their substrate and individually placed into each exposure solution.

Diazinon was obtained from the commercial product Spectracide: Lawn and Garden Insect Control, which is 25% active ingredient (diazinon) and 75% inert ingredients. Diazinon solutions were prepared daily by micropipetting the diazinon product into 1 liter filtered sea water. Prepared solutions were placed into 1 liter Wheaton bottles for transfer. Exposures were performed in 500 ml Carolina dishes that were aerated with air-stones and covered. Four replicates of ten mussels were exposed at each of the seven diazinon concentrations and control. The diazinon concentrations were replaced daily. The diazinon concentrations were confirmed via GC analysis (Appendix 1).

Mortality was observed (failure of valve closure upon prodding) and recorded at 24-hours, 48-hours, 72-hours, and 96-hours. EPA Probit Analysis was performed on the data if there were at least two partial kills and a monotonically increasing response. If those assumptions were not met, the

Trimmed Spearman-Karber analysis was used to analyze the data. The data consisted of the exposure of forty mussels (four replicates of ten) to each of the seven exposure concentrations and control.

Chlorpyrifos

The chlorpyrifos exposures were performed in the same manner as those used for the diazinon test. Chlorpyrifos was obtained from the commercial product ORTHO: Dursban Lawn Insect Spray which was labeled to contain 5.3% chlorpyrifos and 94.7% inert ingredients. Exposure solutions were prepared by the same method used for diazinon. The chlorpyrifos concentrations were confirmed via GC analysis (Appendix 1). The data from the chlorpyrifos exposures were analyzed in the same manner as the preceding diazinon test. Data for seven concentrations and control consisting of four replicates of ten mussels per concentration were analyzed.

Toxicant Growth Exposures

Diazinon

Following static toxicity testing, four sublethal concentrations (estimated from the preceding diazinon LC50 determination) and one control were prepared for relative growth evaluation exposures. Four groups of 30 mussels were

measured for overall length at the beginning and end of the 21-day trial. Each exposure group was exposed in two replicates of 15 mussels for 21-days to one of the determined sublethal concentrations. The two groups of fifteen mussels were immobilized on plexiglass squares with Super Glue and marked for measurement as before for each exposure concentration. Each group of fifteen mussels was placed in an aerated and covered 1.5L shallow glass aquarium. One aquarium was used to expose each replicate of 15 mussels. In addition, one aquarium was renewed with the second highest pesticide concentration tested, but no mussels were exposed to this solution. This was done to observe the difference in pesticide concentration resulting from physical factors compared to those attributed to the presence of mussels.

Exposures were performed at 24°C(±1°C). Diazinon exposure solutions were prepared daily in 4L Erlenmeyer flasks with filtered sea water. The concentration of diazinon was measured at the initiation of the exposure and 24-hours later prior to the daily renewal. Concentrations were determined with GC analysis (Appendix 1).

Chlorpyrifos

The exposure conditions and procedures used to expose mussels to sublethal chlorpyrifos concentrations (estimated from the preceding chlorpyrifos LC50 determination) for relative growth analysis were the same as those for diazinon. Again, one mid-range concentration solution contained no mussels. This solution was used to record the relative chlorpyrifos concentration. The concentration of chlorpyrifos was measured at the initiation of the exposure and 24-hours later prior to the daily renewal. Concentrations were determined with GC analysis (Appendix 1).

Chlorpyrifos Bioaccumulation

Mussels were exposed to chlorpyrifos concentrations approximately equal to those used in the chlorpyrifos growth study. Four sublethal concentrations and control were used in the bioaccumulation evaluation exposures.

Large Carolina dishes (round bowl, 1.5L) were used to expose 60 mussels to 1.2 liters of each chlorpyrifos concentration and control. The chlorpyrifos solutions were prepared using the same protocol stated above for the chlorpyrifos growth analysis. The dishes were aerated and covered during the exposure. Tissue samples were taken at 24

hours, 72 hours, and 7-days of exposure. Tissue samples were taken by removing the mussel soft tissues from the valves but excluding the byssus. The tissues were combined to make two samples of at least five grams for each exposure solution. Each sample consisted of 8 to 12 mussels' soft tissues. Samples were frozen until analyzed (Appendix 2).

Linear regression analysis was run to measure the degree of association and relation of the tissue concentration with the exposure concentration. Bioconcentration factors and percent increase in tissue concentration were also calculated.

I. recurvum Upper Temperature Tolerance for Temperature Acclimations

Mussels (n=30), removed from rocks by breaking their byssal threads, were acclimated to 20, 25, 30, and 35°C for a period of at least three weeks. These were the same mussels used in the final three temperature and feeding growth analyses. Mussels were placed into 10-gallon flow through aquaria which were continually renewed with filtered sea water. Each acclimation was performed in separate aquaria. Temperatures were maintained within 1°C of the desired temperature with Haake heaters for the duration of the acclimation. The 20°C exposure, which was less than

ambient temperature, was placed into a cooling bath and heated to appropriate temperature with a Haake heater. Mussels were fed 50 ml of *Isochrysus* marine algae at a concentration of approximately 1.0 X 10^6 cells per ml and 10 ml Roti-Rich invertebrate food daily.

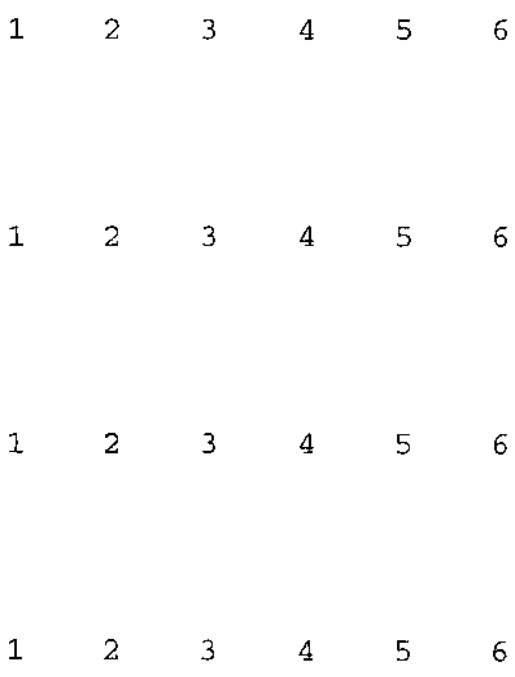
Upper temperature tolerance was determined at the end of the 21-day acclimation period. Mussels were removed from the plexiglass square on which they were mounted for the growth tests and individually placed on a viewing rack composed of two inch PVC halves forming four numbered shelves (Figure 2). The rack facilitated viewing and rapid introduction/removal from the water bath. After the mussels were placed on the viewing rack, they were submerged into a 10-gallon water bath at a temperature approximately equal to the respective acclimation. The Haake heater was then turned on at the highest setting. The heater warmed the 9-10 gallons of water at a rate of 0.3 to 0.5°C per minute. Temperature was monitored with a YSI SCT Model 30 temperature probe. Mussels remained open for a short time, but closed as the temperature increased (~40°C). As the temperature approached the respective upper temperature tolerance, the mussels opened and decreasingly resisted closure upon prodding with a stainless steel probe tipped with a syringe needle. The upper temperature tolerance

endpoint defined as heat coma temperature was the point when mussels were not capable of closure upon prodding (Spidle et al., 1995).

The temperature at which the mussels were not able to close was recorded individually for each mussel exposed as was overall length. Mussels from each acclimation temperature were measured for upper temperature tolerance in two replicates of fifteen mussels. Summary statistics were calculated for each acclimation temperature's respective temperature tolerance data. Analysis of variance, analysis of variance on ranked data, Kruskal-Wallis chi square approximation, median 1-way chi square approximation, linear regression, and multiple range tests for statistically significant differences were performed. The resultant regression line was used to estimate deviations in upper temperature tolerance resulting from ambient stressors. Important differences for bio-monitoring uses were determined to occur at a minimum of 1°C from that of the control.

Figure 2

Bivalve Viewing Rack



I. recurvum Temperature Tolerance for Organophosphate Exposure

Mussels were removed from the their substrate by breaking the byssal attachments. Sixty mussels were exposed, in two replicates of thirty, to five sublethal diazinon or chlorpyrifos concentrations in filtered sea water. Diazinon exposures were performed after the completion of the chlorpyrifos exposures. Exposures were performed in ten gallon aquaria under continuous flow conditions using a diluter system and a wet table at the Environmental Protection Agency's Gulf Ecology Division in Gulf Breeze, Florida. Mussels were exposed to the respective toxicant and concentration at approximately 27°C for 72-hours. Mussels were not fed during the exposure. After the exposure, the mussels were rinsed for 24-hours in the same aquaria by removing the toxicant from the injector and allowing the diluter system to continually flow filtered sea water through the aquaria. Mortality during and after the exposure was recorded.

Upper temperature tolerance was determined at the end of the 72-hour exposure period. Mussels were placed on a viewing rack and arranged by exposure concentration. Mussels placed on the viewing rack, were submerged into the 10-gallon water bath at a temperature approximately equal to

the respective acclimation temperature. The Haake heater was then turned on at the highest setting. The heater warmed the 9-10 gallons of water at a rate of 0.3 to 0.5°C per minute. Temperature was monitored with a YSI SCT Model 30 temperature probe. Mussels remained open for a short time, but then closed as the temperature increased. As the temperature reached the respective upper temperature tolerance, the mussels opened and decreasingly resisted closure upon prodding with a stainless steel probe tipped with a needle. The upper temperature tolerance endpoint was determined as described for the temperature acclimation experiments.

Three replicates of six mussels for each exposure concentration were tested for upper temperature tolerance for chlorpyrifos and then diazinon exposures. Each concentration's mussels were place on a different shelf for each replicate of upper temperature tolerance determination. This procedure was repeated for two replicates of six mussels after the 24-hour rinse period for both pesticides.

The temperature at which each individual mussel was unable to close was recorded, along with overall length and wet weight. The data were analyzed by the same methods as the temperature acclimation tests. The resultant means were used to estimate deviations in upper temperature tolerance

from the temperature acclimation regression line. Exposure concentrations were determined by the same GC methods as mentioned above the toxicant growth exposures.

LC50 Determination for *Corbicula fluminea*

Utilizing existing LC50 data for bivalve molluscs, including those calculated for the hooked mussel, multiple range finding tests were performed. Static toxicity tests to determine LC50 concentrations were performed following range finding assays to reveal an accurate sublethal concentration range of diazinon for *C. fluminea*. Twenty-four mussels were exposed to each diazinon concentration and a reconstituted hard water control. Diazinon was obtained from the commercial product Spectracide: Lawn and Garden Insect Control, which is 25% active ingredient (diazinon) and 75% inert ingredients. Diazinon stock solutions were prepared daily by micropipetting the diazinon product into 2L reconstituted hard water. Four serial dilutions were made daily from 1L of the diazinon stock for a total of five diazinon exposure solutions. Prepared solutions were placed into 1L Wheaton bottles for transfer.

Static exposures were performed in covered 500 ml Carolina dishes. Four replicates of six clams were exposed to each of the five diazinon concentrations and control. The

diazinon concentrations were replaced daily two hours after feeding each dish one ml of Roti-Rich. Mortality was recorded daily upon static renewal and dead clams were removed. The diazinon concentrations were estimated via linear regression of the Millipore Immunoassay test results for the diazinon temperature tolerance assays which followed. Mortality was observed (failure of valve closure upon prodding) and recorded. Probit analysis was performed on the data if there were at least two partial kills and a monotonically increasing response. If those assumptions were not met, the Trimmed Spearman-Karber method was used to analyze the data. The data came from the exposure of twenty-four clams (four replicates of six) to each of the five exposure concentrations and control.

The response of the clams to diazinon exposure was further examined under behavior regulated exposures. Ninety-six clams were used in this assay. Twenty-four clams were exposed in four replicates of six clams to the highest concentration used for the 96-hour LC50 determination. A second set of twenty four mussels were exposed simultaneously to the same concentration. Individuals in the second group of mussels, however, were held closed with cross-sections of 0.5 inch tygon tubing. The tubing was forced over the shell perpendicular to the hinge. The second

group was bound after the initial 24-hours of exposure to eliminate periodic "re-sampling" of the exposure solution by the mussels. The same procedure was followed for 48 clams exposed to reconstituted hard water. Twenty-four clams were exposed in four groups of six for the duration of the 96-hour exposure as before in the LC50 determination. The second group of 24 clams exposed to reconstituted hard water were bound with tygon tubing cross-sections after the initial twenty four hours of exposure.

All replicate solutions were replaced daily two hours after feeding each dish one ml of Roti-Rich by the same methods used for the LC50 determination. Mortality was recorded daily upon static renewal and dead clams were removed. After the 96-hour exposure was complete, all replicates in each concentration were placed in clean reconstituted hard water. At this time all bound clams were unbound by removing the tygon cross-sections. The clams were left in the reconstituted hard water rinse for 24-hours. After the 24-hour rinsing period, the clams were re-examined for mortality which was defined as failure to close upon prodding. Mortality was recorded and compared between bound and unbound clams in each of the two exposure concentrations to determine the influence voluntary "re-sampling" on diazinon toxicity.

The extreme tolerance of *C. fluminea* to diazinon, even when the behavior was not restricted, prompted an additional test to examine the LC50 of *C. fluminea* over 21-days of exposure. The assay was designed to observe survival differences among the diazinon exposures and reconstituted hard water control. The exposures were carried out in the same manner as was the 96-hour LC50 test (static renewal in covered 500 ml Carolina dishes).

Diazinon and control solutions were prepared and replaced daily two hours after feeding each dish one ml of Roti-Rich. Six diazinon concentrations were used for exposure. The highest concentration was doubled in volume and used as a stock solution for serial dilutions to achieve the five lower concentrations. The highest concentration/stock was approximately equal to the 96-hour diazinon LC50 concentration for *C. fluminea* determined by the LC50 test. Mortality was recorded daily and dead clams were removed before static renewals. Probit analysis was performed on the data if at least two partial kills and a monotonically increasing response occurred. If these assumptions were not met, the Trimmed Spearman-Karber analysis was used to analyze the data. The experiment exposed 18 clams (3 replicates of 6 clams) to six diazinon concentrations and a control.

C. fluminea Upper Temperature Tolerance for Temperature Acclimations

Forty *C. fluminea* were acclimated to 10, 20, and 30°C for a period of at least three weeks in dechlorinated Denton, TX tap water. Attempts to acclimate clams to 1 and 40°C were unsuccessful. Acclimations were performed in a shallow water wet table approximately eight inches deep with aquarium gravel covering the bottom. Flow was provided to simulate a moving stream using an underwater pump and the outflow from the heating unit. Temperatures were maintained with Haake heaters for the duration of the acclimation within 1°C of the desired temperature. The 1°C and 10°C exposures were accomplished by placing a cooling coil into the wet table from which the cooled water could be heated to the desired temperature with a Haake heater. Clams were fed 50 ml of Roti-Rich invertebrate food daily.

Upper temperature tolerance was determined at the end of the 21-day acclimation period. Clams were transferred to covered 500 ml Carolina dishes in an incubation chamber to maintain the respective acclimation temperature for the 24-hour control exposures. This was done to maintain consistency with the following toxicant exposure assays. After 24-hours, clams were placed upon a viewing rack composed of 2 inch PVC halves forming four numbered shelves

(same as *I. recurvum* trials). The rack facilitated viewing and introduction/removal from the water bath. After the mussels were placed on the viewing rack, they were submerged into a 10-gallon water bath at a temperature approximately equal to the respective acclimation. The Haake heater was then turned on at the highest setting. The heater warmed the 9-10 gallons of water at a rate of 0.3 to 0.5°C per minute. Temperature was monitored with a Cole and Parmer thermistor thermometer. Clams remained open for a short time, but then closed as the temperature continued to increase. As the temperature approached the respective upper temperature tolerance, the individual clams opened and decreasingly resisted closure upon prodding with a stainless steel probe tipped with a syringe needle. The upper temperature tolerance endpoint was defined as the point when clams were not capable of closure upon prodding (Spidle et al., 1995).

The temperature at which the clams were not able to close was recorded for each clam exposed. Overall length was also recorded for each clam. The clams from each acclimation temperature were measured for upper temperature tolerance in four replicates of six mussels. Standard water chemistry measurements were made for all three control acclimation temperatures at the beginning and end of the 24-hour exposure in the incubation chamber. The data were analyzed

by the same methods as the *I. recurvum* temperature acclimation tests. Important differences for bio-monitoring uses were determined to occur at a minimum of 1°C from that of the control.

C. fluminea Temperature Tolerance for Organophosphate Exposure

Forty-eight clams were exposed, in two replicates of six to four sublethal diazinon concentrations, for 24-hours. This procedure was repeated for three acclimation temperatures of 10, 20 and 30°C. Concentrations were prepared by micropipetting commercial diazinon into dechlorinated Denton, TX tap water from the shallow water simulated stream used in the acclimation of the clams to each respective acclimation temperature. Serial dilutions were made from the highest diazinon concentration to prepare the three lower exposure solutions. Exposures were performed in covered 500 ml Carolina dishes in an incubation chamber set to maintain the respective acclimation temperature for the duration of the exposure. Any mortality occurring during the exposure was recorded. Standard water chemistry measurements were made for the medium and high concentration as well as the control acclimation at the beginning and end of the 24-hour exposure.

Upper temperature tolerance was determined at the end of the 24-hour exposure period for each concentration at each acclimation temperature. Clams were placed on a viewing rack and arranged by exposure concentration. After the clams were placed on the viewing rack, they were submerged into a 10-gallon water bath at a temperature approximately equal to the respective acclimation. The Haake heater was then turned on at the highest setting. The heater warmed the 9-10 gallons of water at a rate of 0.3 to 0.5°C per minute. Temperature was monitored with a Cole and Parmer thermistor thermometer. The clams remained open for a short time, but then closed as the temperature continued to increase. As the temperature approached the respective upper temperature tolerance, the clams opened and decreasingly resisted closure upon prodding with a stainless steel probe tipped with a needle. The upper temperature tolerance endpoint was determined as before. This procedure was repeated for two replicates of six clams for each exposure concentration.

The temperature at which the mussels were not able to close was recorded individually for each mussel exposed. Overall length was recorded for each clam as well. The data were analyzed by the same methods as the *I. recurvum* temperature acclimation tests.

Exposure concentrations were determined with a diazinon

immunoassay test. Standards were prepared in high quality water (ASTM II) using diazinon standard solution provided with the test kit at concentrations of 500 ng/L, 100 ng/L and 30 ng/L diazinon in 100 ml volumetric flasks. A negative control was prepared using high quality water (ASTM II). A 200 ul pipette was used to inject 100 ul of each calibrator and the negative control into two antibody plated wells each. Diazinon-enzyme conjugate solution was then injected into each well at a volume of 100 ul using an Eppendorf repeater pipette. The contents of the cells were mixed thoroughly by rotating the strip holder in a circular motion on the bench top. The wells were then covered with parafilm and incubated for one hour at room temperature (~23°C) on an orbital mixer at 200 rpm. After the incubation, the parafilm was removed and the contents of the wells were discarded. The wells were then rinsed with cool tap water five times. All water was then removed from the wells by tapping the wells firmly on a hard surface. Using the Eppendorf repeater pipette, 100 ul of substrate solution was then added to each well beginning with the negative control and then in ascending concentration order. The contents of the wells were then mixed by rotating the strip holder in a circular motion on the bench top. The wells were then placed upon the orbital mixer for 30 minutes at 200 rpm. The Eppendorf

repeater was then used to inject 100 ul of 1 N HCl stop solution to each well and mixed thoroughly as before. The wells were then read with the microwell strip reader after calibration at 450 nm and 650 nm.

The same procedure was followed for each of the five exposure solutions. Dilutions were made for each of the four diazinon solutions to bring them into the range of the standard curve.

The optical density (color) in this method is inversely proportional to the concentration of diazinon. The higher the concentration, the lighter the optical density. The test uses polyclonal antibodies which bind either to diazinon in the sample or a diazinon-enzyme conjugate. The antibodies are immobilized on the walls of the test wells. Diazinon in the samples competes with the diazinon-enzyme conjugate for a limited number of antibody binding sites.

Results were calculated by first averaging the optical density of the two replicates for each standard. The average optical density was then used to calculate %Bo which is the average optical density of the sample divided by the average optical density of the negative control multiplied by 100. The %Bo was then regressed against the log of the diazinon concentration to determine the fit of the standard curve. The concentrations were determined by interpolating the %Bo

value of each sample using the model produced by the regression. The concentration determined was then multiplied by the dilution factor for each respective sample.

C. fluminea Temperature Tolerance for Heavy Metal Exposure

Seventy-two clams were exposed, in two replicates of six, to six sublethal cupric sulfate ($CuSO_4 \cdot 5H_2O$) concentrations for 24-hours. Exposure concentrations were based upon available toxicity data for *C. fluminea* and other bivalves. This procedure was repeated for the three acclimation temperatures 10, 20 and 30°C. Concentrations were prepared by micropipetting known 250 milligram/ liter stock cupric sulfate solution prepared from 1 gram $CuSO_4 \cdot 5H_2O$ in 1L high quality water into dechlorinated water from the shallow water simulated stream used in the acclimation of clams to each respective acclimation temperature. Serial dilutions were used to make the five lower nominal concentration solutions. Exposures were performed in covered 500 ml Carolina dishes in an incubation chamber to maintain the respective acclimation temperature for the duration of the exposure. Any mortality occurring during the exposure was recorded. Upper temperature tolerance determinations and analyses were performed as the diazinon exposures were. Standard water chemistry

measurements were made for the medium and high concentrations as well as the control acclimation at the beginning and end of the 24-hour exposure. Copper (Cu^{++}) concentrations were determined using graphite furnace atomic absorption spectrometry.

The spectrometer was a Perkin-Elmer, model 2380, with a HGA-400 programmer for graphite furnace operation. The minimum detection level was 1.0 ug/L copper. Instrument parameters were set as follows: drying time and temperature 30 seconds-130°C, ashing time and temperature 30 seconds-900°C, atomizing time and temperature 10 seconds-2,700°C, purge gas atmosphere argon, and wavelength 324.7 nm.

Standards were prepared from 1,000 ppm certified reference solution in reconstituted hard water. Standards were prepared at concentrations of 12.5, 25.0, 50.0, and 100.0 ug/L copper in volumetric flasks with a diluted stock made from the certified standard solution. A negative control was also prepared consisting of concentrated nitric acid (HNO_3) acidified reconstituted hard water. Standards were acidified to a pH below 2 with concentrated HNO_3. Samples estimated to have concentrations above 100.0 ug/L copper were diluted to a concentration within the range of the standard curve. Samples were acidified to a pH of <2 using a concentrated HNO_3. A standard curve was established

using the standard solutions prepared. Samples were analyzed in triplicate and concentrations were determined using mean sample absorbance relative to the absorbance recorded for the standard curve.

C. fluminea Temperature Tolerance for Chlorine Exposure

Seventy-two clams were exposed, in two replicates of six, to six sublethal sodium hypochlorite (NaOCl) concentrations for 24-hours. Exposure concentrations ranging from 0.09 mg/L to 5.7 mg/L were based upon available toxicity data for *C. fluminea* and other bivalves. This procedure was repeated at three acclimation temperatures of 10, 20 and 30°C. Concentrations were prepared by micropipetting 100 ul/L stock NaOCl solution into dechlorinated water from the shallow water simulated stream used to acclimate clams to each respective acclimation temperature. Serial dilutions were used to make the five lower concentration solutions. Exposures were performed in covered 500 ml Carolina dishes in an incubation chamber to maintain the respective acclimation temperature for the duration of the exposure. Any mortality occurring during the exposure was recorded. Upper temperature tolerance determinations and analyses were performed as the diazinon exposures were. Standard water chemistry measurements were

made for the medium and high concentration as well as the control acclimation at the beginning and end of the 24-hour exposure. Total residual chlorine concentrations were determined with a Hach amperometric titrator.

Sample volumes of 200 ml were used to determine the chlorine concentration in exposure solutions, which were estimated to be from 0 to 2.5 mg/L chlorine. Two samples from each exposure solution were poured into 250 ml beakers. The tip of the platinum wire probe was then immersed in the sample. The contents of one potassium iodide powder pillow was added to each sample along with 1.0 ml of pH 4 acetate buffer solution. The contents were then stirred with a TitraStir titration stand. The LED reading was adjusted to 1.00 after fluctuation ceased. The 0.0564 N POA titrant was then dispensed slowly in the sample. The LED reading decreased as the titrant was added. The endpoint was reached when the LED reading stopped decreasing. The concentration of total residual chlorine was equal to ul of the titrant (0.0564 N POA) used times 0.01.

RESULTS AND DISCUSSION

Growth in Various Media and Food

The potential use of mussel growth for biological monitoring purposes was evaluated. The growth of *I. recurvum* (juvenile and mature) was examined in the laboratory under three exposure media, three flow conditions, and four feeding regimes. All laboratory exposures were performed at four acclimation temperatures. Growth of both mature and juvenile mussels was measured in the field at sites representing differing water quality. The data from the growth experiments are presented in appendix tables A1-A7.

The results of the analysis of the growth data from the initial growth trial of *Ischadium recurvum* in synthetic sea water and fed *Isochrysus galbana* are shown in Table 1. The initial length of the mussels among the four temperature exposures were not significantly different at an α level of 0.05. Growth over the 21-day period was statistically significant among the acclimation temperatures 20, 25 and 30°C (Table 2). However, growth in the 35°C exposure was not significant. The growth among the four temperature groups was statistically significantly different.

Table 1

Growth of *I. recurvum* in Synthetic Sea Water Fed *Isochrysus* for 21-Days

Acclimation temperature (°C), mean initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS), two-tailed variance ratio test (Zar 122), and mean growth (Gr) presented. Analysis of variance, Kruskal-Wallis X^2 approximation, and Median 1-Way analyses results for initial size and actual growth immediately follow.

Temp Group	Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Gr (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
35°C	11.82	2.92	0.0423	4.34 / 2.92 = 1.493 P(F≥1.49) >0.20	-0.013	0.00	0.3110	0.0117 / 0.0018 = 5.94 Pr> 0.001
30°C	11.21	3.39	0.7532		0.06	0.00	0.2968	
25°C	11.58	4.34	0.5106		0.10	0.01	0.0001	
20°C	10.78	3.07	0.0430		0.04	0.00	0.0885	

ANOVA	Initial Size	F = 2.99	Pr>F = 0.0868
ANOVA	Growth	F = 9.22	Pr>F = 0.0030
Kruskal-Wallis	Initial Size	X^2 = 5.12	Pr>X^2 = 0.1632
Kruskal-Wallis	Growth	X^2 = 32.68	Pr>X^2 = 0.0001
Median 1-Way	Initial Size	X^2 = 5.18	Pr>X^2 = 0.1590
Median 1-Way	Growth	X^2 = 19.11	Pr>X^2 = 0.0003

Table 2

The significance of the difference in size of mussels in synthetic sea water and fed *Isochrysus* at Day-1 and Day-21.

Temperature Group	$\bar{x}$ Growth (mm)	Paired T- test T Value	Prob>|T|
35	-0.01	-1.21	0.2389
30	0.06	5.44	0.0001
25	0.10	5.30	0.0001
20	0.04	5.44	0.0001

Detecting significant differences with the small amount of growth observed was probably more attributable to the large sample sizes (n=30 per exposure temperature) and more normal distributions of the initial mussel sizes (relative to subsequent trials) than the actual change in overall mean length for the 20, 25 and 30°C exposures. The actual change in overall length was 0.06 mm, 0.10 mm and 0.04 mm respectively. The predetermined difference necessary for qualified growth was much more than observed growth.

Growth determined to be useful for the purpose of bio-indication was defined as a minimum increase in overall length of 0.5 mm. Growth less than the defined minimum was not thought to provide a conclusive basis for comparison. The fragile nature of the mussel valves did not lend them to repetitive measurements. Reproducible measurements were not confidently attained below 0.5 mm because of the irregularly shaped, ribbed, brittle valves. The overall greatest length to the nearest 0.5 mm was used previously in the study of mussels for the same reasons (Morton, 1988).

Morton (1988) reported growth rates in a Hong Kong mangrove for another mussel specie ranging from 0.9 to 1.4 mm per month and in some cases as much as 2.1 mm per month. Field data suggest that growth in excess of the 0.5 mm qualified minimum is attainable over this time period. In

field growth evaluations performed on *I. recurvum*, growth was achieved from 1.6 to 2.1 mm in 14-days during summer months.

The inconsistency in repetitive measures was realized during this trial, which resulted in the marking of the valves with a paint pen to alleviate some of the measurement variation in subsequent trials. And although the growth was statistically significantly different among the four temperature exposures, the actual growth was not indicative of biologically useful differences for the above mentioned reasons.

The results of the analysis of the growth data from the second trial, growth of *I. recurvum* in filtered sea water and fed *Isochrysus galbana*, are shown in Table 3. The initial length of the mussels among the four temperature exposures were not significantly different at an α level of 0.05. Growth over the 21-day period was significant among the acclimation temperatures 20, 30 and 35°C (Table 4). However, growth in the 25°C exposure was not significant. The growth among the four groups was not significantly different.

Table 3

Growth of *I. recurvum* in Filtered Sea Water Fed *Isochrysus* for 21-Days

Acclimation temperature (°C), mean initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS), two-tailed variance ratio test (Zar 122), and mean growth (Gr) presented. ANOVA, Kruskal-Wallis X^2 approximation, and Median 1-Way analyses results for initial size and actual growth immediately follow.

Temp Group	Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Gr (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F(α=0.05)
35°C	24.41	4.44	0.6733	5.09 / 4.34 = 1.17 P(F≥1.17) >0.50	0.18	0.09	0.0001	0.1185 / 0.0358 = 3.31 0.01>Pr >0.005
30°C	24.77	4.52	0.8152		0.30	0.12	0.0002	
25°C	24.91	5.09	0.0152		0.07	0.04	0.0029	
20°C	24.71	4.34	0.3860		0.15	0.09	0.0002	
ANOVA			Initial Size		F = 0.37		Pr>F =0.5464	
ANOVA			Growth		F = 1.42		Pr>F =0.2367	
Kruskal-Wallis			Initial Size		X^2 = 1.02		Pr>X^2 = 0.7958	
Kruskal-Wallis			Growth		X^2 = 6.20		Pr>X^2 = 0.1022	
Median 1-Way			Initial Size		X^2 = 2.62		Pr>X^2 = 0.4545	
Median 1-Way			Growth		X^2 = 3.52		Pr>X^2 =0.3180	

Table 4

The significance of the difference in size of mussels in filtered sea water and fed *Isochrysus* at Day-1 and Day-21.

Temperature Group	$\bar{x}$ Growth (mm)	Paired T- test T Value	Prob>|T|
35	0.18	3.22	0.0031
30	0.3	4.61	0.0001
25	0.07	1.87	0.0739
20	0.15	2.66	0.0131

Exposures in flow through filtered sea water yielded slightly more growth. The initial trial under these conditions, in which the mussels were fed *Isochrysus,* also produced significant growth for three of the four exposure temperatures. Mean growths of 0.18 mm, 0.30 mm and 0.15 mm were significant for the temperatures 35, 30 and 20°C respectively. Mean growth in the 25°C exposure was not significant at 0.07 mm. These changes in overall length fell short of the 0.5 mm predetermined growth minimum. The growth among the four groups was not statistically significantly different. In addition, the growth was less than practical for evaluation of potential use as a bio-indicator. Growth was not found to be useable for the intended purpose.

The results of the analysis of the growth data from the third trial, growth of *I. recurvum* in filtered sea water and fed Roti-Rich, are shown in Table 5. The initial length of the mussels among the four temperature exposures were not significantly different at an α level of 0.05. Growth over the 21-day period was significant among the acclimation temperatures 20, 25, 30 and 35°C (Table 6). The growth among the four temperature groups was not significantly different.

Table 5

Growth of *I. recurvum* in Filtered Sea Water Fed Roti-Rich for 21-Days
Acclimation temperature (°C), mean initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS), two-tailed variance ratio test (Zar 122), and mean growth (Gr) presented. ANOVA, Kruskal-Wallis X^2 approximation, and Median 1-Way analyses results for initial size and actual growth immediately follow.

Temp Group	Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Gr (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
35°C	24.61	4.33	0.6733	5.5253/ 3.7425 = 1.48 P(F≥1.48) >0.20	0.16	0.09	0.0001	0.0918 / 0.0666 = 1.38 0.50> Pr > 0.20
30°C	25.07	3.90	0.9542		0.17	0.07	0.0001	
25°C	24.98	5.53	0.0143		0.12	0.06	0.0001	
20°C	25.04	3.74	0.2063		0.12	0.06	0.0001	

ANOVA	Initial Size	F = 0.28	Pr>F = 0.8384
ANOVA	Growth	F = 0.54	Pr>F = 0.4639
Kruskal-Wallis	Initial Size	X^2 = 1.27	Pr>X^2 = 0.7365
Kruskal-Wallis	Growth	X^2 = 1.37	Pr>X^2 = 0.7120
Median 1-Way	Initial Size	X^2 = 3.12	Pr>X^2 = 0.3731
Median 1-Way	Growth	X^2 = 1.46	Pr>X^2 = 0.6906

Table 6

The significance of the difference in size of mussels in synthetic sea water and fed Roti-Rich at Day-1 and Day-21.

Temperature Group	$\bar{x}$ Growth (mm)	Paired T- test T Value	Prob>|T|
35	0.16	2.82	0.0087
30	0.17	3.52	0.0016
25	0.12	2.39	0.0247
20	0.12	2.60	0.0150

The second trial in filtered sea water, in which the mussels were fed Roti-Rich invertebrate food, also yielded more growth than the initial trial in synthetic sea water. Mean growth in the four exposure groups was 0.12 mm at 20°, 0.12 mm at 25°, 0.17 mm at 30° and 0.16 mm at 35°C. However, the growth among the four groups was not statistically significantly different. The growth again fell well short of the qualified 0.5 mm minimum for growth. The growth achieved was not thought to be useful for bio-indication purposes because of the relatively small change in overall length.

The results of the analysis of the growth data from the fourth trial, growth of *I. recurvum* in filtered sea water and fed *Isochrysus galbana* and Roti-Rich, are shown in Table 7. The initial lengths of the mussels among the four temperature exposures were not significantly different at an α level of 0.05. Growth over the 21-day period was statistically significant among the acclimation temperatures 20, 30 and 35°C (Table 8). However, growth in the 25°C exposure was not significant. The growth among the four groups was not significantly different.

Table 7

Growth of *I. recurvum* in Filtered Sea Water Fed *Isochrysus* and Roti-Rich for 21-Days

Acclimation temperature (°C), mean initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS), two-tailed variance ratio test (Zar 122), and mean growth (Gr) reported. ANOVA, Kruskal-Wallis X^2 approximation, and Median 1-Way analyses results for initial size and actual growth immediately follow.

Temp Group	Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Gr (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
35°C	24.78	4.67	0.7279	5.9779/ 2.5308 = 2.36	0.12	0.03	0.0001	0.1211 / 0.0346 = 3.50
30°C	25.17	4.04	0.9766		0.20	0.12	0.0001	
25°C	25.11	5.98	0.0318	0.05> P(F≥2.36) >0.02	0.08	0.05	0.0001	0.001> Pr > 0.002
20°C	25.17	2.53	0.2194		0.11	0.07	0.0001	

ANOVA	Initial Size	F = 0.39	Pr>F = 0.5320
ANOVA	Growth	F = 0.30	Pr>F = 0.5835
Kruskal-Wallis	Initial Size	X^2 = 1.03	Pr>X^2 = 0.794
Kruskal-Wallis	Growth	X^2 = 1.64	Pr>X^2 = 0.651
Median 1-Way	Initial Size	X^2 = 2.94	Pr>X^2 = 0.401
Median 1-Way	Growth	X^2 = 1.80	Pr>X^2 = 0.615

Table 8

The significance of the difference in size of mussels in synthetic sea water and fed *Isochrysus* and Roti- Rich at Day-1 and Day-21.

Temperature Group	$\bar{x}$ Growth (mm)	Paired T- test T Value	Prob>|T|
35	0.12	3.50	0.0016
30	0.20	2.93	0.0070
25	0.08	1.93	0.0655
20	0.11	2.27	0.0317

In the third trial in filtered sea water the mussels were fed both *Isochrysus* and Roti-Rich in increased amounts. As in the previous three trials, the growth (0.08-0.2 mm) was much less than the predetermined minimum of 0.5 mm deemed important for evaluating mussels for use as biological monitors.

The filtered sea water used in these experiments was achieved by filtering raw sea water through a sand filter followed by a 20 um mesh filter. The filtering process is designed to remove detritus and planktonic organisms as well as other suspended substances. The resulting filtered sea water was assumed to not contain the necessary constituents to maintain a bivalve culture.

The necessary constituents needed for laboratory bivalve culture, however, have not been determined. Foe and Knight (1986) reported that no studies of the bivalve *Corbicula fluminea* had involved long-term laboratory maintenance, partially because there were no known laboratory culture techniques. They further stated that determining an adequate diet was a major problem in the development of laboratory culture methodology. Artificial foods such as nine-grain cereal, denatured brewers yeast, and trout chow among others provide some nutrition to bivalves like *C. fluminea*, but none would support positive

clam growth. However, some trialgal and one dialgal culture of green algae did produce positive tissue growth over one month (Foe and Knight, 1986). In their experiments none of the trialgal or dialgal culture food sources provided for shell growth which met the 0.5 mm determined minimum for the purposes proposed here, but algal combinations consisting of five or six different algae did, in some cases, produce growth in excess of 0.5 mm in 30 days.

The complexity of the food resources required to achieve useful growth (defined as a minimum of 0.5 mm of increased shell length deemed necessary for the evaluation of bio-indication potential) for *C. fluminea* may suggest that the foods used to achieve growth of *I. recurvum* were inadequate. Unialgal *I. galbana* cultures and the artificial food Roti-rich were utilized separately and together as food sources for the mussels. The study by Foe and Knight (1986) indicates that any combination of these two food resources would be inadequate to achieve the desired growth in the 21-day exposure period if *I. recurvum* has similar nutritional requirements to *C. fluminea*.

The determination of the optimal temperature range for laboratory growth of *I. recurvum* was not achieved because of the minimal growth observed in the laboratory growth tests. However, mussel studies have repeatedly illustrated a strong

direct relationship between temperature and growth. A study performed on the Mediterranean mussel (*Mytilus galloprovincialus*) demonstrated that growth and development were greatest from 20-25°C (His and Dinet, 1989). Blue mussels achieve optimal growth at 20°C (Brenko and Calabrese, 1969) and *Mytilus californianus* Conrad, 1837 was reportedly most successful in growth from 18-20°C (Widdows, 1991). Widdows (1991) also recorded the influence of salinity, temperature, dissolved oxygen, and toxicant variations on the growth and development of *Mytilus*. He reported that, although salinity had little effect over a large range, temperature dependent growth is relative to latitude.

Identification of mussel culture methodologies which produce more growth may allow for optimal growth temperature determinations for *I. recurvum*. Extended exposure periods (>21-days) and more adequate foods would likely provide more information about the optimal growth temperature for *I. recurvum* than the results presented.

No growth was observed for the mussels in raw sea water (Table 9). Mortality was much higher than expected, where twenty of the thirty mussels died within the fourteen day exposure period. This was the same water body as was used for the Santa Rosa Sound (SR) field location, in which no

mussels suffered mortality.

Table 9

Growth of *I. recurvum* in Raw Sea Water for 21-Days
Initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS) for mean initial size (Init Lth), and mean growth (Gr) reported.

Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Gr (mm)	Var
27.18	3.68	0.6587	0	-

The exposure of mussels to raw sea water yielded no growth and considerable mortality. Twenty of the thirty mussels exposed to the flow through raw sea water died. This was contradictory to the field exposure in the same waters at the location of the sea water intake. The field exposures at this location suffered no losses due to mortality. High mortality and lack of growth may be attributable to the high flow rate (relative to the filtered sea water exposures) in the shallow aquaria used for exposure. The shallow aquaria may have heightened even more the effect of the increased flow by placing the mussels directly in the current (versus being deeper in an aquarium below direct flow). Failure to achieve growth in the laboratory in useful amounts for

biological monitoring purposes prompted laboratory investigation into the use of upper temperature tolerance as a endpoint in subsequent tests.

Growth in Field Exposures

Growth of both mature and juvenile mussels was tested in the field at sites representing differing water quality. The growth of mussels in the field was compared among sites and to the laboratory growth tests to evaluate its potential use for biological monitoring purposes. Comparisons among field sites were based on measured organophosphate (diazinon and chlorpyrifos) concentrations and the qualified growth minimum determined necessary for bio-indication purposes.

Adult Mussels

The temperature, dissolved oxygen, and salinity were measured at the four field locations at high and low tide three times during the 14-day exposure period (Table 10). All measurements were taken during the day.

Table 10

Range of temperature (°C), dissolved oxygen (ppm), and salinity (parts/thousand) measured at four field locations at high and low tide three times during a 14-day exposure period.

Field Group	Temperature	Dissolved Oxygen	Salinity
BC01	30.1 - 31.9	6.1 - 7.0	16.1 - 19.0
BC02	29.8 - 30.8	5.9 - 6.8	9.6 - 14.4
BC03	29.7 - 30.0	6.1 - 6.6	16.1 - 19.0
SR	28.9 - 30.5	6.4 - 6.6	22.4 - 22.9

The diazinon and chlorpyrifos concentrations were determined from single samples taken at the field locations following a rainfall event by the previously mentioned GC methods. Samples were analyzed in duplicate and met relative percent difference (RPD) limits of >30% as prescribed by the Quality Assurance Protocol Procedures (QAPP). At the time the sample was taken, no chlorpyrifos or diazinon was found at the SR site (at a detection limit of 0.1 ug/L). This may have been expected from the minimal freshwater input into this water body which predominantly consists of a relatively small amount of storm water run-off. An analysis of the water sample taken at BC01 showed the presence of diazinon at a concentration of 0.119 ug/L and chlorpyrifos at 0.116 ug/L. BC03 had slightly higher concentrations of both diazinon (0.174 ug/L) and chlorpyrifos (0.159 ug/l). The

highest concentration of both pesticides was detected in the water sample taken from BC02 with a determined diazinon concentration of 0.240 ug/L and a chlorpyrifos concentration of 0.332 ug/L.

The results of the analysis of the growth data from the *I. recurvum* field exposures at the four field locations are shown in Table 11. Three of the field locations are in Bayou Chico and represent contaminated sites; one is in the relatively clean Santa Rosa Sound. The initial length of the mussels exposed at the four field sites were not statistically significantly different from one another at an α level of 0.05. Average growth over the 21-day exposure period at each field site was statistically significant (BC01, BC02, and SR; Table 12). Growth of the mussels exposed at the BC01 site were not analyzed because all but one mussel were lost from the rack during the exposure period. There were no statistically significant differences between the median growth of the mussels at the three field sites.

Table 11

Growth of *I. recurvum* in Field Exposures for 21-Days

Field location, mean initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS), two-tailed variance ratio test (Zar 122), and mean growth (Gr) reported. ANOVA, ANOVA on Ranked Data, Kruskal-Wallis X^2 approximation, and Median 1-Way analyses results for initial size and actual growth immediately follow.

Field Group	Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Gr (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
BC01	20.0	-	-	15.484/ 2.5623 = 6.04	2.1	-	-	0.8476 / 0.2573 = 3.29
BC02	18.69	6.43	0.0001		1.8	0.52	0.1136	
BC03	18.24	15.48	0.3992	0.01> P(F≥6.04) >0.005	1.84	0.85	0.0018	0.10> Pr > 0.05
SR	16.33	2.56	0.8192		1.62	0.26	0.3800	
ANOVA			Initial Size		F = 1.55		Pr>F = 0.2107	
ANOVA			Growth		F = 0.24		Pr>F = 0.8684	
Kruskal-Wallis			Initial Size		X^2 = 7.27		Pr>X^2 = 0.0638	
Kruskal-Wallis			Growth		X^2 =0.97		Pr>X^2 = 0.8091	
Median 1-Way			Initial Size		X^2 = 6.16		Pr>X^2 = 0.1040	
Median 1-Way			Growth		X^2 = 1.16		Pr>X^2 = 0.7626	

Table 12

The significance of the difference in size of mussels in field exposures at Day-1 and Day-21.

Field Group	$\bar{x}$ Growth (mm)	Paired T- test T Value	Prob>|T|
BC01	2.10	-	-
BC02	1.80	13.42	0.0001
BC03	1.84	10.55	0.0001
SR	1.62	10.099	0.0001

The mussel at all field locations grew well in excess of the established 0.5 mm biologically useful minimum. The mussels were, in addition, only exposed for two weeks (14 days in contrast with the 21 days for the lab exposures). Storm events in the area prompted the early retrieval of the field exposure racks. However, in only two-thirds the exposure time the field mussels grew well in excess of 1.5 mm over that of the laboratory growth. The growth of the mussels among the four field locations was biologically useful but not statistically different.

The field data did not present an evident correlation between growth and diazinon and chlorpyrifos concentration. The least growth occurred at the SR site where pesticide concentrations were undetectable (below 0.1 ug/L). Among the Bayou Chico sites, the growth differed very little in the presence of concentrations of measured pesticides which approximately tripled between sites BC01 (0.119 ug/L diazinon and 0.116 ug/L chlorpyrifos) and BC02 (0.240 ug/L diazinon and 0.332 ug/L chlorpyrifos). The concentrations were, however, very low relative to the estimated 96-hour LC50 values for diazinon (1,355 ug/L) and chlorpyrifos (960 ug/L) which was determined to provide background toxicological data for these mussels for laboratory exposures which follow these tests. The field concentrations

are even orders of magnitude lower than the 96 hour estimated LC01 values of 686 ug/L diazinon and 200 ug/L chlorpyrifos (based on the same LC50 determination which follows). However, the single sample for each site analyzed for the target organophosphates were taken after only one rain event and may not represent continuous or even repetitive exposure levels. The tolerance of mussels to organophosphorus pesticides in terms of growth may not translate well into a realistic indicator of environmental concentrations if these measured concentrations are typically as different among sites as measured in these singular samples. The growth of the mussels did meet the predetermined biologically useful criteria, however, it was not indicative of the differences among the field concentrations.

Juvenile Mussels

The results of the analysis of the data from the field exposures for the growth of juvenile *I. recurvum* at four field locations are shown in Table 13. The initial length of the mussels among the three field exposures were significantly different at an α level of 0.05. Growth over the exposure period was significant at the three field locations BC02, BC03 and SR (Table 14). However, growth in

the BC01 field group was not analyzed because all mussels were lost from the rack during the exposure period. Growth among the field groups was not significantly different despite significant differences in initial size.

Table 13

Growth of juvenile *I. recurvum* in Field Exposures for 21-Days
Field location, mean initial length (Init Lth), variance, Shapiro-Wilks normality test (SAS), two-tailed variance ratio test (Zar 122), and mean growth (Gr) presented. ANOVA, Kruskal-Wallis X^2 approximation, and Median 1-Way analyses results for initial size and actual growth immediately follow.

Field Group	Init Lth (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Gr (mm)	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
BC01	-	-	-	1.9312/ 0.5479 = 3.5247 0.10> P(F≥3.52) >0.20	-	-	-	0.1713 / 0.1068 = 1.6039 P(F≥1.6) >0.5
BC02	5.48	0.55	0.2964		0.76	0.17	0.1091	
BC03	7.67	0.68	0.8891		0.97	0.11	0.6220	
SR	6.43	1.93	0.8941		0.66	0.17	0.4399	
ANOVA			Initial Length		F = 10.00		Pr>F = 0.0006	
ANOVA			Growth		F = 1.69		Pr>F = 0.2044	
Kruskal-Wallis			Initial Length		X^2 = 13.27		Pr>X^2 = 0.0013	
Kruskal-Wallis			Growth		X^2 = 3.75		Pr>X^2 = 0.1534	
Median 1-Way			Initial Length		X^2 = 13.89		Pr>X^2 = 0.0010	
Median 1-Way			Growth		X^2 = 2.41		Pr>X^2 = 0.2996	

Table 14

The significance of the difference in size of juvenile mussels in field exposures at Day-1 and Day-21.

Field Group	$\bar{x}$ Growth (mm)	Paired T- test T Value	Prob>|T|
BC01	-	-	-
BC02	0.76	5.21	0.0012
BC03	0.97	9.39	0.0001
SR	0.66	5.11	0.0006

Juvenile mussels, at the same field location for the same exposure period as the mature mussels, also grew significantly at sites BC02, BC03, and SR. The juveniles did not grow as much as the adults. The mean growth of the juveniles was 0.76 mm at BC02, 0.97 mm at BC03, and 0.66 mm at SR. The growth was only slightly greater than predetermined biologically useful minimum of 0.5 mm. The sample sizes in these exposures were reduced (n=10) and the initial lengths were significantly different which may have reduced the power of the test. However, as in the mature mussel exposure, the juveniles exposed to water without organophosphorus pesticides grew the least. This more likely to be the result of factors other than pesticide concentration. The smaller amount of growth at the same sites as the adult mussels may indicate that the juveniles are less well suited as bio-indicators for the short

exposure time under these conditions.

The use of mussels as indicators of water quality may vary in usefulness among seasons. Both growth and reproduction exhibit pronounced seasonal variation (Banse and Mosher, 1980; Morton, 1988; Mallet and Carver, 1989). According to these studies, the greatest growth is achieved by many species in the summer and fall. The use of mussel growth for *in situ* exposures to assess ambient water quality may provide the most information during these seasons if the exposure time is relatively short (30 days). The use of juvenile mussels for this purpose may require a better understanding of their life history. According to Morton (1988), newly recruited mussels sometimes grow during different seasons than do the adults because of energy demands attributed to sexual maturation.

Ischadium recurvum LC50 Determination

Toxicant exposures followed the temperature trials to provide toxicity data for the mussels. The data were used to determine the sensitivity of mussels to organophosphates relative to available toxicity data for other organisms. The data were also generated to provide an estimation of chronic pesticide concentrations. These concentrations were utilized in exposures (tests which follow) which would provide for

relative growth comparisons with the preceding field test.

Diazinon LC50

The LC50 data for diazinon was calculated from the survival of forty mussels exposed to each of seven diazinon concentrations and a control (Table A-8). The medium used in this toxicity test was filtered sea water. Each group of forty mussels was subdivided into four replicates of ten for each exposure concentration. The diazinon 96-hour LC50 for the hooked mussel was estimated to be 1,020 ug/L with a 95% UCL of 1614 ug/L and 95% LCL of 0.0 ug/L with the Probit method (LC01 = 200 ug/L). The analysis, however, violated the assumption of homogeneity (X^2=14.971; X^2(0.05)=11.070). The confidence limits about this estimation were large (95% UCL = 1,614 ug/L and 95% LCL = 0.0 ug/L) because the assumption of monotonic response was violated resulting in heterogeneity. The Probit method was run again with only the lower four concentrations and the control which showed the 96-hour LC50 estimation to increase to 1,354 ug/L with a 95% UCL of 1,501 ug/L and 95% LCL of 1,041 ug/L (LC01 = 686 ug/L). The high concentration in this analysis was the lowest concentration which caused 100% mortality. The data then showed a monotonic response and were homogeneous (X^2= 0.407; X^2(0.05)=5.991). This analysis increased the

estimated 96-hour LC50 by more than 300 ug/L and the LC01 by approximately 500 ug/L.

The variation in the data could possibly be explained by the ability of bivalves to partially isolate themselves from a toxic environment by simply closing their valves and respiring anaerobically. Variation in the mussels ability to effectively detect toxicants and isolate themselves in the presence of stressors may account for the heterogeneity and the non-monotonically increasing data. Experiments reported later were designed to examine the ability of bivalves to isolate themselves from toxic environments.

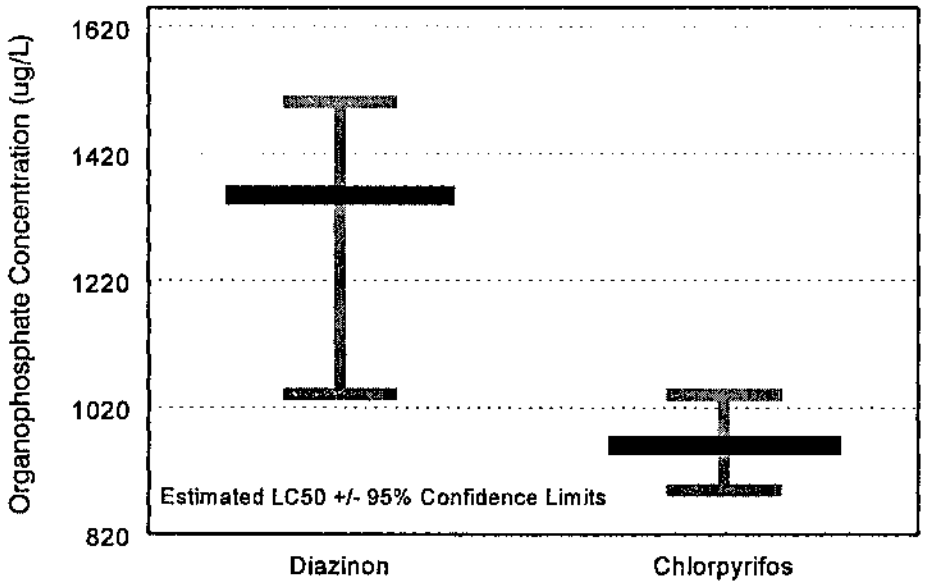
Chlorpyrifos LC50

The exposure data for the forty mussels in four replicates of ten exposed to each of the six chlorpyrifos concentrations and a control (Table A-9) also violated the homogeneity assumption. The data were analyzed with the Trimmed Spearman-Karber analysis with a 20% trim. The chlorpyrifos 96-hour LC50 for the hooked mussel was estimated to be 960 ug/L with a 95% UCL of 1,040 ug/L and a 95% LCL of 890 ug/L. The upper 95% confidence in the chlorpyrifos LC50 estimate was approximately equal to the lower 95% confidence about the diazinon LC50 estimate (1041 ug/L), which may suggest that the estimated LC50 values are

different (Figure 3). However, the unidentified pesticide carriers ("inert" organic solvents) in which these active ingredients (diazinon and chlorpyrifos) are sold may have affected the results of the toxicity tests. If these carriers were toxic to the mussels or affected the toxicity of the active ingredients, the results may reflect the activity of these anonymous participants to some unknown extent.

Figure 3

Comparison of the Diazinon and Chlorpyrifos LC50 Values for *I. recurvum*.



The heterogeneity of the variances (caused by a non-monotonic response) was again likely attributed to the unique abilities of mussels to isolate themselves from a toxic exposure to varying degrees, while still in the presence of that toxicant. Exposure is a function of the magnitude, duration and frequency with which an organism interacts with a biologically available toxicant. Bivalves have some control over the frequency and duration of such an interaction, providing the initial toxic insult is not acute. Mortality occurs under these conditions when bivalves cannot completely isolate themselves, periodically sample the environment, or have incurred levels of toxic insult sufficient to cause mortality prior to isolation. The latter situation can be complicated by the fact that the isolated environment often already includes the toxicant, which is the trigger to isolate. Bivalves need be exposed to a toxic substance for a certain duration within a given frequency to have an acute reaction to it.

Although acute organophosphate concentrations are high for the hooked mussel, this is not uncommon for many bivalve species. The U.S. Environmental Protection Agency's Aquatic Toxicity Information Retrieval database (AQUIRE) reports 96-hour EC50 values for *Crassostrea virginica* (American or virgin oyster) as high as 1,000 ug/L diazinon and 10,200

ug/L chlorpyrifos. Another bivalve, *Corbicula fluminea*, has an estimated 96-hour LC50 value for diazinon of 4,067 ug/L with a 95% UCL of 5,814 ug/L and 95% LCL of 2,847 ug/L (provided by subsequent tests). High values are also reported for other resistant species which inhabit estuaries. For example, the estimated 96-hour LC50 for juvenile *Cyprinodon variegatus* (Sheepshead minnow) exposed to diazinon is 1,400 ug/L (Goodman et al., 1979) and juvenile *Opsanus beta* (Gulf toadfish) exposed to chlorpyrifos has an estimated 96-hour LC50 concentration of 520 ug/L (Hansen et al. 1986). Despite the resistant nature of the freshwater clam *C. fluminea*, freshwater organisms are often much less resistant to organophosphates. *Lepomis machrochirus* (Bluegills) and *Salmo gairdneri* (Rainbow trout) have estimated 24-hour LC50 values of 52 ug/L and 380 ug/L diazinon respectively (Cope 1965) and some invertebrates are even more sensitive (*Ceriodaphnia dubia* 48-hour LC50, 0.5 ug/L diazinon and *Hyalella azteca* 0.29 ug/L chlorpyrifos; AQUIRE, 1997).

Toxicant Exposure Growth for *Ischadium recurvum*

Following static toxicity testing, sublethal concentrations (estimated from the preceding LC50 determinations) and a control were utilized for relative

growth evaluation exposures. The growth in these tests was used for comparison with the growth in the field exposures relative to measured organophosphate concentrations.

Diazinon Growth

The 21-day growth exposure in the four concentrations of diazinon and one control group were run for only 14-days and were not analyzed for growth. The concentrations were much higher than appropriate for a 21-day exposure ranging from 1,281 ug/L to 5,163 ug/L diazinon (96-hour LC50 1,354 ug/L ;95% UCL 1,501 ug/L, 95% LCL of 1,041 ug/L). A great deal of mortality was observed, and the days to death were recorded (Table A-10). Even the controls experienced a large degree of mortality at a concentration which was measured to be from 1.81 to 182 ug/L diazinon. The 96-hour diazinon LC01 was estimated to be 686 ug/L for *I. recurvum*. The extended exposure (14-days) to a concentration which reached almost a third of the 96-hour LC01 may suggest that mortality would be expected in these exposures if the higher measured concentration (T-24) was typical in the control. An extended (21-day) toxicity test to evaluate the relative toxicity of diazinon to bivalves (*Corbicula fluminea*) in prolonged exposures follows.

The measured diazinon concentrations were: DIAZ-Control

as new solution was added (T0)=1.82 ug/L, 24-hours later prior to static renewal (T24)=182 ug/L, DIAZ-01 (T0)= 1,281 ug/L, (T24)= 1,153 ug/L, DIAZ-02 (T0)= 1,929 ug/L, (T24)= 1,612 ug/L, DIAZ-03 (T0)= 2,600 ug/L, (T24)= 1,500 ug/L, DIAZ-04 (T0)= 5,163 ug/L, (T24)= 1,673 ug/L, and the solution to which mussels were not exposed was nominally the same as DIAZ-03 and confirmed to be DIAZ-NM (T0)= 3,265 ug/L and (T24)= 1,689 ug/L. The concentrations decreased over the twenty hour period for all exposures but the control, which increased (Table 15).

Table 15

Diazinon Concentration Data for Growth Exposures of *Ischadium recurvum*

Concentration number, Diazinon concentration at T0 (ug/L), Diazinon Concentration at T24 (ug/L), and Percent change reported.

Conc No.	T0 Diaz Conc	T24 Diaz Conc	Percent Change
DIAZ-CON	1.81	182	+ 9,900
DIAZ-01	1,281	1,153	- 9.99
DIAZ-02	1,929	1,612	- 16.43
DIAZ-03	2,600	1,500	- 42.31
DIAZ-03-NM	3,265	1,689	- 48.27
DIAZ-04	5,163	1,673	- 67.60

Biologically useful growth was not expected, in retrospect, based upon the growth observed in the previous test using temperature, media, and food as variables. In addition, exposure concentrations were much higher than was intended for this chronic exposure. High mortality in all exposures, including the control, was recorded for possible acute uses. The high mortality in the control may have been partially caused static conditions under which they were exposed (versus the previous flow through exposures without pesticides). This would, however, contradict the findings discussed by Foe and Knight (1986) that growth is independent of flow and substrate, indicating that flow would not be a beneficial factor for bivalves.

Information that may be useful from this failed growth assay is the apparent transfer of the pesticide into the adjacent control solution, despite efforts to minimize this problem by covering the aerated shallow aquaria. The initially contaminated control samples (1.81 ug/L) increased 100 fold over 24-hours to a concentration of 182 ug/L diazinon.

In addition to the increase in concentration in the control from the diazinon exposure, all other concentrations decreased over the 24-hour renewal period (Table 15). Decreases in concentration ranged from 1,281 ug/L to 1,153

ug/L in the lowest concentration to 5,163 ug/L to 1,673 ug/L in the highest concentration. Higher concentration solutions proportionately decreased in concentration more rapidly than lower concentration solutions over the 24-hour period.

Decreases in concentration may have been, in part, caused by the atomization of pesticide via aeration. In addition, hydrolysis, a large part of the breakdown process (Morgan, 1989), likely played a role. To some extent, photolysis may have been involved despite efforts to minimize this effect with an opaque covering. The biological activity (respiration, filtration, circulation, and consequently bioaccumulation) of the mussels cannot be ruled out as a participant in the breakdown of the pesticide. The biological interaction of mussels with the pesticide was examined by duplicating the DIAZ-03 conditions and placing no mussels in it. The concentration in this solution was 3,265 ug/L at initiation and 1,689 ug/L diazinon at the conclusion of the 24-hour exposure period. This was similar to the percent decrease in approximately the same concentration to which mussels were exposed (2,600 ug/L to 1,500 ug/L diazinon at 24-hours). This may suggests that the decrease in diazinon concentration was the result of physical interactions of the pesticide, and not biological interaction.

Chlorpyrifos Growth

The 21-day growth exposure in the four "sublethal" concentrations of chlorpyrifos and one control group were run for only 14-days and were not analyzed for growth. The concentrations were again much higher than appropriate for a 21-day exposure as concentrations ranged from 345 ug/L to 5,495 ug/L chlorpyrifos (96-hour LC50 960 ug/L; 95% UCL 1,040 ug/L, 95% LCL 890 ug/L). A great deal of mortality was again observed, and the days to death were recorded (Table A-11). Even the control concentration, which was measured at a high of 0.557 ug/L chlorpyrifos, and the lowest concentration exposure of 345 ug/L chlorpyrifos showed mortality. The lowest concentration (345 ug/L) is more than a third of the estimated 96-hour LC50 value (960 ug/L). Mortality resulting from a 14-day exposure to this relatively high concentration would be expected. However, mortality in the control may have been influenced by the static conditions under which the exposures were performed or the prolonged exposure to a low chlorpyrifos concentration (as in the diazinon exposure).

It is possible that the measured concentrations in the control do not truly represent the level of exposure. The presence of mussels seemed to be involved in lowering chlorpyrifos concentration by some biological interaction

(discussion to follow in chlorpyrifos growth and bioaccumulation). The data provided from subsequent tests to compare 96-hour and 21-day diazinon LC50s for *Corbicula fluminea* suggests that if the measured chlorpyrifos concentration (0.557 ug/L) is accurate, little mortality would have been caused by chlorpyrifos during this 14-day exposure. The 96-hour estimated diazinon LC50 for *C. fluminea* of 4,067 ug/L decreased to 548 ug/L in the 21-day LC50 estimate. If the relationship is similar for *I. recurvum* exposed to chlorpyrifos, the 21-day LC50 would be approximately 130 ug/L chlorpyrifos. It is unlikely that exposure to 0.557 ug/L chlorpyrifos would result in 67% mortality after only 14-days of exposure if chlorpyrifos exposure was the cause and the concentrations were accurate.

The determination of chlorpyrifos concentration resulted in the following concentrations: CHLO-Control(T0)=0.557 ug/L, (T24)=0.304 ug/L, CHLO-01 (T0)= 345 ug/L, (T24)= 82 ug/L, CHLO-02 (T0)= 1,059 ug/L, (T24)= 102 ug/L, CHLO-03 (T0)= 2,400 ug/L, (T24)= 197 ug/L, CHLO-04 (T0)= 5,495 ug/L, (T24)= 530 ug/L. The concentration without mussels was nominally the same as CHLO-03 and confirmed to be CHLO-NM (T0)= 2,272 ug/L and (T24)= 573 ug/L. The concentrations decreased over the 24-hour period for all exposures solutions (Table 16).

Table 16

Chlorpyrifos Concentration Data for Growth Exposures of *Ischadium recurvum*

Concentration number, Chlorpyrifos concentration at T0 (ug/L), Chlorpyrifos Concentration at T24 (ug/L), and Percent change (%)reported.

Conc No.	T0 Chlo Conc	T24 Chlo Conc	Percent Change
CHLO-CON	0.557	0.304	- 45.42
CHLO-01	345	82	- 76.23
CHLO-02	1,059	102	- 90.37
CHLO-03	2,400	195	- 91.88
CHLO-03-NM	2,272	573	- 74.78
CHLO-04	5,495	530	- 90.35

The problem of diffusion into the control was not as obvious in this exposure as it was in the diazinon exposure (Table 16). The initially contaminated control exposure (0.557 ug/L) decreased to a concentration of 0.304 ug/L chlorpyrifos at the time of the 24-hour static renewal.

The decrease in the control was less than would be expected from examining the subsequent four chlorpyrifos exposures (Table 15). The lowest concentration decreased from 345 ug/L to 82 ug/L and the highest concentration decreased from 5,495 ug/L to 530 ug/L. All chlorpyrifos exposure concentrations decreased within the range of 76% to

92%. This decrease was much higher than was observed for the control (45%) indicating that diffusion was possibly occurring in competition with the degradation or atomization processes. Another possibility is that the lower concentrations degrade slower as may be evident by the 76% decrease in the lowest concentration (the only exposure concentration decreasing less than 90% in chlorpyrifos concentration).

The effect of biological interaction was also examined by comparing one concentration with mussels with a similar concentration not containing mussels. The exposure concentration CHLO-03 decreased from 2,400 ug/L to 195 ug/L over the 24-hour period. However, the approximate same exposure concentration to which mussels were not exposed decreased from 2,272 ug/L to 573 ug/L chlorpyrifos. The concentration of the solution to which mussels were not exposed decreased 17% less than the approximately same concentration to which mussels were exposed. This may indicate that, unlike diazinon, chlorpyrifos decomposition was influenced by biological interaction. The decomposition may have been enhanced by enzymatic action, physical interaction involved with filtration, and/or bioaccumulation of the lipophilic pesticide into the tissues of the mussels. However, the relative differences in concentration are based

on single samples taken for each exposure solution at T0 and T24 and no indication of variability in these measures is available to determine if they are actually different. The possibility of chlorpyrifos storage in mussels prompted the following assay to measure the degree of bioaccumulation of chlorpyrifos by the hooked mussel.

Chlorpyrifos Bioaccumulation

The difference observed in the relative chlorpyrifos concentrations between solutions to which mussels were exposed and solutions to which mussels were not exposed prompted the examination of the bioaccumulation capacity of *I. recurvum*. The chlorpyrifos tissue concentration of mussels exposed to five chlorpyrifos concentrations increased with time (Table 17). The mussel tissue concentration was significantly related to the exposure concentration (ANOVA, $F=13.012$, $p=0.0036$, $R^2=0.5202$, [Chlorpyrifos Tissue] = 6.07 + [Exposure] · 24.58; Figure 4). Percent increase in tissue concentration was calculated by subtracting the preceding chlorpyrifos tissue concentration from the current chlorpyrifos tissue concentration, dividing that number by the preceding tissue concentration, and then multiplying by 100. The 24-hour calculation of percent increase used the exposure solution

concentration in place of the preceding tissue concentration.

Concentration in the tissues in all exposures, including the control, increased within a range of 49 and 106,000% within the first 24-hours (assuming preceding tissue concentration = exposure concentration), increasing on average 22,000%. Bioaccumulation increased tissue concentration at an average of 734% (367% increase per day), represented by mussel tissue concentrations increasing within the range of 85 and 2,600%, between 24 and 72 hours of exposure. The increase in tissue concentration over the next four days (at 7-days) increased within the range of -10 to 151%. The average daily increase was 13% for the days four through seven.

Table 17

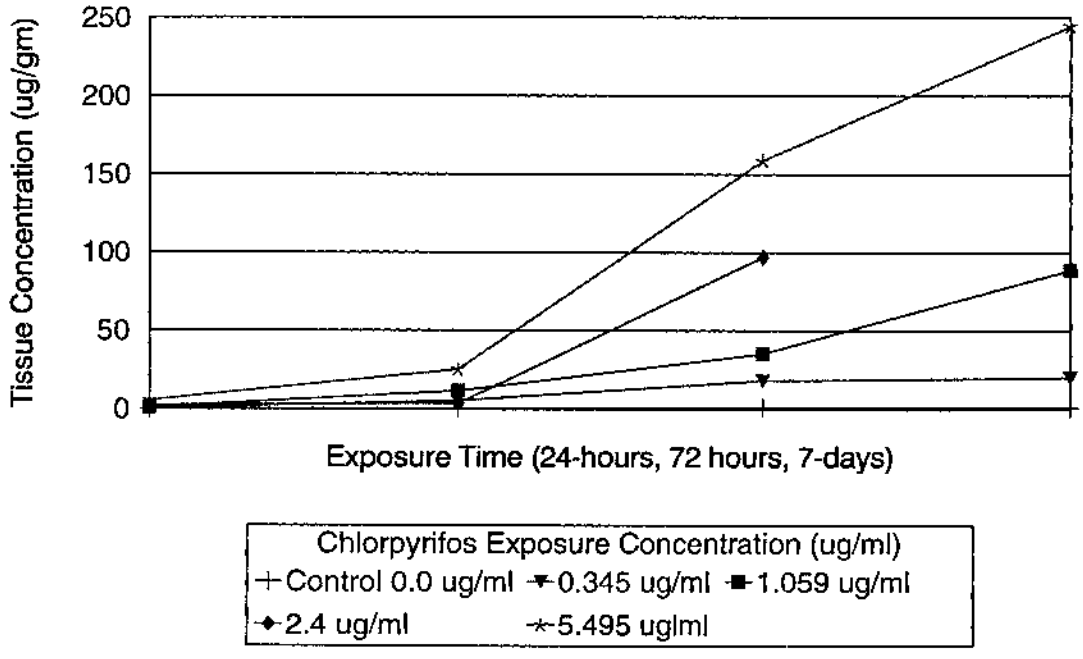
Chlorpyrifos Bioaccumulation by *I. recurvum* Over 7-Days.

Exposure concentration (ug/ml), tissue concentration (ug/g) after 24 hour exposure, tissue concentration (ug/g) after 72-hours, and tissue concentration (ug/g) after seven days, and percent increase in tissue concentration between measurements shown.

Conc No.	[Exposure]	T24-Hr [Tissue]	%Incr	T72-Hr [Tissue]	%Incr	T7-Day [Tissue]	%Incr
CHLO-CON	0.000557	0.59	105,825	1.09	84.7	0.98	-10.1
CHLO-01	0.345	5.46	1,483	18.23	233.9	20.07	10.1
CHLO-02	1.059	11.70	1,005	35.37	202.3	88.91	151.4
CHLO-03	2.400	3.57	48.8	97.14	2,621	-------	-----
CHLO-04	5.495	25.32	360.8	158.71	526.8	244.50	54.1

Figure 4

Mussel chlorpyrifos tissue concentration (ug/g) over time in exposure to five chlorpyrifos concentrations (ug/ml).



The largest increase on average occurred over the first 24-hours for all concentrations, however, high variation in the percent increase was evident in the higher concentrations. The trend seems to indicate that the higher the exposure concentration the lower the relative percent increase (assuming that the initial tissue concentrations were equal to the exposure concentration). The second trend indicated by the results is the decrease in percent increase of tissue concentration with exposure time. One possible reason for the decrease over time may have been a behavioral or physiological compensation made by the mussel to mediate the exposure to the toxicant. Saturation of the tissues was unlikely to be the cause of decrease in the rate of bioaccumulation as could be seen by the 7-day tissue concentrations ranging from a low of 0.98 ug/g (in the lowest concentration) to a high of 244.5 ug/g (in the highest exposure concentration).

The mechanisms by which mussels regulate their tissue concentration independent of saturation, in part, may be better represented by bioconcentration factors (BCF, tissue concentration/ exposure concentration; Table 18). The 72-hour factors ranged from 53 (lowest exposure concentration) to 29 (highest exposure concentration). A great deal of non-monotonic variation was evident in the 7-day

bioconcentration factors with the highest factor resulting from the second lowest exposure concentration (26 higher than the lowest exposure concentration BCF). The most noteworthy bioaccumulation was calculated for the control having a 24-hour value of 1,059 increasing to a 7-day value of 1,759, which decreased slightly from the maximum value at 72-hour of 1,957.

No chlorpyrifos bioaccumulation data for invertebrates was available in the literature when Montanes and Hattum (1995) reported that freshwater isopods (*Asellus aquaticus*) accumulated sufficient levels of chlorpyrifos in low concentration exposures to generate an average BCF of 1,715 in 14-days. The chlorpyrifos BCF's for vertebrates were comparable to these values at 1,700 for *Pimephales promelas* (Jarven et al., 1983), 1,575 for *Poecilia reticulata* (Deneer, 1993) and 1,050 for *Gasterosteus aculeatus* (Deneer, 1994).

The larger BCF values for the control may reflect the greater gradient between exposure concentration and tissue concentration. The greater the difference, the larger the potential for uptake. In addition, the very low levels in the control may be below that which the mussels could detect or identify as harmful. If that were the case, the filtration rates may have been greater for controls

(relative to higher concentration exposures) increasing the interaction occurring with the pesticide making it more available to the tissues. This condition may persist until the mussel has accumulated sufficient levels of the pesticide to trigger defense mechanisms such as valve closure.

Laughlin and French (1988) conducted experiments where mussels accumulated bis(tributyl)tin (TBT) at concentrations which provided no evidence of acute stress modifying health and behavior. Mussels exposed to 23 and 45 ng/L TBT accumulated the substance until day four or six, and then tissue concentrations declined to an apparent steady state. In contrast, mussels exposed to higher concentrations (63, 141 or 670 ng/L TBT) exhibited increasing TBT tissue concentrations with no apparent approach to a steady state over the 2 to 6 week exposure period. This relationship was also observed for *I. recurvum* exposed to chlorpyrifos when the contaminated control BCFs were compared to that of exposure concentrations.

Accumulation of TBT was said to increase in all exposures as a function of exposure concentration. The highest tissue concentrations were consistently found in the gills (Laughlin and French, 1988). After reviewing a study on bioaccumulation in oysters (*Crassostrea virginica;* Lee,

1985: as cited by Laughlin and French, 1988), Laughlin and French proposed that the action of physiological/biochemical mechanisms influence structural modification and excretion of xenobiotics. They suggested that these inducible clearance mechanisms are active with respect to TBT at low concentrations, but higher concentration exposures overwhelm these mechanisms not allowing a steady state to be reached. The activity of inducible clearance mechanisms may have also been involved in *I. recurvum* exposed to the contaminated control were a steady state was observed between days four and seven. And like the TBT exposures, the clearance mechanisms may have been overwhelmed by the higher exposure concentrations not allowing a steady state to be reached. Mussel bio-accumulation studies are not new, but studies are often designed to measure metal concentrations in the environment (Balogh, 1988; Hutagalung, 1989; Lakshmanan and Nambisan, 1989; Cain and Luoma, 1990; Riedel et al., 1995).

Table 18

Chlorpyrifos Bioaccumulation Rates of *I. recurvum* Over 7-d.

Exposure concentration (ug/ml), tissue concentration (ug/g) after 24 hour exposure, tissue concentration (ug/g) after 72 hours, and tissue concentration (ug/g) after seven days, and corresponding bioconcentration factors for the tissue measurements shown.

Conc No.	[Exposure]	T24-Hr [Tissue]	BC Factor	T72-Hr [Tissue]	BC Factor	T7-Day [Tissue]	BC Factor
CHLO-CON	0.000557	0.59	1,059	1.09	1,957	0.98	1,759
CHLO-01	0.345	5.46	16	18.23	53	20.07	58
CHLO-02	1.059	11.70	11	35.37	33	88.91	84
CHLO-03	2.400	3.57	1.5	97.14	40	--------	-----
CHLO-04	5.495	25.32	5	158.71	29	244.50	44

***I. recurvum* Temperature Tolerance for Temperature Acclimations**

The failure to achieve growth in the laboratory that would be useful for bio-indication led to the examination of a different bio-monitoring endpoint. The upper temperature tolerance of *I. recurvum* was then tested for possible use as a bio-indicator of relative water quality.

The length and heat coma temperature of each individual mussel was recorded for 27 mussels at 35°C acclimation, 27 mussels at 30°C acclimation, 23 mussels at 25°C acclimation, and 22 mussels at 20°C acclimation (Table A-12). The results of the analysis of the temperature tolerance data from the

acclimation of *I. recurvum* to four temperatures are shown in Table 19. The mean heat coma temperature (HCT) decreased between acclimation to 35 and 30°C and acclimation to 25 and 20°C resulting in mean HCTs of 45.7, 45.4, 43.4, and 43.7°C respectively. The HCT of hooked mussels was significantly related to acclimation. The HCTs for the different acclimation temperatures were significantly different. Further analysis showed that the 20 and 25°C acclimation's HCTs were not different, nor were the 30 and 35°C acclimation's HCTs. However, the 30 and 35°C HCTs were different from the 20 and 25°C HCTs.

Table 19

Upper Temperature Tolerance of *Ischadium recurvum* at Four Acclimation Temperatures

Acclimation Temperature (Ac Tp), Mean Heat Coma Temperature (HCT), Variance (Var), and Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test, SNK Multiple Range test, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analysis Reported.

Ac Tp	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
35°C	45.7	0.561	0.0009	3.063/0.40 =7.66	A	A	HCT= (0.16) Accl Temp+40.36
30°C	45.4	0.708	0.5244	0.10 > P(F≥7.66) >0.2	A	A	
25°C	43.4	3.063	0.0001		B	B	R^2= 0.5225 F= 54.931 Pr>F= 0.0001
20°C	43.7	0.400	0.3051		B	B	

Analysis of Variance	F = 59.43	Pr>F = 0.0001
Kruskal-Wallis X^2 Approximation	X^2= 64.144	Pr>X^2 = 0.0001
Median 1-Way Analysis X^2 Approximation	X^2 = 44.656	Pr>X^2 = 0.0001

Liu and Morton (1994) reported the HCT and LT50 (lethal temperature for 50% of the population) for a another subtidal mussel *Septifer virgatus* acclimated to 26°C for 24-hours to be 45°C and 44.5°C respectively (mean HCT of *I. recurvum* acclimated to 25°C for 21-days was 43.4 ± 1.8°C). Urban (1994) evaluated the temperature tolerance of ten commercially important South American bivalve species (*Gari solida, Semele solida, Semele corrugata, Prothaca thaca, Venus antiqua, Tagelus dombeii, Ensis macha, Aulacomya ater, Choromytilus chorus,* and *Argopecten purpuratus*) off Peru and Chile to study some of the effects of El Nino. He found that higher temperatures in Peru made LT50 values for mussels sampled there higher than in Chile. Six of the eight species studied in Chile varied by only 1.2°C which may have been the result of similar temperatures and living conditions in the habitats of these species (Urban, 1994).

The trend evident from the 120 mussels sampled among four acclimation temperatures suggests that expected HCTs could be estimated (Figure 5). The trend in HCT is not dissimilar from the relationship of critical thermal maximum (CTMax) or preferred temperature and acclimation temperature for other aquatic organisms.

Rodriguez et al., (1996) reported that 98% of the variability about the temperature at which *Macrobrachium*

tenellum suffered total disorientation (CTMax) could be explained by acclimation temperature (Figure 6). The thermal stress response was examined by a multiple range test (SNK) which showed that disorientation temperatures from acclimation to 22 and 25°C were different from acclimations to 28 and 32°C. The degree of resistance of *M. tenellum* diminished with increasing acclimation temperature in terms of the difference between acclimation temperature and the temperature at which total disorientation occurred (Rodriguez, et al., 1996).

Stauffer and Boltz (1994) measured the effect of salinity on the final preferred temperature (Figure 7), which is an accurate indicator the optimal temperature for growth (Gift, 1977: as cited by Stauffer and Boltz, 1994). The study was designed to determine if the range expansion of the introduced population of the Mayan cichlid in southern Florida would be limited by salinity and temperature. Salinity altered the preferred temperature of the fishes. Preferred temperature models based on acclimation temperature for each respective salinity concentration achieved coefficients of determination ranging from 0.50 to 0.64 and were used to estimate the potential for range expansion.

Models based upon a regression of determined HCTs by

acclimation temperature for *I. recurvum* could be used to estimate HCTs of specimens in the field if exposure temperatures are known. The regression performed on the data collected from the four acclimation temperatures calculated that 52% of the variation about the HCT was a result of acclimation temperature. The inclusion of more acclimation temperatures in generating this line would likely result in a regression which accounted for more than 52% of the variation. Several additional acclimation temperatures from 20 to 30°C would be of most use in improving this particular regression line and would likely enhance the coefficient of determination. However, the data would not be complete without investigating HCTs resulting from acclimation lower temperatures (<20°C). It is not likely that HCT could be entirely explained by acclimation temperature because of the numerous other factors which play a role in the determination of HCT (e.g. genetics, health, dissolved oxygen, population density, contaminants).

Figure 5

Ischadium recurvum heat coma temperature (°C) relation to acclimation temperature(°C).

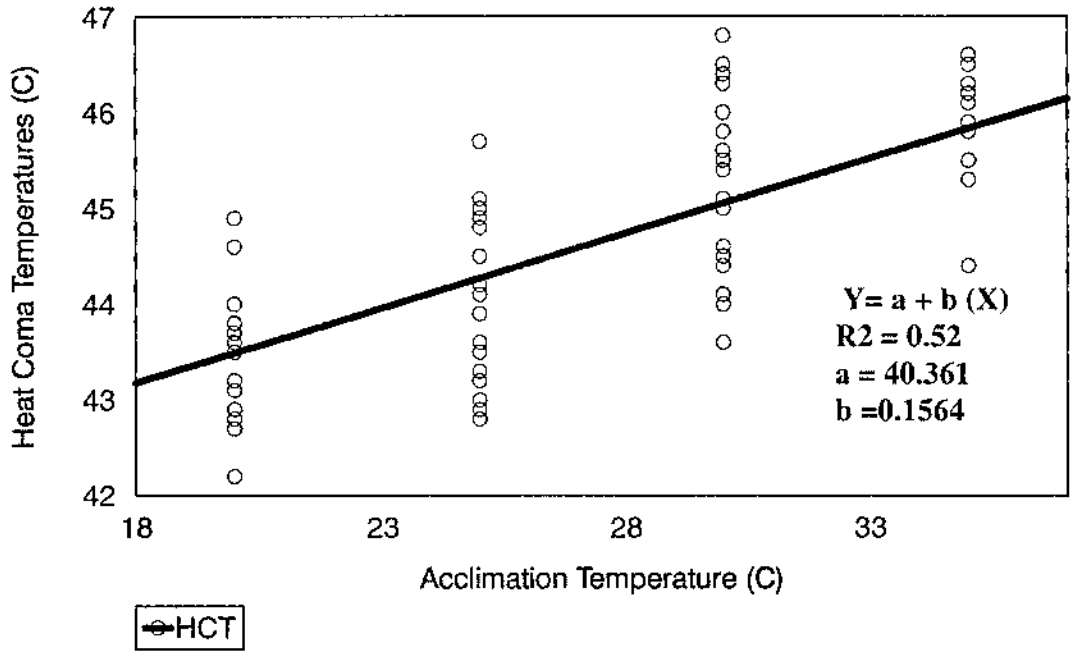


Figure 6

Figure from Rodriguez et al., 1996.

Figure 7

Figure from Stauffer and Boltz, 1994.

Statistically significant differences between exposures or from expected HCTs calculated from the model may or may not be useful. Large sample sizes may result in significant differences when the data are not different enough to be useful for bio-monitoring purposes. Small sample sizes may cause statistical analyses to show no difference between HCTs when the differences are biologically useful. The established minimum 1°C difference from the control may provide for a comparison with the statistical results.

A 1°C change in the temperature of a poikilothermic organism was thought to be biologically important. The physiological rates of these organisms double with every 10°C increase in temperature within certain limits (Q_{10}). A 1°C change in temperature then represents a 10% change in the rates of metabolism, oxygen consumption, and respiration. Failure of an organism to cope with an increase in its rate functions with respect temperature increase causes incapacitation and death. A 10% difference (1°C) in the ability of a mussel to cope with an increase in its rate functions from that of a control (or calculated value) was thought to be useful for bio-monitoring purposes.

I.recurvum Temperature Tolerance for Organophosphate Exposure

After establishing baseline data on the upper temperature tolerance of mussels at different acclimation temperatures, the target organophosphates were tested for effects on upper temperature tolerance. The influence of pesticide exposure and the persistence of the effects on upper temperature tolerance were tested at a temperature between baseline acclimation temperatures (27°C).

Chlorpyrifos Exposure for 72-hours

The heat coma temperature (HCT), length, and weight of each individual mussel was recorded for 18 mussels exposed to each of six chlorpyrifos exposure concentrations (Table A-13). The results of the analysis of the upper temperature tolerance data for *I. recurvum* exposed to chlorpyrifos for 72-hours are shown in Table 20. The mean HCT temperatures were 43.8°C for 0.6 ug/L, 42.5°C for 78 ug/L, 42.4°C for 86 ug/L, 42.6°C for 165 ug/L, 42.2°C for 344 ug/L, and 40.0°C for exposure to 727 ug/L chlorpyrifos for 72-hours. HCTs of the respective exposures were significantly different. Multiple range test on ranked HCTs showed that HCTs resulting from exposures ranging from 78 ug/L to 344 ug/L chlorpyrifos to be significantly different than the control

exposure (0.6 ug/L chlorpyrifos). The multiple range tests further showed that exposure to the highest concentration of chlorpyrifos (727 ug/L) resulted in HCTs significantly different from HCTs resulting from all lower concentration exposures. A regression performed on these data showed the relationship of chlorpyrifos exposure concentration and HCT to be significant. Further investigation of this relationship utilizing a forward selection stepwise regression showed that the chlorpyrifos concentration was the most determinant factor.

Table 20

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Chlorpyrifos Exposure

Chlorpyrifos Concentration ([CHLO] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var) ,and Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test for Ranked Data, SNK Multiple Range test for Ranked Data, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analysis Reported.

[CHLO] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT Rank	SNK MRT Rank	Model
0.6	43.81	0.282	0.2791	6.66 / 0.282 = 23.62 0.002 > P(F≥23.62) >0.005	A	A	HCT= (-0.004) [CHLO] + 43.20 R^2= 0.2234 F= 35.38 Pr>F= 0.0001
78	42.50	3.69	0.0011		B	B	
86	42.41	2.88	0.3332		B	B	
165	42.59	3.55	0.0505		B	B	
344	42.24	2.80	0.1516		B	B	
727	39.96	6.66	0.1970		C	C	
Analysis of Variance					F = 8.44		Pr>F = 0.0001
Analysis of Variance on Ranked Data					F = 8.43		Pr>F = 0.0001
Kruskal-Wallis X^2 Approximation					X^2= 32.43		Pr>X^2 = 0.0001
Median 1-Way Analysis X^2 Approximation					X^2 = 29.60		Pr>X^2 = 0.0001

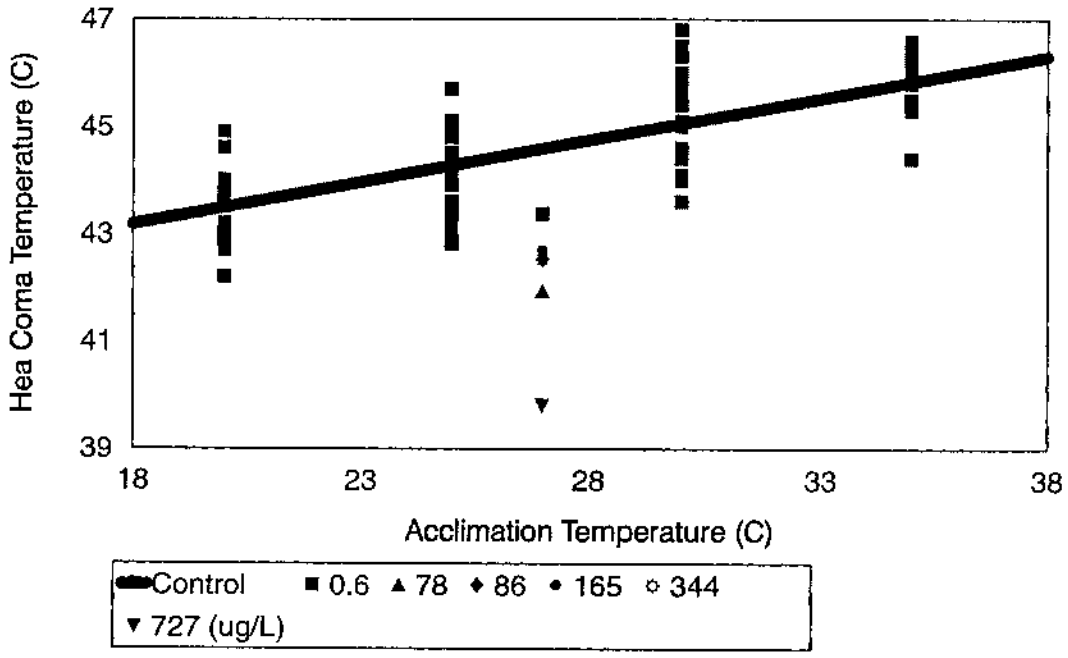
* Forward selection stepwise regression selected chlorpyrifos concentration first (F= 19.21, Pr>F= 0.0001, partial R^2= 0.1917), weight was selected second (F= 1.7598, Pr>F=0.1884, partial R^2= 0.0455), and selected overall length third (F= 4.8215, Pr> 0.0310, partial R^2= 0.0074).

Exposures of hooked mussels to chlorpyrifos influenced their upper temperature tolerance. Increasing the concentration of chlorpyrifos from 0.6 ug/L to 78 ug/L resulted in a 1.3°C decrease in upper temperature tolerance (decrease from 43.8° to 42.5°C) in terms of mean heat coma

temperature for the 18 mussels exposed. This level of loss of upper temperature tolerance may serve as a biologically useful indicator of relative water quality and deviations from the expected HCT could be estimated using the acclimation temperature model. However, the decrease in heat coma temperature was not more pronounced as chlorpyrifos concentrations continue to increase until concentrations reached acute levels (727 ug/L chlorpyrifos in 72-hours in flow through exposures; Figure 8). Concentrations which began to cause mortality decreased mean upper temperature tolerance by 3.85°C (39.96°C) in 72-hours.

Figure 8

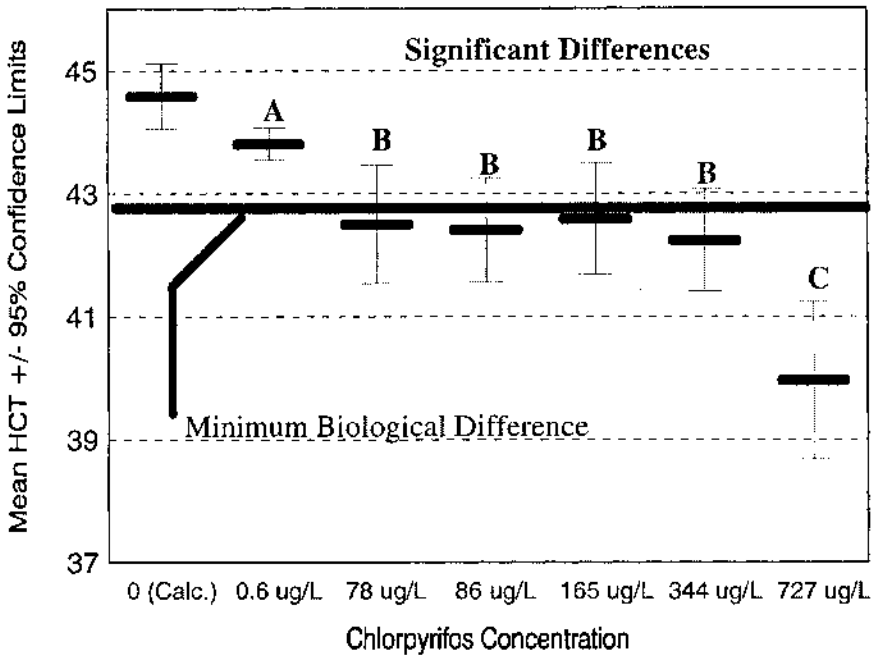
Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour chlorpyrifos exposures (0-727 ug/L).



The influence of sublethal concentrations of chlorpyrifos on HCT exhibit what may be a threshold affect (Figure 9). Chlorpyrifos concentrations which may result in biologically useful differences in upper temperature tolerance may occur below the observed 78 ug/L chlorpyrifos, however, variability about the endpoint, which would likely increase in environmental exposures, may mask such effects. The chlorpyrifos 96-hour LC01 for the hooked mussel was estimated at approximately 200 ug/L and LC10 at 400 ug/L. Exposure concentrations ranging from 78-344 ug/L produced deviations from the mean control HCT that were approximately equal (1.2-1.6°C). All deviations in this range exceeded the 1°C determined minimum for use as biological indicators and were as large or larger as those reported for some other invertebrates studies (Poulton et al., 1989, Moulton et al., 1993). The variations in the estimates observed were also consistent with other CTMax studies (Poulton et al., 1989, Moulton et al., 1993, Stauffer and Boltz, 1994).

Figure 9

Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour chlorpyrifos exposures (0-727 ug/L and a control calculated from the model). Multiple range test significant differences, biomonitoring significance level, mean HCT and 95% confidence limits shown.



Poulton et al. (1989) reported on the affect of hexavalent chromium on the critical thermal maximum (CTMax) of insect nymphs (*Clioperla clio*). They found that nymphs exposed to sublethal levels of chromium (Cr^{+6}) had significant (Non-parametric ANOVA, $p < 0.0001$) reductions in upper temperature tolerance in terms of CTMax (SNK, $CTM_{CONTROL} > CTM_{LC10} > CTM_{LC30} = CTM_{LC50}$; range 31.5-29.5°C). The 96-hour

chromium (Cr^{+6}) LC50 for *C. clio* was estimated to be 101.3 mg/L (C.I.95%= 88.9-118.0 mg/L) with the probit method (Poulton et al., 1989). The difference between the control CTMax and the LC10 CTMax was 0.8°C, and the difference between the control and LC30 CTMax was 1.7°C. The deviations from control HCT (=CTMax) for *I. recurvum* exposed to chlorpyrifos concentrations less than the estimated LC01 were larger than those for *C. clio* exposed to chromium concentrations at the LC10 level. The results may suggest that mussel upper temperature tolerance in terms of HCT may serve as a valid indicator of chlorpyrifos exposure for the range of concentrations tested if field exposures produce similar deviations from the expected (calculated from model for acclimation temperature) HCT.

The ability of mussels to cope with temperature stresses in the event of simultaneous exposure to chlorpyrifos may be of importance when considering the high temperatures reached by some estuaries in the summer months. More sensitive species would be more severely impacted by such a relationship. Non-point source pollutants such as chlorpyrifos are under significant scrutiny (Bernabei et al., 1991; Doggett and Rhodes, 1991; Allender and Britt, 1994; Hanratty and Stay, 1994; Hill et al., 1994; Wilcock et al., 1994) and this may add to the list of concerns

currently being investigated. One such area of interest may be the persistence of such a temperature tolerance relation as the storms, which appear to result in pesticide runoff to some receiving systems, subside and the temperature of the estuaries rise. The persistence of the reduction in HCT of mussels induced by chlorpyrifos exposure was examined in the following test.

I. recurvum Temperature Tolerance for Organophosphate Exposure

Chlorpyrifos Exposure After 24-hour Rinse

The heat coma temperature (HCT), length, and weight of each individual mussel was recorded for 12 mussels 24-hours after exposure to each of four chlorpyrifos exposure concentrations (Table A-14). The 165 ug/L and 727 ug/L chlorpyrifos exposures were not included in this assay. The results of the analysis of the upper temperature tolerance data for *I. recurvum* 24-hours after a 72-hour chlorpyrifos exposure are shown in Table 21. The mean HCTs were 44.2°C for 0.6 ug/L, 42.5°C for 78 ug/L, 42.4°C for 86 ug/L, and 41.4°C for previous exposure to 344 ug/L chlorpyrifos. HCTs from the respective exposures were not statistically different.

Table 21

Upper Temperature Tolerance of *Ischadium recurvum* After 24-Hour Rinse Following Chlorpyrifos Exposures

Chlorpyrifos Concentration ([Chlo] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var) ,and Shapiro-Wilks Normality test, Variance Ratio test, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analysis Reported.

[Chlo] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
0.6	44.17	0.2933	0.0369	8.4875 / 0.2933 = 28.94 0.01 > P(F≥28.94) >0.02
78	42.51	5.1469	0.0731	
86	42.44	4.6136	0.1533	
344	41.38	8.4875	0.3851	

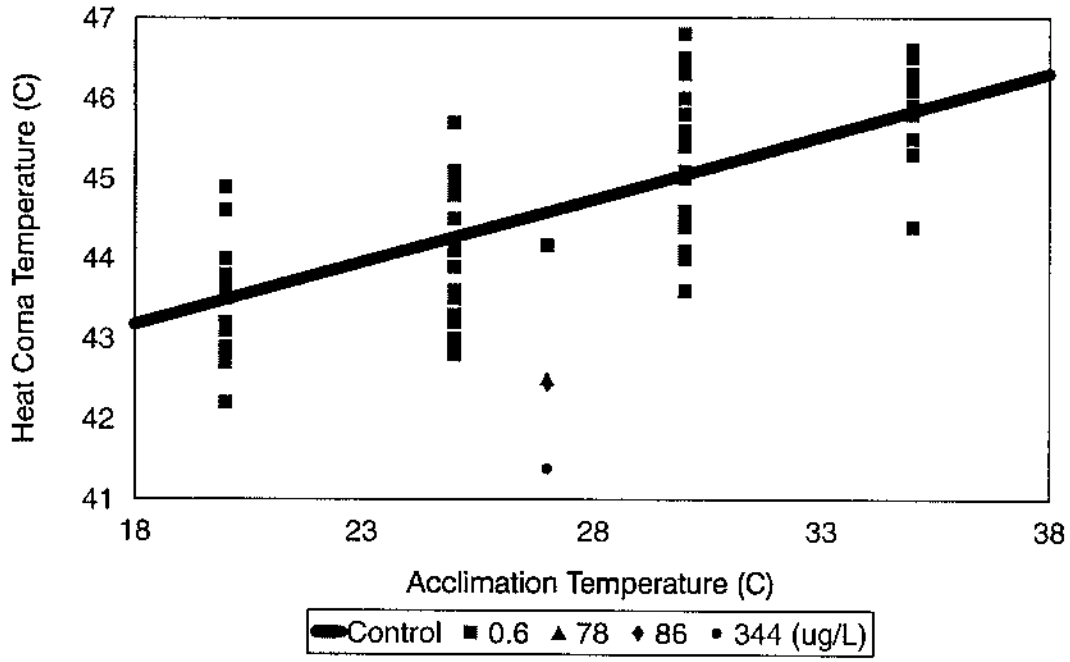
Analysis of Variance	F = 3.50	Pr>F = 0.023
Analysis of Variance on Ranked	F = 2.40	Pr>F = 0.0806
Kruskal-Wallis X^2 Approx	X^2= 6.6064	Pr>X^2 = 0.0856
Median 1-Way Analysis X^2 Approx	X^2 = 3.2532	Pr>X^2 = 0.3542

Although statistical differences were not shown, exposures of hooked mussels to chlorpyrifos continued to influence their upper temperature tolerance after the toxicant had been removed for 24-hours (Figure 10). The relationship demonstrated by the 72-hour exposures persisted as of 24-hours after the exposure. The removal or washing through of the toxicant did little to lessen the initial insult of chlorpyrifos exposure. The deviations from the control and expected HCT (to larger degree) suggest that the HCT of mussels may serve as an appropriate biological

indicator of a chlorpyrifos exposure after the actual exposure has ceased.

Figure 10

Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour chlorpyrifos exposures (0-344 ug/L) followed by a 24-hour rinse period.

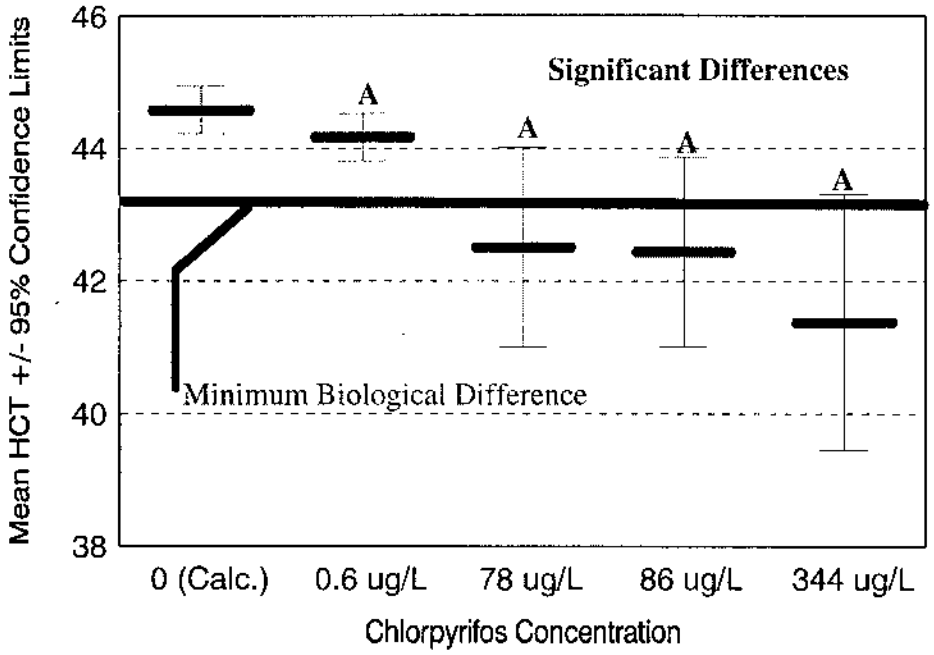


The statistical analysis showed no distinct differences between these exposure influenced HCTs, however, the differences were actually the same or greater than the 72-hour exposure analyses (Table 20). A decreasing HCT trend was still evident in HCT after the 24-hour rinse. The

decrease in HCT, relative to the control, for previous chlorpyrifos exposure in the LC01-LC10 range was from 1.7°C to 2.8°C. Although the differences were not statistically significant, they do meet the 1°C difference from control exposure which was the defined criteria for use as bio-indicators (Figure 11). The true difference between the two analyses may have been a result of sample size. The initial analyses were performed on groups of 18 mussels per exposure concentration and this 24-hour rinse analysis consisted of 12 mussel replicates per exposure. The variances associated with the groups in each respective analysis were approximately equal.

Figure 11

Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour chlorpyrifos exposures (0-344 ug/L a control calculated from the model) followed by a 24-hour rinse period. Multiple range test significant differences, bio-monitoring significance level, mean HCT and 95% confidence limits shown.



I. recurvum Temperature Tolerance for Organophosphate Exposure

Diazinon Exposure for 72-hours

The heat coma temperature (HCT), length, and weight of each individual mussel was recorded for 18 mussels exposed to each of six diazinon concentrations (Table A-15). The mean HCTs were 43.95°C for 0.024 ug/L, 43.78°C for 100 ug/L,

43.66°C for 136 ug/L, 43.23°C for 257 ug/L, 42.24°C for 496 ug/L, and 39.11°C for exposure to 1,314 ug/L diazinon for 72-hours. The results of the analysis of the upper temperature tolerance data from the exposure of *I. recurvum* to diazinon for 72-hours are shown in Table 22. HCTs from the respective exposures were significantly different. Multiple range tests on ranked HCTs showed that HCTs resulting from exposures to 496 ug/L diazinon were significantly different than the control (0.024 ug/L diazinon). The multiple range tests further showed that HCTs from exposure to 1,314 ug/L diazinon were different from those resulting from all other diazinon exposures. A significant relationship between diazinon concentration and HCT was determined using a linear regression. Forward selection stepwise regression showed that the diazinon concentration was the most determinant factor.

Table 22

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Diazinon Exposure

Diazinon Concentration ([Diaz] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test for Ranked Data, SNK Multiple Range test for Ranked Data, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Diaz] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT Rank	SNK MRT Rank	Model
0.024	43.95	0.66	0.0749	5.345 / 0.6626 = 8.067 0.02 > P(F≥8.07) >0.05	A	A	HCT= (-0.0039) [Diaz] + 46.8849 R^2= 0.4555 F= 18.733 Pr>F =0.0001
100	43.78	4.38	0.0001		AB	A	
136	43.66	2.63	0.0005		AB	A	
257	43.23	2.42	0.2221		AB	AB	
496	42.24	5.35	0.0194		B	B	
1314	39.11	4.57	0.1208		C	C	

Analysis of Variance	F = 18.33	Pr>F = 0.0001
Analysis of Variance on Ranked Data	F = 14.29	Pr>F = 0.0001
Kruskal-Wallis X^2 Approximation	X^2= 43.915	Pr>X^2 = 0.0001
Median 1-Way Analysis X^2 Approximation	X^2 = 35.485	Pr>X^2 = 0.0001

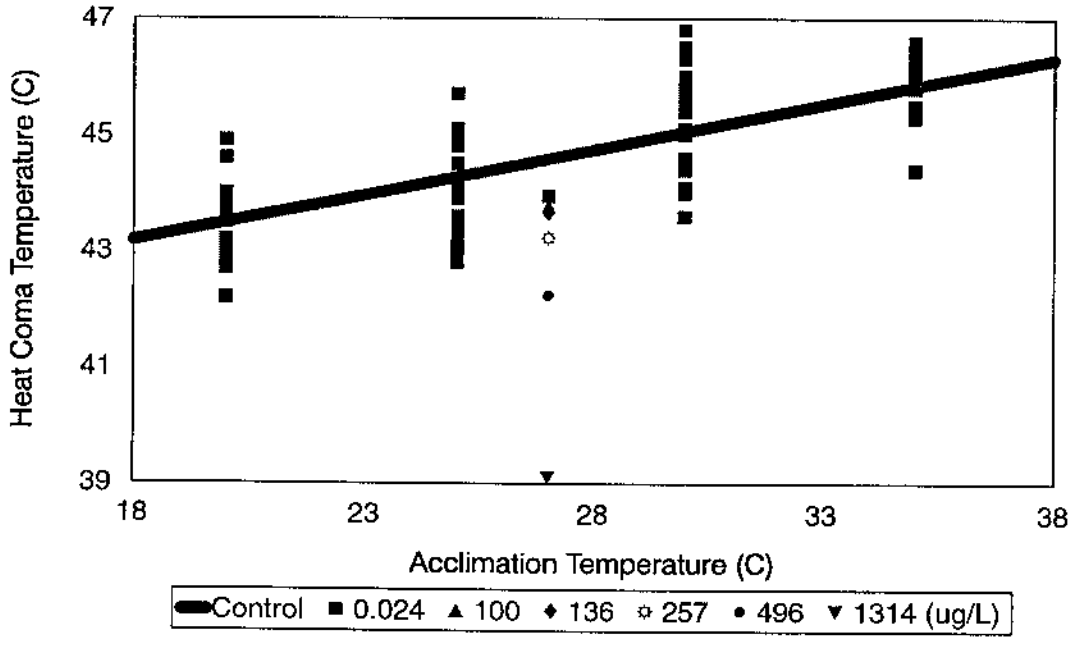
* Forward selection stepwise regression selected diazinon concentration first(F= 94.86, Pr>F= 0.0001, unadjusted partial R^2= 0.4746).

Exposures of hooked mussels to diazinon influenced their upper temperature tolerance, however, this occurred only in exposures to higher concentrations. An increase in the concentration of diazinon to 257 ug/L played a part in the reduction the mean HCT by 0.7°C relative to the control (or somewhat more from the expected value calculated from the model for this acclimation temperature). The deviations

in HCT from the control or expected HCT was much more subtle than in the preceding chlorpyrifos exposures (Figure 12). For example, increasing the concentration of diazinon by the above mentioned 257 ug/L decreased the upper temperature tolerance of the hooked mussel by approximately half as much as the addition of 78 ug/L of chlorpyrifos relative to their respective controls. However, diazinon concentrations which began to cause mortality (1,314 ug/L ~ 96-hour LC50) decreased upper temperature tolerance to the same extent as that observed for lethal concentrations of chlorpyrifos (~4°C).

Figure 12

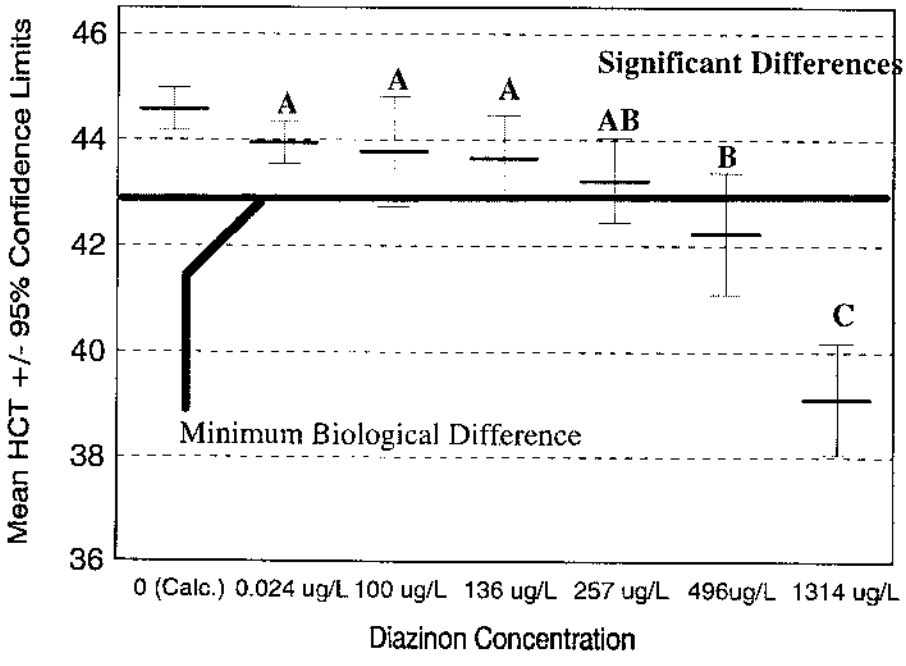
Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour diazinon exposures (0-1,314 ug/L).



Deviations from control HCTs did not meet the defined useful level until mussels were exposed to ≈500 ug/L diazinon, but they did show a decreasing trend as exposure concentration increased (Figure 13). However, this concentration was below the 96-hour diazinon LC01 estimated for the hooked mussel of ≈700 ug/L diazinon. The data suggest that mussel HCT may not be an appropriate biological indicator of diazinon exposure during the actual time of exposure if concentrations are below the estimated LC01.

Figure 13

Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour diazinon exposures (0-1,314 ug/L a control calculated from the model). Multiple range test significant differences, bio-monitoring significance level, mean HCT and 95% confidence limits shown.



A possible reason for the difference in response of hooked mussels to the two organophosphates may be related to the process of transformation to a more toxic form. Organophosphates are transformed in most organisms by liver microsomes to the more toxic -oxon form (Morgan, 1989). If the rate or process of transformation differs in mussels between diazinon and chlorpyrifos, their effect on upper temperature tolerance may differ. However, the specific mode of transformation of these or other organophosphates in bivalves is not known.

It is also possible that the differences in HCT from exposure to diazinon and chlorpyrifos may be less related to the active ingredients than the "inert" ingredients. The diazinon product contained approximately 75% "inert" ingredients and the chlorpyrifos product contained approximately 95% "inert" ingredients. The identity of these ingredients is not known and so their toxicity to bivalves is also not known. Provided with the identity of the pesticide solvents ("inert" ingredients), solvent control could be investigated to provide information about their role in effecting mussel upper temperature tolerance. Examination of the HCT relationship with diazinon after the exposure had ceased was examined in the following test.

I.recurvum Temperature Tolerance for Organophosphate Exposure

Diazinon Exposure After 24-hour Rinse

The heat coma temperature (HCT), length, and weight of each individual mussel was recorded for 12 mussels previously exposed to each of four diazinon concentrations and then rinsed for 24-hours in filtered sea water (Table A-16). The 257 ug/L and 1,314 ug/L diazinon exposures were not included in this assay. The results from the analysis of the upper temperature tolerance data for *I. recurvum* 24-hours after a 72-hour diazinon exposure are shown in Table 23. The mean HCTs were 43.55°C for 0.024 ug/L, 42.84°C for 100 ug/L, 41.94°C for 136 ug/L, and 42.23°C for previous exposure to 496 ug/L diazinon. Median HCTs were significantly different at an α-level of 0.05. This analysis was the only one which showed significant differences of the analyses performed on these data.

Table 23

Upper Temperature Tolerance of *Ischadium recurvum* After 24-Hour Rinse Following Diazinon Exposures

Diazinon Concentration ([Diaz] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), and Shapiro-Wilks Normality test, Variance Ratio test, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Diaz] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)
0.024	43.55	1.3047	0.0046	7.5806 / 1.3047 = 5.8102 0.1 > P(F≥5.8102) >0.2
100	42.84	6.1305	0.0036	
136	41.94	3.6681	0.3005	
496	42.23	7.5806	0.1744	

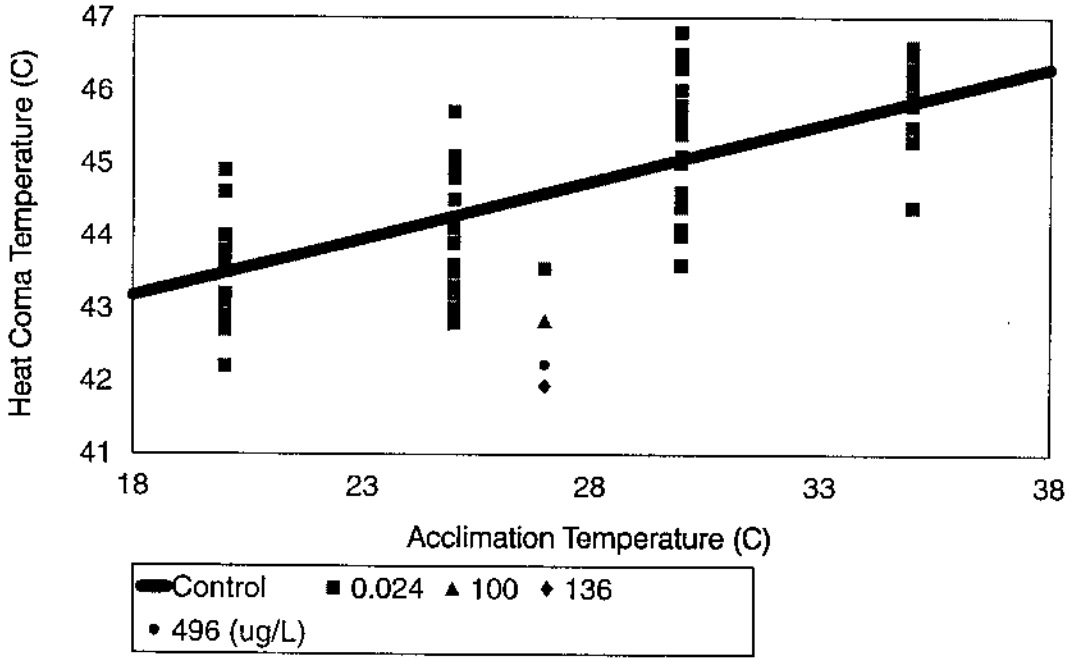
Analysis of Variance	F = 1.45	Pr>F = 0.2420
Analysis of Variance on Ranked Data	F = 1.50	Pr>F = 0.2280
Kruskal-Wallis X^2 Approximation	X^2= 4.3584	Pr>X^2 = 0.2253
Median 1-Way Analysis X^2 Approximation	X^2 = 8.7461	Pr>X^2 = 0.0329

Exposures of hooked mussels to diazinon appeared to be more toxic to mussels, in terms of upper temperature tolerance, after the toxicant had been removed for 24-hours in that deviations in mean HCT from the expected and control HCT were larger (Figure 14). The relationship demonstrated by the 72-hour exposures escalated to a more pronounced deviation from control upper temperature tolerance in the lower two concentrations (100 and 136 ug/L). However, only previous exposure to 136 and 496 ug/L diazinon produced

differences in HCT (1.6 and 1.3°C respectively) from that of the control in excess of 1°C defined as biologically useful for bio-monitoring. The washing through of the toxicant did little to lessen the initial insult of diazinon exposure. This was observed in the preceding chlorpyrifos test as well.

Figure 14

Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour diazinon exposures (0-496 ug/L) followed by a 24-hour rinse period.



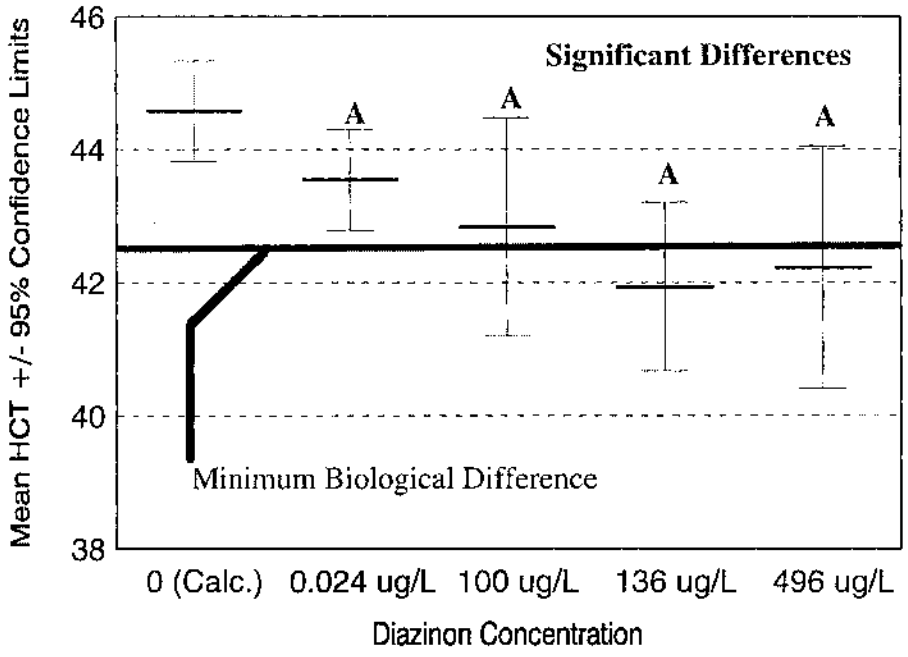
The statistical analysis again showed less distinct differences between these exposure influenced HCTs than the 72-hour exposure analyses, however, the differences were actually the same or greater than at the conclusion of the 72-hour exposure. As in the chlorpyrifos assay the true difference between the two analyses may have been the result of sample size. The initial analyses were performed on groups of 18 mussels per exposure concentration and this 24-hour rinse analysis consisted of 12 mussel replicates per exposure. The actual differences from the control in mean HCT increased 0.5°C in the 100 ug/L exposure and 1.3°C in the 136 ug/L exposure during the course of the rinse period.

During the exposure to diazinon, a larger decrease upper temperature tolerance (~4°C) was observed in concentrations which began to cause mortality (1,314 ug/L ~ 96-hour LC50). Deviations from control HCTs began to meet the defined biologically useful level when mussels were exposed to ~500 ug/L diazinon which was below the 96-hour diazinon LC01 estimated for the hooked mussel of ~700 ug/L diazinon. After the exposure had ceased, however, concentrations as low as 136 ug/L met the defined useful level for bio-monitoring (Figure 15) with a mean HCT 1.6°C lower than the control and even lower still than the calculated (expected) HCT from the acclimation temperature

model (a 2.6°C deviation).

Figure 15

Deviations from the acclimation temperature trend of *Ischadium recurvum* accounted for by 72-hour diazinon exposures (0-496 ug/L period and a control calculated from the model) followed by a 24-hour rinse. Multiple range test significant differences, bio-monitoring significance level, mean HCT and 95% confidence limits shown.



The affect of diazinon on the upper temperature tolerance of the hooked mussel was somewhat delayed in lower concentration exposures. After the exposure had ceased, more evident impacts on upper temperature tolerance could be

seen. A difference (from chlorpyrifos) in the location of transformation or rate of transformation of diazinon into its more toxic -oxon form may have caused such a relation. And the role of the "inert" ingredients cannot be dismissed as not involved. However, as mentioned above for chlorpyrifos exposures, such persistent affects on mussels could make them useful bio-indicators after the occurrence of run-off episodes.

The development of the baseline upper temperature tolerance data for these mussels was based on HCT data for four acclimation temperatures. The data showed a trend of increasing HCT as acclimation temperature increased (R^2=0.52). Deviations from expected HCTs could have been estimated using the acclimation temperature model because exposures were performed at a temperature (27°C) between baseline acclimation temperatures. The control exposures for all the preceding organophosphate tests were contaminated with low organophosphate levels. The mean HCT of the control for each test was higher than organophosphate induced HCTs and the variability was often lower. However, the control HCTs in each test were less than that which would have been expected (~1°C) according to calculations using the acclimation temperature model for estimating HCTs.

Comparisons were made with the control in the preceding

discussion. If the comparisons of the exposure induced HCTs were made with expected values from the model, larger differences between exposure induced HCTs and the control would have been reported. In addition, differences between the contaminated control HCTs and the expected HCTs were, in some cases, at a level which could be useful for bio-monitoring purposes (1°C difference).

Comparisons were not made with expected HCTs for two reasons. The very low levels of contamination in the controls (0.6 ug/L chlorpyrifos and 0.024 ug/L diazinon) were assumed not to effect the HCT of these mussels because of their relatively tolerant nature (96-hour LC50; 960 ug/L chlorpyrifos and 1,354 ug/L diazinon). The inclusion of more acclimation temperatures in establishing the baseline model would likely provide better estimates of expected HCTs for direct comparison. The controls were run to test the accuracy of the model estimations, however, it is not known if the low levels of organophosphate contamination in the controls effected the HCTs. If the HCTs were affected by the low contamination levels, the control estimations may have greatly underestimated the usefulness of mussel upper temperature tolerance as a biological indicator.

LC50 Determination for *Corbicula fluminea*

The data from mussel exposures to organophosphates prompted the evaluation of another bivalve. The upper temperature tolerance relationships observed during and after mussel organophosphate exposures led to the examination of the effects of diazinon on *C. fluminea* upper temperature tolerance. The toxicity of diazinon to these clams was not known and toxicity tests were performed to provide that information. These toxicity tests were utilized to establish a sublethal range of diazinon concentrations for exposures that follow. Variations of the 96-hour LC50 toxicity tests, performed on the mussels, were also performed for these clams. These tests were designed to provide information about the ability of bivalves to isolate themselves from exposure and the effects of extended exposures.

Multiple range finding tests were performed on *C. fluminea* to determine appropriate exposure concentrations for the LC50 determination. Data for the determination of the 96-hour diazinon LC50 for *C. fluminea* consisted of the exposure of 144 clams to six exposure solutions (five diazinon concentrations and a reconstituted hard water control; Table A-17). Exposures were performed in four replicates of six clams to each exposure concentration.

Mortality was defined as failure to close upon prodding.

Resources did not allow for the analysis of the numerous samples required for the diazinon toxicity evaluation of *C. fluminea*. In the interest of reserving the concentration analyses for subsequent experiments, concentrations of diazinon were determined using a simple linear regression of known concentrations (subsequent experiment results) by the microliters of product used to make those solutions from the following diazinon upper temperature tolerance test. The relationship of microliters product to diazinon concentration was highly significant (ANOVA; $F= 85.64$; $Pr>F= 0.0027$; $R^2= 0.97$) yielding the following diazinon concentration model: Diazinon concentration = (152.86) • (ul product) - 35.76. Checking the model against the data from which it was developed resulted in the concentrations shown in Table 24.

Table 24

Diazinon Concentration Model

Microliters diazinon product, determined diazinon concentration (ug/L) from Millipore Immunoassay test, calculated diazinon concentrations (ug/L) utilizing the regression model, and deviations from the determined concentration (ug/L) reported.

Microliters Product	Determined Concentration	Calculated Concentration	Deviation from Determined Concentration
5.00	771.5	728.51	-42.99
2.50	250.75	346.40	+95.65
1.25	177.45	155.32	-22.13
0.63	54.55	59.78	+5.23

Determination of concentration for the 96-hour LC50 required extrapolation beyond the limits of the data from which the model was made. Exposure concentrations were calculated to be 920 ug/L, 1,875 ug/L, 3,786 ug/L, 7,607 ug/L, and 15,251 ug/L plus a reconstituted hard water control containing no diazinon.

The 96-hour diazinon LC50 for *C. fluminea* was estimated to be 4,067 ug/L with a lower 95% confidence of 2,847 ug/L and an upper 95% confidence of 5,814 ug/L diazinon based on the calculated diazinon concentrations. The concentrations calculated for the diazinon 96-hour LC50 for *C. fluminea* were only an estimation of the concentrations that were actually used for exposure. The five diazinon concentrations

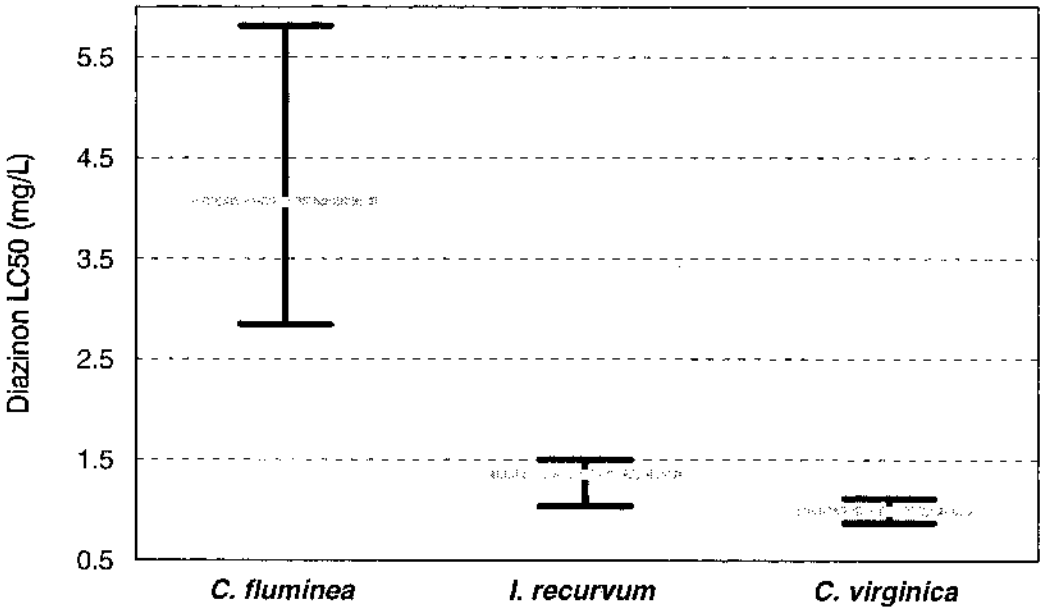
were all calculated to be well above the higher extreme of the data used to calculate the model. The calculated concentrations, however, did correspond well with the microliters of diazinon product used to make each respective concentration upon inspecting the reference data (data used to generate the model). For example, the concentration determined to correspond to five microliters of diazinon product was 771.5 ug/L diazinon and the concentration of diazinon from 6.3 microliters of product was calculated to be 919.64 ug/L. Further inspection of this relation showed that 100 microliters of product was calculated to create a 15,251 ug/L diazinon solution which is very close to multiplying the determined five microliter product concentration (771.5 ug/L) by twenty yielding 15,430 ug/L.

Regardless of the exact concentration, the LC50 estimation is likely to be within 200 ug/L of the actual concentration. The estimated 96-hour diazinon LC50 for *C. fluminea* was extremely high even when compared to other bivalve species such as the hooked mussel (preceding test) and the American oyster (AQUIRE; Figure 16). With some confidence the estimation could place the LC50 between three and six mg/L. The ability of mussels to isolate themselves from their immediate environment may, in part, explain the very high resistance and the non-monotonic response as the

concentrations increased by large amounts. This level of exposure is unlikely to be present in the environment even in the systems in which *C. fluminea* inhabit.

Figure 16

Comparison of diazinon LC50 values and 95% confidence limits for the bivalves *C. fluminea* and *I.* recurvum, and the reported range of LC50 estimations for *C. virginica.*



The ability of *C. fluminea* to incur such high levels of diazinon insult may have been partially explained by the ability to isolate themselves from the unsuitable environment through valve closure. The results of the toxicity test prompted an examination of the ability of *C.*

fluminea to protect themselves from adverse exposure. This was examined in a behavior regulation study in which clams were forced closed for 72-hours after the initial 24-hours of exposure to diazinon.

Behaviorally regulated exposures (clams manually closed with tygon tubing) demonstrated noteworthy changes in the tolerance of *C. fluminea* exposed to 15,251 ug/L diazinon. The clams were exposed for 24-hours to 15,251 ug/L diazinon. Prior to binding half the clams, four mortalities occurred for each group of twenty-four clams in the high diazinon concentration exposures. No mortality was observed for the total forty-eight clams in reconstituted hard water. Half of the clams in diazinon solutions and reconstituted hard water were bound at 24-hours for the remainder of the 96-hour exposure. Clams that were not bound in both exposure solutions were examined for mortality daily upon renewal of the exposure solution. Mortality was recorded daily.

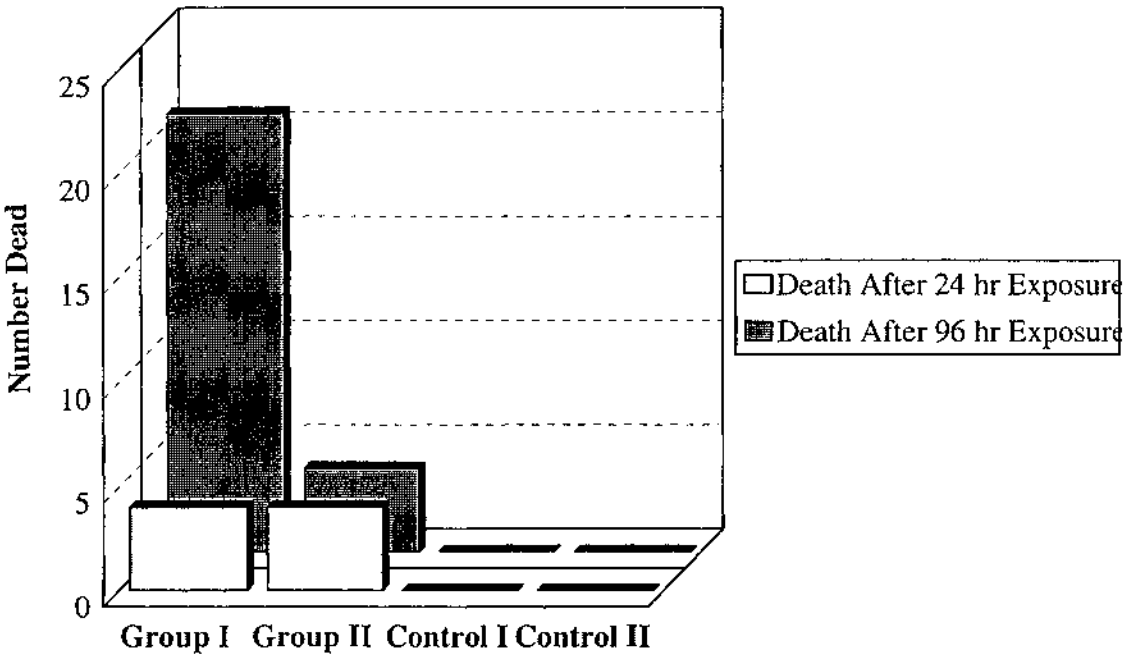
After a total of 96-hours of exposure, bound clams were unbound and were placed into reconstituted hard water for an additional 48-hours as were clams that were not bound. Clams were examined daily for mortality after they were placed in hard water. Unbound clams in diazinon exposures continued to suffer mortality throughout the diazinon exposure. Twenty-one unbound clams died during the 96-hour exposure to 15,251

ug/L diazinon. However, no deaths occurred in the bound clams in the 48-hour post exposure period. No mortality of bound or unbound control clams occurred in reconstituted hard water during the 96-hour exposure or in the 48-hour post exposure period.

When comparing manually closed clam survival to those which were able to open at will, less mortality occurred in the behaviorally regulated clams (Figure 17). In fact, no mortality occurred after the clams were closed manually even though they were exposed to high diazinon concentrations for 24-hours prior to forced closure. No mortality occurred in control exposures for bound or unbound clams.

Figure 17

Corbicula fluminea exposure to diazinon (15,251 ug/L) in a behaviorally regulated study.



Group I and Control I were left unbound for 96-hour.
Group II and Control II were bound after the initial 24-hour exposure.

Both bound exposures suggested that the clams could cope anaerobically for the 72-hour period during which they were manually closed. Isani et al. (1989) exposed the bivalve *Scapharca inaequivalvis* to sea water flushed with nitrogen to promote anaerobiosis for up to 96-hours without inducing mortality. They found that the anaerobic metabolic adaption is mainly active during the first hours of anoxia, but that the duration depends on the specie. The combined

results indicate that the clams were capable of isolating themselves from the unsuitable environment by means of voluntary valve closure. Some clams which were not bound were observed actively siphoning the exposure solution. This was assumed based on the occurrence of gaped valves with siphons protruding in an open and active manner. The high mortality which did occur in unbound clams in diazinon solutions may have been the result of periodic "re-sampling" of the environment over the exposure period.

The 21-day diazinon LC50 estimation for *C. fluminea* was based on the exposure of 126 clams to seven diazinon exposure solutions which were renewed daily. Three replicates of six clams were examined daily for mortality during feeding and renewal. Mortality was defined as failure to close upon prodding and was recorded daily as the dead clams were removed. Concentrations were determined using the same model as before for the 96-hour LC50 test. Trimmed Spearman-Karber analysis was used to analyze these data (Table A-18). The 21-day diazinon LC50 for *C. fluminea* was estimated to be to 548 ug/L with a 95% lower confidence of 455 ug/L and a 95% upper confidence of 658 ug/L diazinon based upon the calculated concentrations.

The concentrations calculated for the estimation of the diazinon 21-day LC50 for *C. fluminea* were again only

calculated estimations of the concentrations that were actually used for exposure (Table 24). The higher two of the six diazinon concentrations were calculated to be well above the higher extreme of the data used to calculate the model. These two calculated concentrations were both within 70 ug/L diazinon of the expected concentration based upon the technique discussed for the 96-hour LC50.

The LC50 estimation is again likely to be within 200 ug/L of the actual concentration. The estimation of diazinon LC50 for *C. fluminea* over this extended time period may represent the toxicity of diazinon to the clams better than that determined for the 96-hour exposure, because it likely precludes their ability to isolate themselves from their environment and operate anaerobically. The levels of diazinon which the bivalves could withstand were still extremely high in comparison to many aquatic species. For example, 96-hour diazinon LC50 values have been reported at 136-500 ul/L for *Lepomis macrochirus* and 100-1,000 ug/L for *Salvelinus fontinalis* and invertebrates such as *Pteronarcys californica* (96-hour LC50 = 25 ug/L) and *Hyalella azteca* (96-hour LC50= 6.5 ug/L) are often less tolerant of diazinon exposure (AQUIRE, 1997). However, this high level of exposure is not likely to be present in the environment in a manner that would persist for three weeks.

C. fluminea Upper Temperature Tolerance for Temperature Acclimations

The upper temperature tolerance of *I. recurvum* was tested for possible use as an indicator of relative water quality at one acclimation temperature. Direct comparisons with the baseline data was not possible because exposures were performed at a temperature not used in generating the baseline data. The temperature tolerance of *C. fluminea* was then examined. The following baseline data were compared to that of *I. recurvum* and were later used to evaluate the effects of three toxicants on the upper temperature tolerance of *C. fluminea*. Exposures were performed at each successful acclimation temperature. This made direct comparisons with the baseline (model) data possible and precluded the need for estimations generated from a model.

The length and heat coma temperature of each individual clam was recorded for 24 clams acclimated to each 10, 20, and 30°C (Table A-19). Clams acclimated to 1 and 40°C suffered numerous mortalities and were excluded from the study for lack of a specific explanation of the stress which caused mortality. Routine water chemistry measurements were taken both before and after the 24-hour control exposure at each temperature. The measurements included dissolved oxygen, pH, hardness, alkalinity and conductivity (Table A-

23).

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to three acclimation temperatures are shown in Table 25. The mean heat coma temperature (HCT) decreased between 20 and 30°C acclimations and acclimation to 10°C resulting in mean HCTs of 44.28, 44.38, and 28.98°C respectively. The HCT of *C. fluminea* was significantly related to acclimation temperature. The HCTs for different acclimation temperatures were significantly different. Further analysis showed that the 20 and 30°C acclimation's HCTs were not different, however, the 10°C acclimation HCTs were different from both of the higher temperature acclimations.

Table 25

Upper Temperature Tolerance of *Corbicula fluminea* Acclimated to 10, 20 and 30°C for 21-days
Acclimation Temperature (°C), Mean Heat Coma Temperature (HCT), Variance (Var), and Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test, SNK Multiple Range test, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analysis Reported.

Accl Temp (°C)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
30	44.28	0.811	0.8900	13.38 / 0.811= 16.50	A	A	HCT= (0.765)(°C Accl) + 23.19 R^2= 0.6828 F= 150.645 Pr>F=0.0001
20	44.38	0.815	0.0040		A	A	
10	28.98	13.38	0.0001	P(F≥16.5) >0.001	B	B	
Analysis of Variance					F = 376.64		Pr>F = 0.0001
Analysis of Variance on Ranked Data					F = 69.41		Pr>F = 0.0001
Kruskal-Wallis X^2 Approximation					X^2= 47.427		Pr>X^2 = 0.0001
Median 1-Way Analysis X^2 Approximation					X^2 = 35.829		Pr>X^2 = 0.0001

* Forward selection stepwise regression selected acclimation temperature first (F= 150.6542, Pr>F= 0.0001, partial R^2= 0.6828) and overall length was selected second (F= 13.9927, Pr>F=0.0004, partial R^2= 0.0535).

Temperature has a pronounced influence of *C. fluminea* as it does for all organisms. Foe and Knight (1987) investigated the use of *C. fluminea* to assess a point source thermal discharge of a power plant. They found that clams transplanted to a site where temperature varied between 25 and 35°C suffered almost complete mortality within 60 days. Clams exposed for six months to water temperatures elevated by 0.5 to 2.0°C (relative to two other sites) achieved less tissue and shell growth (Foe and Knight, 1987).

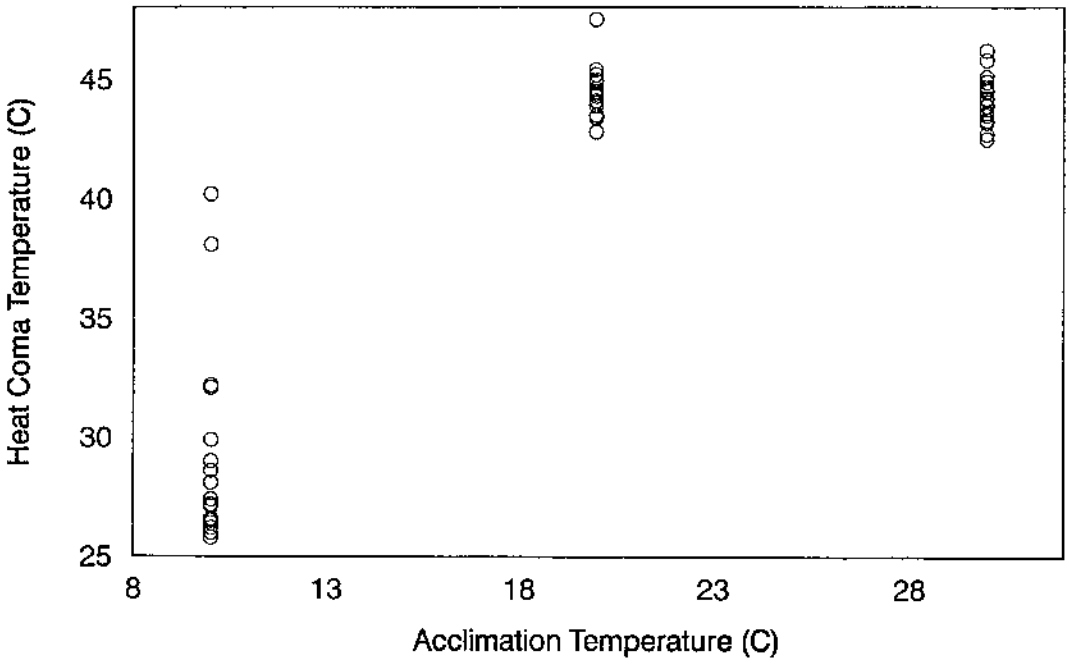
The trend evident from 72 clams sampled among three acclimation temperatures of 10, 20 and 30°C showed decreasing upper temperature tolerance as acclimation temperature decreased to 10°C. In addition, the mean HCT was somewhat inflated at 10°C by relatively few particularly resistant clams (Figure 18). This drove the variation about the HCT up markedly in comparison to the 20 and 30°C acclimation exposures. Clams acclimated to 30°C did not show an increase in upper temperature tolerance when compared to 20°C acclimations. The upper temperature tolerance achieved by *C. fluminea* seemed to reach a maximum during the acclimation to 20°C.

The acclimation temperature to HCT relationship suggested that expected HCTs could be estimated (Figure 18). Models based upon a regression of determined HCTs could be used to estimate HCTs of specimens in the field if exposure temperatures are known (as before for the hooked mussel). The regression performed on the data collected from the three acclimation temperatures calculated that 68% of the variation about the HCT was a result of acclimation temperature. The inclusion of more acclimation temperatures in generating the data for this relationship would likely result in a regression which accounts for more than 68% of the variation about the HCT with acclimation temperature.

Several additional acclimation temperatures from 10 to 20°C would be of most use to this particular regression in enhancing the coefficient of determination. In addition, the 30°C acclimation did not provide an increase in upper temperature tolerance relative to the 20°C acclimation. The model based on the linear fit to these data drastically underestimates HCTs for the temperature range from 10 to 20°C. The data from the 30°C acclimation detracted from a linear fit to the data and replacing the 30°C acclimation data with an acclimation to 15°C may provide for a better linear relationship. However, it is not likely that the HCT will be entirely explained by acclimation temperature because of the numerous other factors which play a role in the determination of HCT (e.g., genetics, health, dissolved oxygen, population density, contaminants).

Figure 18

Corbicula fluminea heat coma temperature (°C) in relation to acclimation temperature(°C).

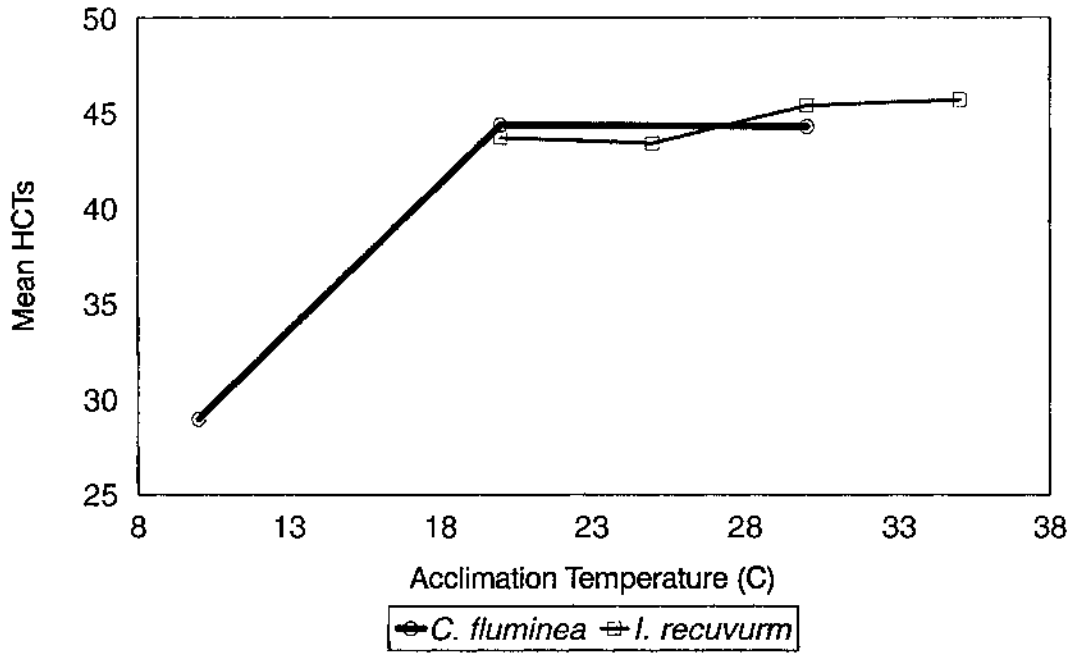


The trend in HCT relative to acclimation temperature was somewhat different between the two bivalves tested (Figure 19). HCTs for *I. recurvum* showed an increasing trend as acclimation temperature increased from 20 t 35°C. The HCTs for *C. fluminea* increased between acclimations to 10°C and 20°C. The 30°C acclimation of *C. fluminea* provided no increase in upper temperature tolerance relative to the 20°C acclimation. The model based on the *I. recurvum* data provided for better HCT estimations than the model based on

C. fluminea data because only two useful acclimation temperatures were available (10 and 20°C) to calculate the *C. fluminea* model. The 30°C acclimation for *C. fluminea* was not helpful in making estimations of HCT because it altered the linear fit to the data. The data for both bivalves would have would have been more complete if lower acclimation temperatures were added (11-19°C).

Figure 19

A comparison of the heat coma temperature (°C) in relation to acclimation temperature(°C) for the two bivalves *Corbicula fluminea* and *Ischadium recurvum*.



The slope of the HCT relation to acclimation temperature for *I. recurvum* was much less than what was observed for *C. fluminea* between 10 and 20°C. The upper temperature tolerance of the mussels increased relatively slowly (2°C) among acclimation to 20°, 25°, 30° and 35°C. The HCTs changed by a larger amount (15°C) for clams acclimated to 10° and 20°C. The difference may have been related to the normal temperature range typically experienced by each bivalve. *I. recurvum* is an intertidal mussel which may be exposed to the air temperature of summer highs or winter lows. C. *fluminea* is less likely to experience temperatures to aerial extremes because they are not sessile or typically exposed to tidal fluctuations. Although differences in natural exposures may influence HCTs, the comparison between the two bivalve species will not be complete without investigating the HCT of *I. recurvum* acclimated to lower temperatures (10-15°).

The data collected for establishing a baseline model for *C. fluminea* upper temperature tolerance was not complete enough to allow for HCTs estimations. The high acclimation temperature altered the slope of the model to a large extent. Upper temperature tolerance comparisons with following toxicant exposures were made with the control exposures because of the anticipated inaccuracies of the

predictions made with the model.

C. fluminea Temperature Tolerance for Organophosphate Exposure

After establishing baseline data on the upper temperature tolerance of clams at different acclimation temperatures, toxicant exposures were performed to test their effects on *C. fluminea* HCT. The influence of diazinon exposure on upper temperature tolerance was tested at each acclimation temperature used in establishing the baseline data. The resulting HCTs were compared to control exposures and the preceding *I. recurvum* diazinon exposures.

The length and heat coma temperature of each individual clam was recorded for 12 clams exposed to each of four diazinon concentrations and a control. Diazinon concentrations were measure to be 772, 251, 177, and 54 ug/L and a 0 ug/L control. Exposures were performed at 10, 20, and 30°C after acclimation to those temperatures over three weeks (Table A-20). Diazinon concentrations were determined based on a standard curve (R^2= 0.9385). Routine water chemistry measurements were made both before and after the 24-hour exposure to each concentration at each acclimation temperature. The measurements included dissolved oxygen, pH, hardness, alkalinity, and conductivity (Table A-23).

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to diazinon at 10°C are shown in Table 26. The mean heat coma temperature (HCT) decreased between 20 and 30°C and 10°C acclimations as in the temperature acclimation trials. Mean HCTs by ascending exposure concentration at 10°C were 29.0, 39.4, 34.9, 34.7, and 34.9°C. The HCT of *C. fluminea* was not significantly (0.05) related to diazinon concentration. The HCTs for the different diazinon exposures were significantly different. This was the result of the much lower HCTs of the control exposed clams which were significantly different than all other exposures.

Table 26

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Diazinon Exposure at 10°C

Diazinon Concentration ([Diaz] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test, SNK Multiple Range test, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Diaz] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
0	28.98	13.38	0.0001	39.08 / 13.38 = 2.92 0.10 > P(F≥2.92) >0.05	B	B	ANOVA F= 2.746 Pr>F=0.102
54.55	39.36	27.03	0.0013		A	A	
177.45	34.93	28.44	0.0632		A	A	
250.75	34.73	26.02	0.2212		A	A	
771.5	34.93	39.08	0.1677		A	A	

Analysis of Variance	F = 9.68	Pr>F = 0.0001
Analysis of Variance on Ranked Data	F = 11.79	Pr>F = 0.0001
Kruskal-Wallis X^2 Approximation	X^2= 29.014	Pr>X^2 = 0.0001
Median 1-Way Analysis X^2 Approximation	X^2 = 13.142	Pr>X^2 = 0.0106

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to diazinon at 20°C are shown in Table 27. Mean HCTs by ascending exposure concentration at 20°C were 44.4, 44.5, 43.9, 45.0, and 44.3°C. The HCT of *C. fluminea* was again not significantly (0.05) related to diazinon concentration. The HCTs for the different diazinon exposures were significantly different. Tukey and Student Newman-Keuls multiple range test showed HCTs of clams exposed to 177.5 ug/L diazinon to be significantly different than all other exposures.

Table 27

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Diazinon Exposure at 20°C

Diazinon Concentration ([Diaz] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range, SNK Multiple Range test, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Diaz] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
0	44.38	0.815	0.0040	0.997 / 0.397 = 2.51 0.20 > P(F≥2.51) >0.10	AB	AB	ANOVA F= 0.051 Pr>F=0.82
54.55	44.53	0.740	0.6474		AB	AB	
177.45	43.85	0.705	0.1508		B	B	
250.75	44.99	0.397	0.0005		A	A	
771.5	44.28	0.997	0.1503		AB	AB	
Analysis of Variance				F = 2.63		Pr>F = 0.0419	
Analysis of Variance on Ranked Data				F = 2.85		Pr>F = 0.0305	
Kruskal-Wallis X^2 Approximation				X^2= 10.313		Pr>X^2 = 0.0355	
Median 1-Way Analysis X^2 Approximation				X^2 = 9.0426		Pr>X^2 = 0.0600	

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to diazinon at 30°C are shown in Table 28. Mean HCTs by ascending exposure concentration at 30°C were 44.3, 45.0, 44.9, 44.7, and 45.0°C. The HCT of *C. fluminea* was, as in the lower acclimation temperatures, not significantly related to diazinon concentration (at the 0.05 α level). The HCTs for the different diazinon exposures were also not significantly different.

Table 28

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Diazinon Exposure at 30°C

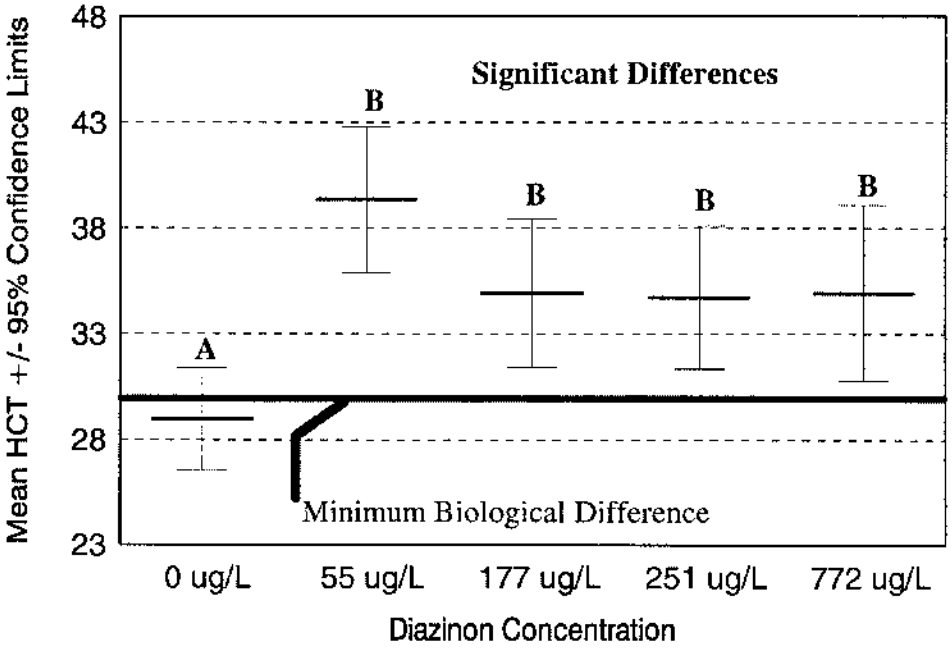
Diazinon Concentration ([Diaz] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range, SNK Multiple Range test, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Diaz] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
0	44.28	0.811	0.8900	4.534 / 0.811 = 6.82 0.005 > P(F≥6.82) >0.002	A	A	ANOVA F=1.076 Pr>F=0.30
54.55	44.98	3.182	0.1409		A	A	
177.45	44.85	0.971	0.3004		A	A	
250.75	44.66	3.912	0.8398		A	A	
771.5	45.01	4.534	0.5894		A	A	
Analysis of Variance				F = 0.70		Pr>F = 0.5973	
Analysis of Variance on Ranked Data				F = 0.90		Pr>F = 0.4692	
Kruskal-Wallis X^2 Approximation				X^2= 3.6201		Pr>X^2 = 0.4599	
Median 1-Way Analysis X^2 Approximation				X^2 = 4.5738		Pr>X^2 = 0.3339	

Exposures of *C. fluminea* to diazinon influenced their upper temperature tolerance, however, this occurred only for 10°C acclimations (Figure 20). An increase in the concentration of diazinon of 54.6 ug/L played a part in increasing the mean HCT by 10.4°C (96-hour LC50 ≅4,000 ug/L). Exposure to concentrations of 177.4, 250.75 and 771.5 ug/L diazinon also increased the upper temperature tolerance of the exposed clams, but by a smaller amount of approximately 6°C in all three exposures.

Figure 20

Deviations from the acclimation temperature control HCT of *Corbicula fluminea* at 10°C accounted for by 24-hour diazinon exposures (0-771.5 ug/L).



The mechanism by which diazinon increased the upper temperature tolerance of clams acclimated to 10°C is unknown, however, the deviation in mean HCT was greater than 1°C defined as being biologically useful as an indicator of water quality. Interactions within the clams may have been involved as the acclimation temperature was closer to the critical acclimation minimum temperature. This may be evident from the attempted acclimation to 1°C during which high levels of mortality occurred. Some organisms posses

physiological processes to respond to cold temperatures (such as slowing metabolism to become inactive or glycoprotein production; Schmidt-Neilsen, 1995) which may account for this reaction to organophosphate exposure. Fluctuations in metabolism in low temperature acclimation or the introduction of biologically active constituents in response to cold may affect the influence of diazinon on clam HCT.

Exposures to diazinon of *C. fluminea* acclimated to 20 and 30°C did not influence upper temperature tolerance by any appreciable amount (Figures 21 and 22), in contrast to the 10°C acclimation. The upper temperature tolerance in terms of HCT of diazinon exposed clams did not vary in useful amounts from the control exposure at 20 or 30°C. Exposure induced deviation from the mean control HCT was not greater than 0.6°C for clams acclimated to 20°C or 0.7°C for clams acclimated to 30°C. In fact, all diazinon exposures and the two controls (20 and 30°C) resulted in mean heat coma temperatures within a range of 1.16°C. This may suggests that diazinon was not toxic in terms of HCT and that the capacity of *C. fluminea* to acclimate to higher temperatures approached a maximum between 20 and 30°C (as discussed for the acclimation temperature data). This may be further supported by the failed acclimation to 40°C,

indicating that upper temperature tolerance was reached for chronic exposure between 30° and 40°C (as found by Foe and Knight, 1987). The diazinon exposures showed that no effects on upper temperature tolerance were experienced by the clams at either 20 or 30°C.

Figure 21

Deviations from the acclimation temperature control HCT of *Corbicula fluminea* at 20°C accounted for by 24-hour diazinon exposures (0-771.5 ug/L).

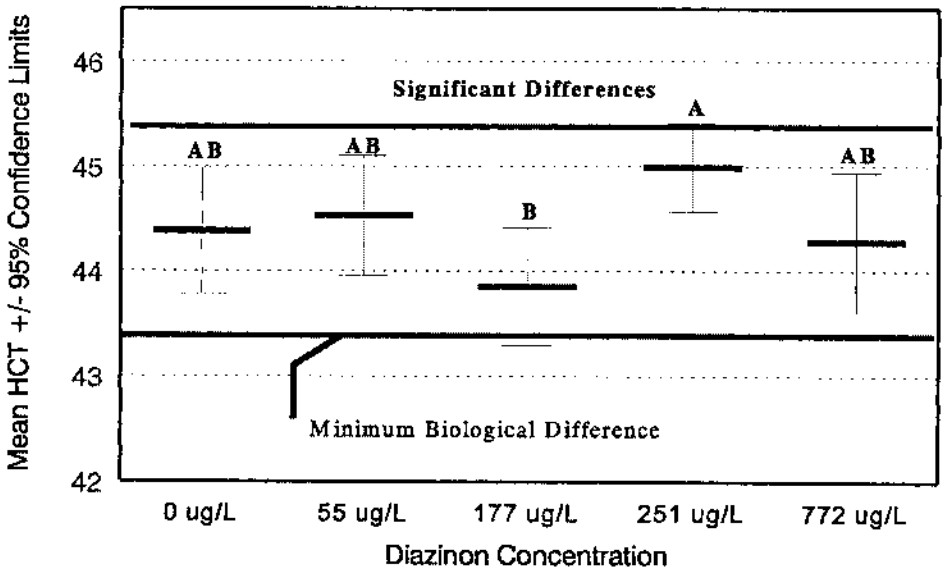
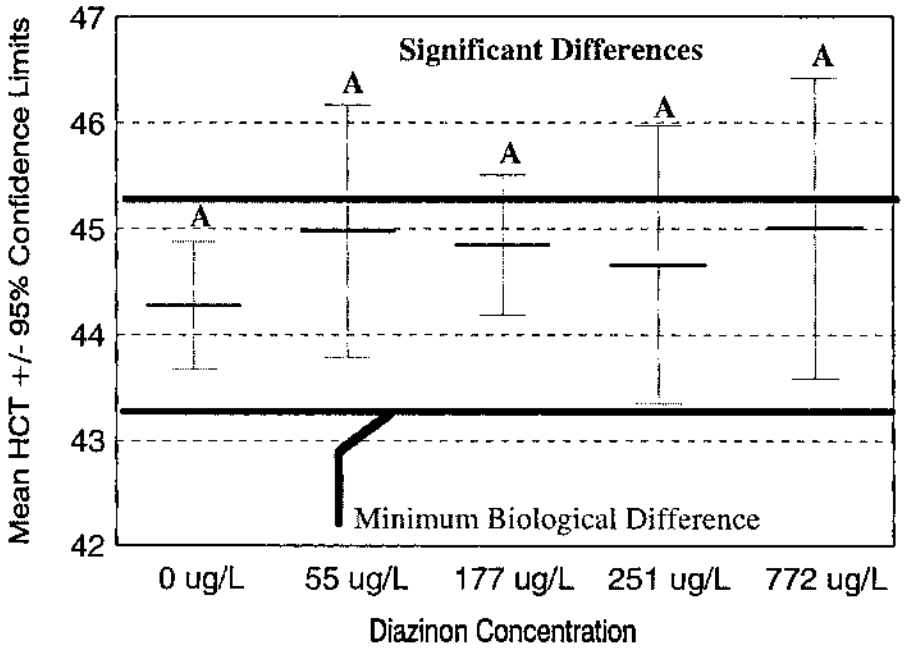


Figure 22

Deviations from the acclimation temperature control HCT of *Corbicula fluminea* at 30°C accounted for by 24-hour diazinon exposures (0-771.5 ug/L).



The significant differences in mean HCT found in statistical analysis of the 20°C exposures were not thought to be large enough to be useful as biological indicators. The difference between the significantly different groups was only 0.53°C between a mid-range concentration (177.45 ug/L diazinon) and the control exposure (0 ug/L diazinon). The differences in *C. fluminea* HCTs are believed to be too small to be of use as an indicator of organophosphate exposure (>1°C).

Exposure of *I. recurvum* to diazinon at 27°C showed useful HCT deviations from control HCTs when exposure concentrations reached ~500 ug/L diazinon. This concentration was below the 96-hour diazinon LC01 estimated for the hooked mussel of ~700 ug/L diazinon. Exposure of *C. fluminea* to diazinon (0-772 ug/L) at 20° and 30°C produced no useful deviations in HCT from that of the control. The concentrations were not expected to effect *C. fluminea* HCT, in retrospect, because of observations made for *I. recurvum*. Exposure concentrations which caused useful deviations in *I. recurvum* HCT were approximately 37% of the 96-hour LC50 estimate of 1,354 ug/L. The highest exposure concentration for *C. fluminea* was 772 ug/L diazinon which is approximately 19% of the 96-hour LC50 estimate of 4,067 ug/L. Exposure concentrations for *I. recurvum* which were 19% (257 ug/L) of the 96-hour LC50 did not induce useful changes in HCT at 27°C.

The differences between the exposure of the two bivalves were the duration and magnitude of exposure and exposure temperature. *I. recurvum* was exposed to diazinon for 72-hours and *C. fluminea* was exposed for 24-hours. The magnitude of exposure was much higher for *I. recurvum* (in higher concentrations) than *C. fluminea*. *I. recurvum* was only exposed at 27°C, but *C. fluminea* was exposed at 10°,

20° and 30°C. The ability to isolate themselves via valve closure may have differed between the two bivalves and influenced the frequency with which they interacted with diazinon. Any of these differences in the conditions of exposure or the ability to isolate may have influenced the differences in the effects of diazinon on the HCT of each bivalve. However, specie specific differences in diazinon toxicity between these bivalves was also likely to have been involved.

C. fluminea Temperature Tolerance for Heavy Metal Exposure

After the influence of diazinon exposure on upper temperature tolerance was tested, copper exposures were performed to test clam HCT sensitivity to heavy metals. Comparison were made with the baseline data on the upper temperature tolerance of clams at different acclimation temperatures.

The length and heat coma temperature of each individual clam was recorded for 12 clams exposed to each of six copper concentrations and a control. Concentrations were determined to be 1,311, 710, 350, 205, 179, and 110 ug/L copper and a 0 ug/L copper control. Exposures were repeated at 10, 20, and 30°C after acclimation to those temperatures over three weeks (Table A-21). Routine water chemistry measurements

were made both before and after the 24-hour exposure to each concentration and temperature. These measurements included dissolved oxygen, pH, hardness, alkalinity, and conductivity (Table A-23).

The results of the analysis of the upper temperature tolerance data for *C. fluminea* exposed to copper at 10°C are shown in Table 29. Mean HCT again decreased between 20 and 30°C and 10°C acclimations as in the temperature acclimation trials. Mean HCTs by ascending exposure concentration at 10°C were 28.98, 27.41, 26.72, 30.19, 29.95, 27.07 and 28.38°C. The HCTs for the different copper exposures were significantly different. The mean rank control HCT was significantly different than HCTs resulting from exposures to 710, 179, and 111 ug/L copper, and HCTs from exposure to 1,311, 351, and 205 ug/L copper were not different from other copper exposure concentrations.

Table 29

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Copper Exposure at 10°C

Copper Concentration ([Cu] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test for Ranked Data, SNK Multiple Range test for Ranked Data, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Cu] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT Rank	SNK MRT Rank	Model
0	28.98	13.38	0.0001	52.06 / 13.38 = 3.89 0.05 > P(F≥3.89) >0.02	A	A	ANOVA F= 0.130 Pr>F= 0.7193
111.0	27.41	29.49	0.0001		B	B	
179.2	26.72	23.36	0.0001		B	B	
204.8	30.19	52.06	0.0016		AB	AB	
350.5	29.95	33.65	0.0018		AB	AB	
710.0	27.07	29.16	0.0001		B	B	
1310.9	28.38	40.06	0.0001		AB	AB	
Analysis of Variance on Ranked Data				F = 4.63		Pr>F = 0.0004	
Kruskal-Wallis X^2 Approximation				X^2= 22.572		Pr>X^2 = 0.0010	
Median 1-Way Analysis X^2 Approximation				X^2 = 24.800		Pr>X^2 = 0.0004	

The results of the analysis of the upper temperature tolerance data for *C. fluminea* exposed to copper at 20°C are shown in Table 30. Mean HCTs by ascending exposure concentration at 20°C were 44.38, 42.98, 42.63, 43.77, 43.97, 43.29, and 44.02°C. The HCTs from the different copper exposures were significantly different. Tukey and Student Newman-Keuls multiple range test showed HCTs of clams under control conditions to be significantly different from the HCTs resulting from exposure to 179.2 and 111.0

ug/L copper.

Table 30

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Copper Exposure at 20°C

Copper Concentration ([Cu] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test, SNK Multiple Range test, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Cu] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
0	44.38	0.815	0.0040	1.726 / 0.664 = 2.60 0.20 > P(F≥2.60) >0.10	A	A	ANOVA F= 0.007 Pr>F= 0.9337
111.0	42.98	1.541	0.1570		BC	BC	
179.2	42.63	0.882	0.1176		C	C	
204.8	43.77	2.202	0.0342		AB	ABC	
350.5	43.97	0.668	0.0278		AB	AB	
710.0	43.29	1.726	0.1083		ABC	ABC	
1310.9	44.02	0.664	0.5341		AB	AB	
Analysis of Variance				F = 4.92		Pr>F = 0.0002	
Analysis of Variance on Ranked Data				F = 4.91		Pr>F = 0.0002	
Kruskal-Wallis X^2 Approximation				X^2= 23.53		Pr>X^2 = 0.0006	
Median 1-Way Analysis X^2 Approximation				X^2 = 17.78		Pr>X^2 = 0.0068	

The results of the analysis of the upper temperature tolerance data for *C. fluminea* exposed to copper at 30°C are shown in Table 31. Mean HCTs by ascending exposure concentration at 30°C were 44.28, 43.71, 42.61, 41.8, 41.35, 40.98, and 41.77°C. The HCT of *C. fluminea* was significantly

related to copper concentration. The HCTs from the different copper exposures were significantly different. Tukey and Student Newman Keuls's multiple range test showed HCTs of clams under control conditions were significantly different from the HCTs resulting from exposure to all concentrations but the lowest exposure of 111.0 ug/L copper.

Table 31

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Copper Exposure at 30°C

Copper Concentration ([Cu] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test, SNK Multiple Range test for Non-Ranked and Ranked Data, Model provided by Linear Regression, Analysis of Variance, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Cu] (ug/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT	SNK MRT	Model
0	44.28	0.811	0.8900	2.834 / 0.811 = 3.49 0.02 > P(F≥3.49) >0.01	A	A	HCT= (-0.00214) (°C Accl) + 43.306 R2= 0.2207 F= 26.048 Pr>F=0.0001
111.0	43.71	1.401	0.2155		AB	A	
179.2	42.61	0.863	0.3494		BC	B	
204.8	41.8	2.195	0.1287		CD	BC	
350.5	41.35	2.457	0.0318		CD	BC	
710.0	40.98	1.440	0.0767		D	C	
1310.9	41.77	2.834	0.2549		CD	BC	
Analysis of Variance				F = 15.55			Pr>F = 0.0001
Analysis of Variance on Ranked Data				F = 17.06			Pr>F = 0.0001
Kruskal-Wallis X^2 Approximation				X^2= 50.277			Pr>X^2 = 0.0001
Median 1-Way Analysis X^2 Approximation				X^2 = 47.514			Pr>X^2 = 0.0001

The influence of copper on some bivalve species has been examined. Belanger et al. (1990) found that adult and juvenile *C. fluminea* achieved less tissue and shell growth when exposed
to copper concentrations ranging from 8.4-26.7 ug/L (temperatures ranging 23.9-24.7°C) in artificial streams. They also found that during these exposures clam tissue copper concentrations increased to levels 2.5-6 times higher than clams not exposed to copper. And clam growth was reduced for *C. fluminea* exposed to 22.5-104.8 ug/L copper (average temperatures ranging 19.6-22.0°C) in studies conducted in the Clinch River in Virginia where the hardness was approximately twice as much as in the artificial streams (Belanger et al., 1990). The researchers proposed that growth patterns of *C. fluminea* are a "clear and interpretable" indicator of copper contamination and are an alternative to contemporary chronic testing protocols.

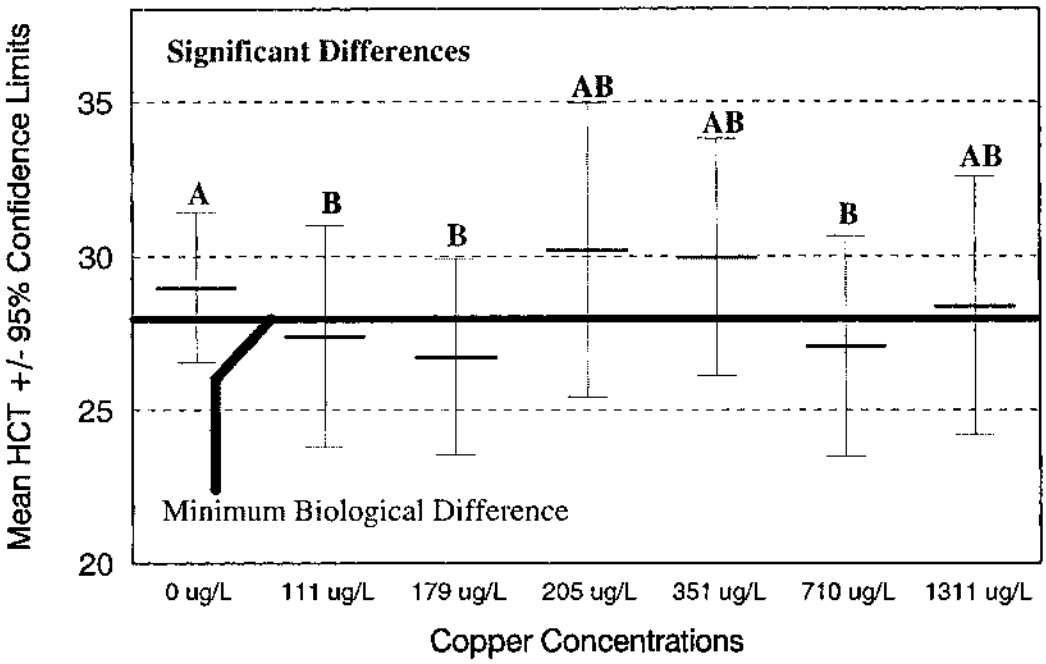
Akberali and Black (1980) observed behavioral responses and mortality of the estuarine bivalve *Scrobicularia plana* exposed to copper concentrations ranging from 10 to 500 ug/L at 10°C. They reported that *S. plana* can detect copper at concentrations as low as 10 ug/L and respond by closing their valves. In copper concentrations of 10, 50, and 100 ug/L *S. plana* opened their valves and interacted with the

exposure solutions 2-3 hours after the initial closure response. In exposure to higher concentrations (500 ug/L copper) *S. plana* remained closed for the duration of the 6-hour exposure at 10°C. Mortality of *S. plana* was reported to reach 50% in 5-7 days when exposed to 500 ug/L copper (Akberali and Black, 1980).

Exposures of *C. fluminea* to copper influenced their upper temperature tolerance at 10, 20 and 30°C acclimations. In contrast to the diazinon exposures, upper temperature tolerance decreased during the 24-hour exposures at 10°C (Figure 23). The decrease in mean HCT was notable in the lower copper concentration exposures. Exposure of clams at 10°C to 179 and 111 ug/L copper showed an impact on upper temperature tolerance. This was evident by a reduction in mean HCT by 2.3 and 1.6°C, respectively, from the control exposure which met the *a priori* assumption for use as a biological indicator (>1°C). However, the differences were not clearly useful until similar relationships were seen in the subsequent 20°C exposures, because of the large amount of variation associated with exposures at 10°C. Exposure of clams to 710 ug/L copper also decreased HCT by 1.9°C meeting the level defined as useful for bio-indication.

Figure 23

Deviations from the acclimation temperature control HCT of *Corbicula fluminea* at 10°C accounted for by 24-hour copper exposures (0-1,311 ug/L).

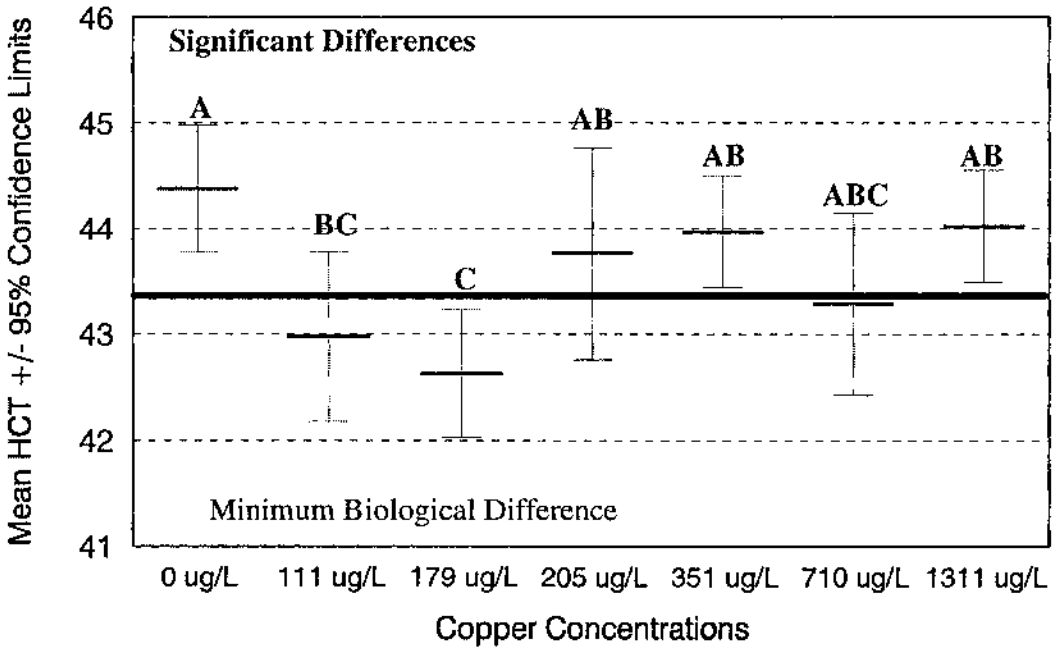


This relation was again present for exposures to copper concentrations at 20°C acclimations (Figure 24). Unlike the diazinon exposures, *C. fluminea* suffered a loss of upper temperature tolerance at 20°C in the presence of copper. Again the decreases in mean HCT which met the defined biologically useful criteria occurred in the lower two copper concentrations of 179.2 and 111.0 ug/L. These exposures lowered the mean HCT of *C. fluminea* by 1.8 and

1.4°C respectively, relative to the control. In addition, the mean HCT of *C. fluminea* showed a decreasing trend as copper exposure concentrations decreased. The exception to this trend was again in the exposure of clams to 710 ug/L copper which decreased HCT by 1.1°C just meeting the useful level for bio-indication.

Figure 24

Deviations from the acclimation temperature control HCT of *Corbicula fluminea* at 20°C accounted for by 24-hour copper exposures (0-1,311 ug/L).



Copper exposures performed at 10° and 20°C showed lower concentrations (111 and 179 ug/L) to induce deviations from control HCTs in amounts that would be useful as biological indicators of relative water quality. However, the variability about the HCT endpoint at 10°C make the deviations less clear than at 20°C. These concentrations are much lower than the reported 24-hour copper LC50 for *Ceriodaphnia dubia* of 649 ug/L. The 48-hour copper LC50 values from contemporary acute testing methods using *C. dubia* range from 17 to 406 ug/L (AQUIRE, 1997). The data from the 20°C exposures (Figure 24) may suggest that relative HCT may be useful as an indicator at concentrations below 111 ug/L copper, which would be even more comparable to common acute *C. dubia* values (30-70 ug/L copper).

The failure of some higher exposure concentrations to induce a response may be the result of selective isolation through valve closure. The detection of a potential toxicant by a bivalve often changes the duration and frequency of exposure (as discussed for the LC50 determinations). This may be supported by the observations made by Akberali and Black (1980) pertaining to the difference in duration of isolation for bivalves exposed to different copper concentrations (as described above). If clams fail to detect a toxicant or are unable to selectively isolate themselves

relative to higher concentration (e.g. *I. recurvum* organophosphate HCT tests), the response would be expected to be monotonic. Tests with other invertebrates, which lack a mechanism for self isolation, have demonstrated this monotonic relationship (e.g. insect nymphs exposed to hexavalent chromium, Poulton et al. 1989; as discussed for *I. recurvum* chlorpyrifos exposures).

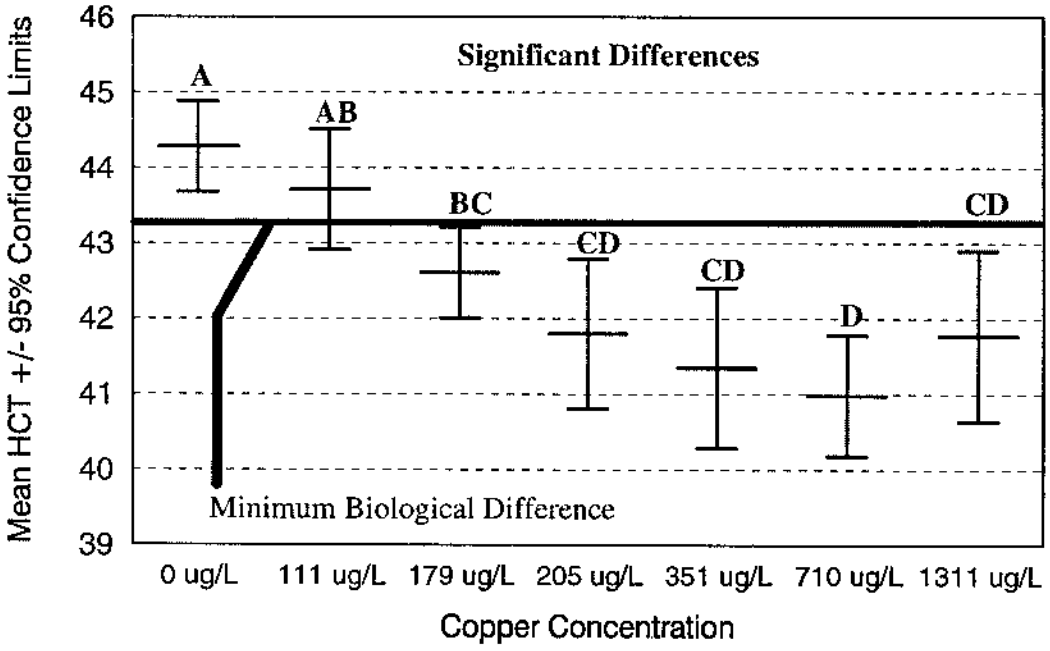
Upper temperature tolerance of *C. fluminea* also suffered decreases during copper exposures at 30°C (Figure 25). Unlike exposures at 10 and 20°C, the greatest loss of upper temperature tolerance occurred in higher concentration exposures of 1,311, 710, 351, and 205 ug/L copper. The deviations of mean HCT from the mean HCT of the control were somewhat larger than previously at 2.5, 3.3, 3.0 and 2.5°C respectively. The mean HCT resulting from exposures to 179 and 111 ug/L copper deviated 1.7 and 0.6°C respectively from the mean HCT of the control.

Exposure to all copper concentrations influenced deviations from the control HCT in excess of the 1°C defined biologically useful minimum with the exception of the lowest exposure concentration (111 ug/L copper). This was contrary to the trend evident in the 10°C and 20°C acclimation exposures of increasingly lower HCT as copper concentrations decreased. However, exposure to 710 ug/L copper again

produced larger deviations from the control relative to copper concentrations both immediately higher and lower than 710 ug/L (1,311 and 351 ug/L copper).

Figure 25

Deviations from the acclimation temperature control HCT of *Corbicula fluminea* at 30°C accounted for by 24-hour copper exposures (0-1,311 ug/L).



The increased toxicity of higher concentrations of copper at 30°C may be the result of the consistently lower dissolved oxygen present in the exposure solutions at the

higher temperature. Higher temperatures become saturated at lower concentrations of dissolved oxygen. The situation is most notable at the 24-hour measurement after the exposure (Table A-23). Lower dissolved oxygen may have decreased the duration which the clams could isolate themselves from their exposure environment via valve closure. Temperature has a direct relationship to metabolism in poikilothermic organisms. Increasing the temperature to 30°C would double the metabolic rate and respiratory and filtration demands relative to the 20°C exposure (as discussed for *I. recurvum* acclimation temperatures). Increasing the temperature to 30°C likely increased the interaction of the clams with exposures from which they would otherwise isolate themselves.

C. fluminea Temperature Tolerance for Chlorine Exposure

After the influence of diazinon and copper on upper temperature tolerance was tested, chlorine exposures were performed to test clam HCT sensitivity to halogens. Comparison were made with the baseline data on the upper temperature tolerance of clams at different acclimation temperatures.

The length and heat coma temperature of each individual clam was recorded for 12 clams exposed to each of six

chlorine concentrations. Chlorine concentrations were measured to be 5.7, 3.1, 1.4, 0.6, 0.2, 0.1 mg/L chlorine and a 0 mg/L control. Exposure were performed at 10, 20, and 30°C after acclimation to those temperatures over three weeks (Table A-22). Routine water chemistry measurements were done both before and after the 24-hour exposure to each concentration at each acclimation temperature. The measurements included dissolved oxygen, pH, hardness, alkalinity, and conductivity (Table A-23).

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to chlorine at 10°C are shown in Table 32. Mean HCT again increased between 10°C acclimations and acclimations to 20 and 30°C as in the temperature acclimation trials. Mean HCTs by ascending exposure concentration at 10°C were 28.98, 28.84, 27.53, 28.34, 27.33, 25.5 and 29.74°C. The HCTs for the different chlorine exposures were significantly different. The mean rank control HCTs were significantly different from exposures to 3.1 mg/L chlorine, and HCTs from exposure to all other chlorine exposures were not different from the 3.1 mg/L exposure at 10°C.

Table 32

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Chlorine Exposure at 10°C

Chlorine Concentration ([Cl] in mg/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test for Ranked Data, SNK Multiple Range test for Ranked Data, Model provided by Linear Regression, Analysis of Variance on ranked data, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Cl] (mg/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT Rank	SNK MRT Rank	Model
0	28.98	13.38	0.0001	44.38 / 0.3418 =129.84 P(F≥129.8) >0.001	A	A	ANOVA F= 0.0022 Pr>F=0.96
0.09	28.84	44.38	0.0001		AB	AB	
0.19	27.53	14.79	0.0002		AB	AB	
0.60	28.38	32.78	0.0001		AB	AB	
1.41	27.33	15.34	0.0001		AB	AB	
3.1	25.5	0.3418	0.3778		B	B	
5.7	29.74	43.35	0.0038		AB	AB	
Analysis of Variance on Ranked Data				F = 3.70		Pr>F = 0.0025	
Kruskal-Wallis X^2 Approximation				X^2= 18.967		Pr>X^2 = 0.0042	
Median 1-Way Analysis X^2 Approximation				X^2 = 24.245		Pr>X^2 = 0.0005	

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to chlorine at 20°C are shown in Table 33. Mean HCTs by ascending exposure concentration at 20°C were 44.38, 43.93, 44.50, 44.54, 44.53, 44.34, and 44.56°C. The HCTs for the different chlorine exposures were not significantly different.

Table 33

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Chlorine Exposure at 20°C

Chlorine Concentration ([Cl] in mg/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Tukey Multiple Range test for Ranked Data, SNK Multiple Range test for Ranked Data, Model provided by Linear Regression, Analysis of Variance on ranked data, Kruskal-Wallis X^2 Approximation, and Median 1-Way X^2 Approximation Analyses Reported.

[Cl] (mg/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Tukey MRT Rank	SNK MRT Rank	Model
0	44.38	0.815	0.0040	1.441 / 0.552 =2.61 0.20 > P(F≥2.61) >0.10	A	A	ANOVA F=0.545 Pr>F=0.46
0.09	43.93	0.742	0.3746		A	A	
0.19	44.5	1.054	0.2382		A	A	
0.60	44.54	0.552	0.5162		A	A	
1.41	44.53	0.588	0.0390		A	A	
3.1	44.34	0.684	0.8651		A	A	
5.7	44.56	1.441	0.0106		A	A	
Analysis of Variance on Ranked Data				F = 1.23		Pr>F = 0.2975	
Kruskal-Wallis X^2 Approximation				X^2= 7.2867		Pr>X^2 = 0.2951	
Median 1-Way Analysis X^2 Approximation				X^2 = 6.8730		Pr>X^2 = 0.3327	

The results of the analysis of the upper temperature tolerance data from the exposure of *C. fluminea* to chlorine at 30°C are shown in Table 34. Mean HCTs by ascending exposure concentration at 30°C were 44.28, 44.48, 44.66, 44.31, 44.24, 44.48, and 44.33°C. The HCT of *C. fluminea* was, as in the both lower acclimation temperatures, not significantly (0.05) related to chlorine concentration. The

HCTs for the different chlorine exposures were also not significantly different.

Table 34

Upper Temperature Tolerance of *Corbicula fluminea* for 24-Hour Chlorine Exposure at 30°C
Copper Concentration ([Cu] in ug/L), Mean Heat Coma Temperature (HCT), Variance (Var), Shapiro-Wilks Normality test, Variance Ratio test, Analysis of Variance, and Model provided by Linear Regression.

[Cl] (mg/L)	HCT	Var	Shapiro-Wilks Pr<W (α=0.05)	Var Ratio Pr>F (α=0.05)	Model
0	44.28	0.811	0.8900	2.438 / 0.811 = 3.01 0.10 > P(F≥3.01) >0.05	ANOVA F=0.013 Pr>F=0.9092
0.09	44.48	1.222	0.4368		
0.19	44.66	0.875	0.5013		
0.60	44.31	1.730	0.4063		
1.41	44.24	2.348	0.6087		
3.1	44.48	1.324	0.5937		
5.7	44.33	1.049	0.6020		
Analysis of Variance				F = 0.23	Pr>F = 0.9673

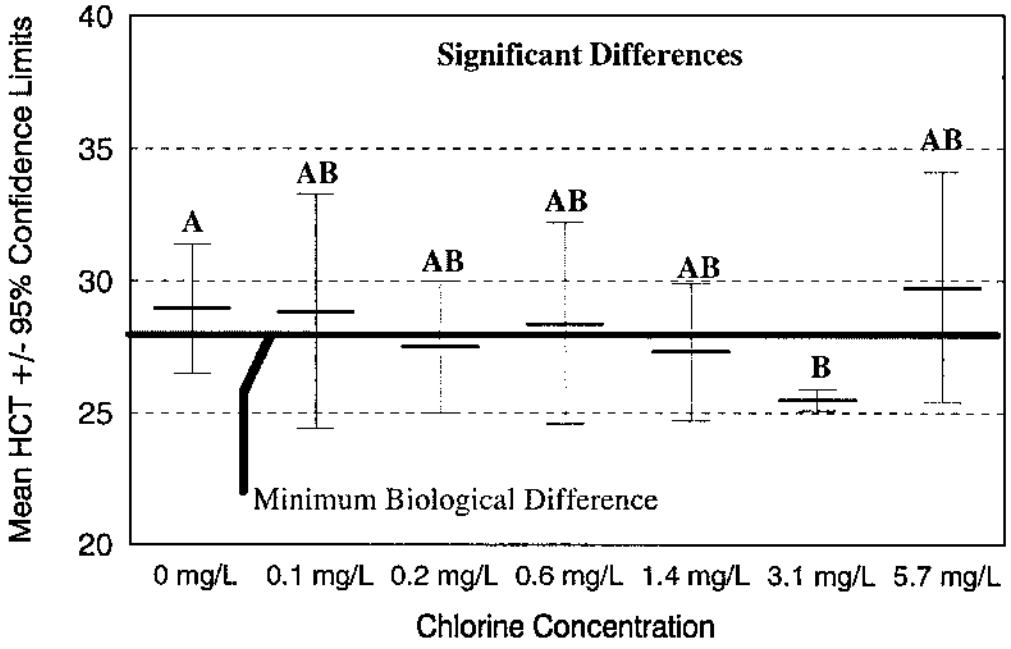
Exposures of *C. fluminea* to chlorine did not influence upper temperature tolerance at 10, 20 or 30°C acclimations (Figures 26, 27 and 28). The significant statistical differences resulting from chlorine exposures of 3.1 mg/L at 10°C were deemed not to be a useful indicator of relative water quality despite being in excess (3.5°C deviation from

the control) of the 1°C defined criteria. The HCTs recorded during the 10°C exposures at this one concentration did not possess the high variance associated with all other exposures at this temperature. The low variability in HCT drove the mean down considerably. Similarly, exposure to 0.2 and 1.4 mg/L chlorine showed mean HCTs which were 1.5° and 1.7°C less than the control. The variability in the HCTs was again much lower than that of other chlorine exposures.

The deviations from mean control HCT were not considered to be useful for bio-indication. They appeared to be the result of differences in the amount of variation in the endpoint for chlorine exposures at 10°C. The HCTs resulting from chlorine exposures seemed not to be influenced by chlorine concentration. And no evident relation between chlorine concentration and HCT was established. Chlorine exposures with higher variability about the measured HCTs showed mean HCTs very close to that of the control.

Figure 26

Deviations from the acclimation temperature trend of *Corbicula* accounted for by 24-hour chlorine exposures (0-5.7 mg/L) at 10°C.



High variation about the HCT endpoint was observed for the majority of the *C. fluminea* exposures at 10°C. The effect of the relative variation in HCT for chlorine exposed clams may have become more evident after observations made at 20° and 30°C. The variability about the endpoint for exposures at these two temperatures was similar among all exposures. The resulting HCTs were very similar to the control HCTs. This may support the assumption that the differences observed in mean HCT among chlorine exposures at

10°C were caused by the differences in variance.

Figure 27

Deviations from the acclimation temperature trend of *Corbicula* accounted for by 24-hour chlorine exposures (0-5.7 mg/L) at 20°C.

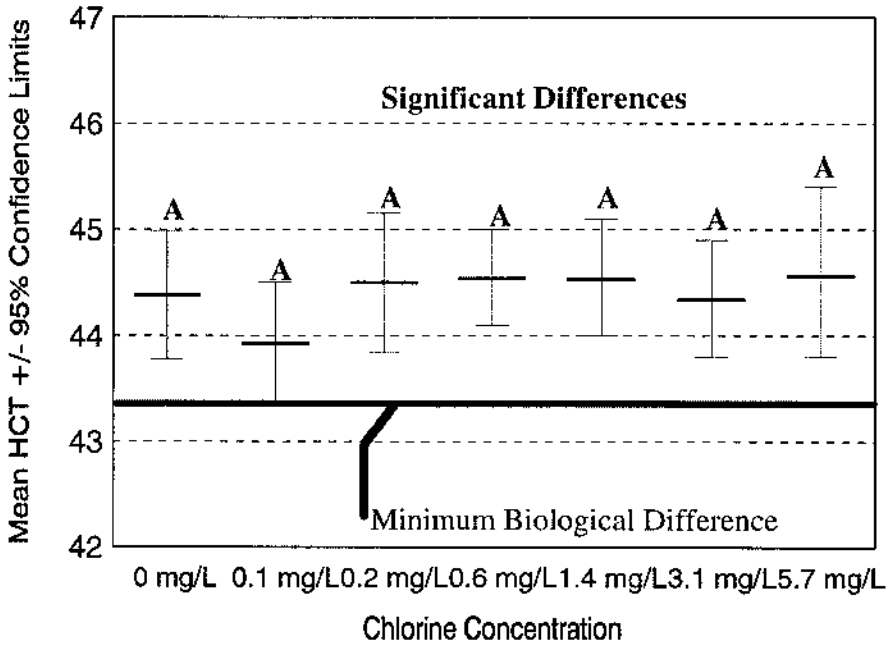
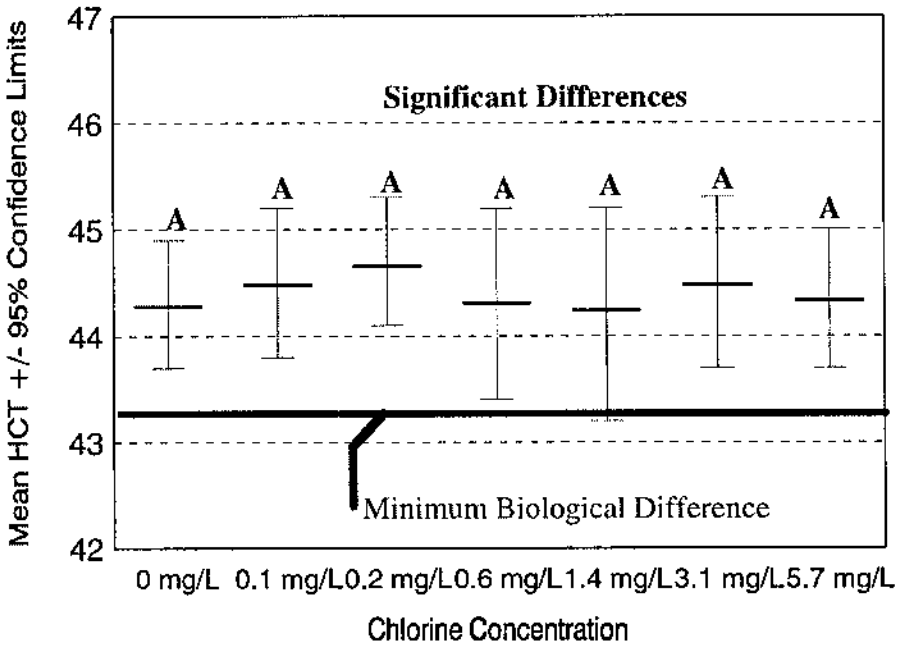


Figure 28

Deviations from the acclimation temperature trend of *Corbicula* accounted for by 24-hour chlorine exposures (0-5.7 mg/L) at 30°C.



The lack of deviation in mean HCT, resulting from chlorine exposure, from that of control exposures suggested that chlorine did not effect *C. fluminea* in terms of upper temperature tolerance. The use of chlorine in treatment for *C. fluminea* fouling in water intakes of municipalities may not be as effective as previously believed unless the mode of action differs from that which was tested for here. However, no mortality was observed during the 24-hour exposures even at the highest concentration of 5.7 mg/L.

This was also true of exposures at 30°C, a temperature at which toxicity increased during the copper exposures.

Mattice et al. (1982) concluded that the application of chlorine (0, 5.0, 7.5, and 10.0 mg/L TRC) combined with heated water (35-46°C) was not more effective than heated water alone (exposure period 30 minutes) in killing *C. fluminea* (as cited by Doherty et al, 1986). However, variations in chlorine concentration (low (0.25 mg/L) and then high concentration (0.5-1.0 mg/L)) or continuous high concentrations were 80-95% successful in killing *C. fluminea* over a 32-day exposure when temperatures remained above 18°C. And 90% mortality was achieved over 32-days with 0.25 mg/L chlorine when temperatures rose from 20 to 25°C. However, mortality levels did not exceed 23% after 28-eight days of exposure to 0.5 mg/L chlorine while temperatures decreased from 20 to 12°C (Doherty, et al., 1986).

CONCLUSIONS

Growth in Various Media and Food

Growth of *I. recurvum* was not determined to be different among the four acclimation temperatures of 20, 25, 30, and 35°C. Growth of *I. recurvum* in synthetic sea water, filtered sea water, and raw sea water in the laboratory resulted in minimal growth regardless of the four acclimation temperatures to which they where exposed. Similarly, the use of *Isochrysus* marine algae, Roti-Rich invertebrate food, and a combination of the two as food sources yielded only that the feeding methods did not produce enough growth to test the usefulness of growth as an indicator of biological health. Raw sea water may have produced more growth if the rate of flow through the test chamber was slower, possibly allowing for more effective filtration.

Field Growth Exposures

Growth of mature and juvenile *I. recurvum* was not significantly different among four field locations. Growth among the field locations was much greater for mature mussels and somewhat greater for juveniles compared to

growth of groups in the laboratory. Growth of mature mussels in the field was sufficient to allow for comparisons among sites. Growth at two sites where elevated organophosphate concentrations were found were not significantly different from that observed for an uncontaminated site. Growth of the juvenile mussels was not great enough to serve as an appropriate indicator. However, the time period of exposure (14-days) to the respective sites was much shorter than desirable for a growth study. Longer field exposures may have shown that juvenile mussels could be used as biological monitors. Chronic pesticide concentrations (based on singular samples after one rainfall event) at the Bayou Chico sites for both mature and juvenile mussels did not effect growth. The least growth occurred at the Santa Rosa Sound site when compared to the Bayou Chico sites even though no pesticides were detected in the Santa Rosa Sound. If the samples taken represent typical conditions, these findings may suggest that mussel growth should not be used as a chronic indicator of ambient organophosphate concentrations.

Ischadium recurvum LC50 Determination for Organophosphates

Determination of the 96-hour LC50 for the hooked mussel exposed to the two organophosphate pesticides, diazinon and chlorpyrifos, was complicated by many factors. The resistant nature of bivalves to these toxicants was evident from toxicological data. The ability of mussels to isolate themselves from their environment for long periods (greater than 24 to 48 hours) may, in part, explain this low level of sensitivity. However, the ability of bivalves to bioaccumulate toxicants to high levels relative to ambient concentrations also shows their resistant nature. Longer exposure times in determining the LC50's may be more indicative of acute concentrations if the time periods exceed the mussels' ability to isolate themselves by "clamming up" and carrying out anaerobic respiration.

Toxicant Growth Exposure

The effects of sublethal concentrations of diazinon and chlorpyrifos on growth of *I. recurvum* was not determined because the concentrations used were acutely toxic. However, biologically useful growth was not expected in these tests (in retrospect) because of the failure to achieve useful growth in the non-toxic flow through exposures where growth was expected to have been greatest. The diffusion of

pesticides into adjacent solutions and the decomposition rates attributed to physical versus biological interaction may prove useful in subsequent research.

Chlorpyrifos Bioaccumulation

Chlorpyrifos tissue concentration of *I. recurvum* is significantly related to chlorpyrifos exposure concentration. The mussel tissue concentrations were markedly different between the (contaminated) control and the intended exposure solutions. Mussels possess the ability to accumulate low levels of chlorpyrifos exposure solutions (0.557 ug/L, diffusion into the control) to tissue concentrations three orders of magnitude higher in only 24-hours (BCF= 1,059) and twice as high in 72-hours (BCF= 1,957). The use of bivalves in monitoring for other toxicants in this manner is not new, however, research on chlorpyrifos bioaccumulation by invertebrates is relatively new (Montanes and Hattum, 1995). The use of mussels to monitor for chlorpyrifos may be useful.

***Ischadium recurvum* Temperature Tolerance for Acclimations**

Upper temperature tolerance of *I. recurvum* is significantly different among four acclimation temperatures. Mussels, like many organisms, show a significant relation

between acclimation temperature and upper temperature tolerance. The higher the temperature to which a mussels is acclimated, the higher its upper temperature tolerance within certain limits. The upper temperature tolerance (HCT) of mussels can be regressed against the acclimation temperature to generate a baseline model for comparison. Comparisons could be made between field organisms from different sites to those of known water quality to determine if differences are present in their ability to cope with heat stress. These differences in upper temperature tolerance may serve as an indicator of relative water quality or the presence of stressors in the environment.

I. recurvum Temperature Tolerance for Organophosphate Exposure Chlorpyrifos Exposure for 72-hours

Upper temperature tolerance of *I. recurvum* is different among a control and sublethal chlorpyrifos concentrations after 72-hours of exposure. The use of the HCT of molluscs may be useful as indicators of ambient water quality. Although mussels are not particularly sensitive to typically acute exposures for some organic contaminants, they may be useful indicators of water quality when other such indicators are lacking. The HCT relationship during exposures is of importance, however, the persistence of

lower tolerance to high temperatures could be cause for concern about the health of an estuary.

I. recurvum Temperature Tolerance for Organophosphate Exposure Chlorpyrifos Exposure After 24-hour Rinse

Upper temperature tolerance of *I. recurvum* is different among a control and sublethal chlorpyrifos concentrations 24-hours after a 72-hour exposure. The persistence of the effects of exposures to chlorpyrifos could serve as an indicator of the severity of insult which a system may have incurred. As previously discussed, mussels bioaccumulate toxins, such as chlorpyrifos, quite efficiently. This situation may cause mussels to show the affects of such an insult some time after the actual exposure has ceased. More sensitive organisms may flee the area or die as a result of the exposure. Mussels are sessile, able to accumulate toxins, and are tolerant of higher levels of some toxins than many organisms. This may create a condition that could provide useful information as to the nature and severity of a run-off episode.

I. recurvum Temperature Tolerance for Organophosphate Exposure Diazinon Exposure for 72-hours

Upper temperature tolerance of *I. recurvum* is different among a control and sublethal diazinon concentrations after 72-hours of exposure. The presence of diazinon had little influence on the upper temperature tolerance of the hooked mussel until concentrations reached very high levels (~500 ug/L). The lessor impact of diazinon, when compared to chlorpyrifos, may be the result of mode of transformation or site of transformation in mussels to the more toxic -oxon form. Information about the toxicity of the carriers of the active ingredients (diazinon and chlorpyrifos) may also provide information about these differences.

I. recurvum Temperature Tolerance for Organophosphate Exposure Diazinon After 24-hour Rinse

Upper temperature tolerance of *I. recurvum* is different among a control and sublethal diazinon concentrations 24-hours after a 72-hour exposure. The effectiveness of lower diazinon concentrations on the upper temperature tolerance of the hooked mussel increased over the 24-hour rinse period. The relationship of the presence of diazinon and the alteration of upper temperature tolerance may serve as indicator of water quality after the occurrence of a run-off

event together with or in the absence of other biological measures.

LC50 Determination of Diazinon for *Corbicula fluminea*

The toxicity of diazinon to *C. fluminea* was found to be relatively low even when compared to other such resistant bivalves. The ability of the clams to isolate themselves from a toxic insult provided variable results. The toxicity of diazinon to *C. fluminea* is dependent on valve closure. The dramatically increased survival for clams which were not provided the opportunity to periodically re-sample their toxic environment may suggest that toxicity for bivalves is a more complicated matter than for other organisms. However, the inability of bivalves to flee such insults may only be somewhat compensated for by their ability to isolate themselves within the unfavorable environment itself.

The tolerance of *C. fluminea* to diazinon is remarkably high even when such isolations are less of a factor as could be seen by the 21-day exposure. Although it is unknown how long *C. fluminea* can voluntarily isolate itself via valve closure, the behaviorally regulated test suggests that such voluntary isolations do not occur for extended periods. The very low susceptibility of *C. fluminea* to organophosphates is likely to be a function of something other than behavior

and permeability alone.

The specific action of organophosphates on bivalves would likely reveal more information about their resistance to such toxicants. It may be in part related to the use of isotonic muscle contractions. These contractions utilize little acetylcholine and thus acetylcholine esterase to regulate their adductor muscle activity, relative to other muscle systems. Organophosphates inhibit the muscle contraction relaxing acetylcholine esterase (Morgan, 1989). With a lessor dependence upon this physiological substance, bivalves may be affected less because they can isolate themselves without continual need for muscle control until more acetylcholine esterase is produced.

The site and mechanism for the transformation of organophosphates to their more toxic -oxon form would likely provide information about their high resistance. In addition, the role of the "inert" carriers of organophosphates in commercial formulas may also aid the understanding the specific action of these products on bivalves.

C. fluminea Upper Temperature Tolerance for Acclimations

The upper temperature tolerance of *C. fluminea* is different among three acclimation temperatures of 10, 20,

and 30°C. Clams, and many organisms such as the hooked mussel, show a significant relation between acclimation temperature and upper temperature tolerance. The higher the temperature to which a clam is acclimated, the higher its upper temperature tolerance within certain limits. The upper temperature tolerance (in terms of heat coma temperature, HCT) of clams can be regressed against the acclimation temperature to generate a baseline model for comparison. Potentially, comparisons could be made between field organisms from different sites to those of known water quality to determine if differences are present in their ability to cope with heat stress. These differences in HCT may serve as an indicator of relative water quality or the presence of stressors in the environment.

C. fluminea Temperature Tolerance for Organophosphate Exposure

The upper temperature tolerance of *C. fluminea* is different among four diazinon concentrations and a control for the acclimation temperature 10°C, but not for acclimations to 20 and 30°C. The presence of diazinon had little influence on the upper temperature tolerance of *C. fluminea* for acclimations to 20 and 30°C. However, diazinon had a profound impact on *C. fluminea* upper temperature

tolerance at 10°C. The upper temperature tolerance of *C. fluminea* in response to exposures to organophosphates may be useful as a bio-indicator at low temperatures. However, the mechanisms by which the upper temperature tolerance of clams exposed to organophosphates deviates from that of control exposures at low temperatures needs further investigation.

C. fluminea Temperature Tolerance for Heavy Metal Exposure

The upper temperature tolerance of *C. fluminea* is different among six copper concentrations and a control for the acclimation temperatures 10, 20, and 30°C. Acclimation to 10 and 20°C showed the lowest concentrations of copper to effect upper temperature tolerance. As mentioned above for *I. recurvum*, the ability of bivalves to isolate themselves from their immediate environment via valve closure may be involved in protecting clams from readily identifiable toxins. The difference between the HCTs resulting from varying concentrations suggests that *C. fluminea* readily detects concentrations above 179.2 ug/L copper and attempt to isolate themselves from the exposure by closing their valves and operating anaerobically. The effects of higher concentrations of copper on *C. fluminea* at 30°C suggests that this mechanism of self isolation is not available to the clams to the same extent as it is at lower temperatures.

The upper temperature tolerance deviations of *C. fluminea* in response to exposures to copper may be useful as a bio-indicator of water quality. However, the mechanisms by which the upper temperature tolerance of clams exposed to copper deviates from that of control exposures needs further investigation.

C. fluminea Temperature Tolerance for Chlorine Exposure

The upper temperature tolerance of *C. fluminea* is not different among six chlorine concentrations and a control for the acclimation temperatures 10, 20, and 30°C. The presence of chlorine did not influence upper temperature tolerance of *C. fluminea*. This was consistent for all exposure concentrations from 0.09 to 5.7 mg/L chlorine. The lack of measurable affects in terms of HCT, prolong valve closure, and mortality suggested that *C. fluminea* upper temperature tolerance may not be a suitable bio-indicator for the presence of chlorine within the duration (24-hours) and range tested (0.09-5.7 mg/L). However, other endpoints may provide for the use of *C. fluminea* as a monitor for the presence of chlorine.

SUMMARY (●) AND RECOMMENDATIONS (○)

● Growth in synthetic sea water, filtered sea water, and raw sea water in the laboratory resulted in minimal information pertaining to the differences produced by these media at any of four acclimation temperatures.

● The use of *Isochrysus* marine algae, Roti-Rich invertebrate food, and a combination of the two as food sources indicated that these feeding methods did not produce enough growth to be useful as indicators at the four exposure temperatures tested.

○ Experiments utilizing a continuous feeding regime in flow through aquaria may produce sufficient growth in the laboratory to be useful for biomonitoring.

- Continuous feeding may more closely simulate environmental conditions in that the food source would be available at all times as it would be in the natural estuarine water column.

- The continuous availability of food may reveal more

information than experiments with other food sources at previously used feeding intervals.

● Growth of mature mussels in the field were sufficient to allow for comparisons among sites.

● Varying concentrations of the target organophosphates among the field sites did not noticeably influence the growth rates of the mussels among the sites.

● Growth of the juvenile mussels in the field was not great enough to serve as an appropriate indicator.

O Field exposures of longer duration (3 to 4 weeks) would likely produce greater increases in overall length during the exposure period.

O Newly recruited mussels (<10 mm in overall length) may be more sensitive to the chronic organophosphate levels detected.

O The examination of the growth of mussels in various salinity exposures may also provide useful information as to the poor growth achieved by mussels in the relatively clean

Santa Rosa Sound when compared to the contaminated Bayou Chico sites.

● Determination of the 96-hour LC50 for the hooked mussel exposed to the two organophosphate pesticides, diazinon and chlorpyrifos, was complicated by some factors.

- The ability of mussels to isolate themselves from their environment for long periods (greater than 24 to 48 hours).

- The ability of bivalves to bioaccumulate toxicants to high levels relative to ambient concentrations.

- Lack of information about the transformation of organophosphates by bivalves.

- Lack of information about the toxicity of the "inert" carriers of organophosphates to bivalves.

O Longer exposure times in determining the LC50's may be more indicative of acute concentrations if the time periods exceed the mussel's ability to isolate itself through valve closure.

O Experiments examining the ability of these mussels to bioaccumulate organophosphates may show to what extent these mussels can cope with organophosphate interaction.

● Growth to be measured in the toxicant exposures was not analyzed because the concentrations used were acutely toxic.

- Growth, in useful degrees, was not expected because of the lack of sufficient growth observed in the non-toxic flow through exposures where growth was expected to have been greatest.

O Diffusion of pesticides into adjacent solutions and the decomposition rates attributed to physical versus biological interaction may prove useful in subsequent research.

O Exposure of mussels to organophosphates may be of minimal value in terms of growth until an appropriate feeding regime and exposure media is identified which will provide for laboratory growth in biologically useful amounts.

O More appropriate levels of exposure could be established using the estimated toxicity data for this and other bivalve species.

○ Extended field exposures may provide more information pertaining to relative growth of mussels in the presence of organophosphates until the laboratory methods are further examined.

● Mussel chlorpyrifos tissue concentrations were markedly different between the (contaminated) control and the intended exposure solutions.

- Mussels possess the ability to accumulate low levels of chlorpyrifos exposure solutions to tissue concentrations three orders of magnitude higher in only 24-hours (BCF= 1,059) and twice as high in 72-hours (BCF= 1,957).

○ Examination of the bioaccumulation ability of the hooked mussel in the presence of different organophosphates or other toxic chemicals may provide useful information for bio-monitoring in the estuaries which they inhabit.

● Mussels show a significant relation between acclimation temperature and upper temperature tolerance.

- The higher the temperature to which a mussels is acclimated, the higher its upper temperature tolerance

within certain limits.

- The upper temperature tolerance (HCT) of mussels can be regressed against a range of acclimation temperatures to generate a baseline model for comparison.

- Comparisons could be made between field organisms from different sites to those of known water quality to determine if differences are present in their ability to cope with heat stress.

- These differences in upper temperature tolerance may serve as an indicator of relative water quality or the presence of stressors in the environment.

O The inclusion of additional acclimation temperatures in the anticipated field temperature range in establishing the acclimation temperature model for estimating upper temperature tolerance would increase the predictive ability of such a model.

● Exposure to chlorpyrifos significantly lowered upper temperature tolerance of the hooked mussel during a 72-hour exposure.

● The lower upper temperature tolerance of the hooked mussel induced by a 72-hour chlorpyrifos exposure remained significant 24-hours after the exposure.

● Exposure to high diazinon concentrations significantly lowered upper temperature tolerance of the hooked mussel during a 72-hour exposure.

● The lower upper temperature tolerance of the hooked mussel induced by a 72-hour diazinon exposure was significant for lower concentration exposures 24-hours after the exposure.

O Extended test examining the persistence of the effects of organophosphates on bivalve upper temperature tolerance may provide useful information.

O Examination of the specific action of organophosphates on bivalves may provide this methodology greater precision for bio-indication.

● The tolerance of *C. fluminea* to diazinon is remarkably high for 96-hour and 21-day exposures.

● *C. fluminea* not provided the opportunity to voluntarily "re-sample" their exposure solution dramatically increases survival in diazinon exposures.

- The very low susceptibility of *C. fluminea* to organophosphates is likely to be a function of something other than behavior and permeability alone.

○ The specific action of diazinon upon bivalves would likely reveal more information about their resistance to such toxicants.

○ Examination of the acetylcholine esterase demands of bivalves during periods of valve closure would provide for a better understanding of their extreme tolerance of organophosphate exposure.

● *C. fluminea* show a significant relation between acclimation temperature and upper temperature tolerance.

- The higher the temperature to which a clam is acclimated, the higher its upper temperature tolerance within certain limits.

- The upper temperature tolerance (HCT) of clams can be regressed against the range of acclimation temperatures to generate a baseline model for comparison.

- Comparisons could be made between field organisms from different sites to those of known water quality to determine if differences are present in their ability to cope with heat stress.

- Differences in HCT may serve as an bio-indicator of relative water quality or the presence of stressors in the environment.

O The inclusion of additional acclimation temperatures in the anticipated field temperature range in establishing the acclimation temperature model for estimating upper temperature tolerance would increase the predictive ability of such a model.

● Diazinon did not significantly influence the upper temperature tolerance of *C. fluminea* for acclimations to 20 and 30°C.

● Diazinon did significantly influence the upper

temperature tolerance of *C. fluminea* for acclimation to 10°C.

O The mechanisms by which the upper temperature tolerance of clams exposed to organophosphates deviates from that of control exposures at low temperatures needs further investigation.

O The mechanisms of cold tolerance of bivalves would also add valuable information.

● The presence of copper significantly influenced the upper temperature tolerance of *C. fluminea* for acclimations to 10, 20 and 30°C.

- Acclimation to 10 and 20°C showed low concentrations of copper to be effective on upper temperature tolerance.

- Higher concentrations of copper influenced the upper temperature tolerance of *C. fluminea* at 30°C.

O The mechanisms by which the upper temperature tolerance of clams exposed to copper deviates from that of control exposures needs further investigation.

- The presence of chlorine did not influence the upper temperature tolerance of *C. fluminea* for acclimations to 10, 20 and 30°C.

APPENDIX 1

Determination of Organophosphate Concentration for Exposure Solutions

Determination of diazinon and chlorpyrifos pesticide concentrations was performed for the field samples, final LC50 exposure concentrations, and growth exposure concentrations. In a graduated cylinder, 250 ml of sample was diluted to 1L and transferred to a 2L separatory funnel. The sample was spiked with 1.0 ml of surrogate solution containing TBPP (Tributyl Phosphate 99% Lot No. 10821 BN) and TPPP (Triphenyl Phosphate 99% Lot No. 03130 KP Aldrich Chemical), 4.0 μg/ml each in acetone, and extracted three time with 50 ml portions of methylene chloride. The extract was transferred to a 500 ml round bottom flask and attached to a rotary evaporator (Buchler Instruments) and reduced to approximately 5 ml. The contents were then transferred to a 25 ml Kuderna-Danish (KD) receiver and solvent exchanger under nitrogen and reduced to 1.0 ml in hexane.

The GC was a temperature programmed HP 5890, Series II equipped with a single heat injector, HP-1701 fused silica capillary column (30m X 0.32mm), and a Nitrogen-Phosphorus detector (NPD) connected via an A/D convertor to a HP 3550 LAS data system.

The analysis used multi-level calibration and a single internal standard peak for identification and

quantification. Standards were prepared at levels expected to span the concentrations of the target compounds in the samples. A calibration curve was constructed for each compound and a relative response factor (RFF) based upon the response of the initial standard was calculated. Compounds were identified by retention time. The concentration for each compound detected in the sample was calculated from the following general equation:

$$\frac{\text{(Target Comp Peak Area)(Volume of Conc. Extract)(Dilution Factor from Calib)}}{\text{(Mean Calibration Factor)(Volume Injected)(Volume of Sample Extracted)}}$$

Standards for diazinon and chlorpyrifos were prepared at 0.2, 0.4, 2.0, and 4.0μg/ml in hexane from neat diazinon (Diazinon 99%, Lot No. LA 57762 Supelco) and concentrated stock (Chlorpyrifos 1000 ug/ml in Methyl tert-Butyl Ether, Lot No. LA52590 Supelco). Linearity of the curve was accepted when a correlation coefficient of 0.990 or better was achieved for all necessary compounds.

Extraction blanks consisting of filtered sea water were prepared with each group of samples. Instrument blanks (1 ml solvent with internal standard added) were injected to check the system cleanliness, stabilize the analytical system operation, and mark the progress of the auto-sampler injections, but were not reported. The sample blanks showed

no diazinon or chlorpyrifos contamination.

One ml of surrogate solution (4.0 μg/ml each in acetone) was prepared from neat stock TBPP (99% Lot No. 10821 BN) and TPPP (99% Lot No. 03130 KP Aldrich Chemical) and was added to each sample at the beginning of the extraction procedure and measured to monitor extraction efficiency. Acceptable surrogate recovery limits as prescribed by the Quality Assurance Protocol Procedures (QAPP) are 40-130%.

Matrix Spike (MS) and Matrix Spike Duplicate (MSD) samples were prepared from sea water to monitor the extraction precision and accuracy. The MS and MSD samples were spiked with 1 ml of the spike solution prepared from the respective diazinon and chlorpyrifos product and carried through extraction and analysis. Acceptable spike recovery limits as prescribed by the QAPP are 40-130% (indicating precision). Acceptable Relative Percent Difference (RPD) limits as prescribed by the QAPP are <30% (indicating accuracy; Avanti Corporation, 1995).

APPENDIX 2

Determination of Organophosphate Concentration for Tissue Samples

Tissue samples were analyzed for chlorpyrifos following the extraction. Five grams of thawed mussel tissue sample were weighed into a 150 mm by 25 mm glass test tube, spiked with 1.0 ml of surrogate solution containing 4.0 ug/ml TBPP and TPPP and blended to a uniform consistency in 10 ml of acetonitrile with a Janke & Kunkel Ultra Turrax T25 sample processor. The test tube was centrifuged, and the liquid layer decanted into a 120 ml oil sample bottle. In the first of two additional extractions, 10 ml of fresh acetonitrile was added to the test tube and the sample sonicated (Tekmar Sonic Disrupter, Model no. TM600-2) for 120 seconds at 50% pulse. After each sonication the sample was centrifuged, the liquid layer was combined with the oil sample contents before the back (liquid) extraction. Approximately 70 ml of 2% sodium sulfate was added to the oil sample bottle, shaken, and allowed to stand 30 minutes. In the first of three back extractions, 10 ml of petroleum ether was added to the oil sample bottle and the contents were shaken for one minute. After each back extraction the contents were allowed to settle, and the upper solvent layer was pipetted into a 50 ml Erlenmeyer flask. A small amount of powdered sodium sulfate was added to the contents of the Erlenmeyer

flask to absorb any water that may have been carried over. Finally, the contents of the Erlenmeyer flask were transferred to a Kunerna-Danish (KD) receiver. The combined solvent extract was concentrated to 1 ml on a nitrogen evaporator in preparation for silica gel clean up.

The clean-up column was prepared by adding 3.5 grams of silica gel (stored at 130°C for at least 24-hours) in a 1% acetic acid/hexane slurry to a 0.9 X 25 cm glass column fitted with a fritted glass disk, a Teflon stopcock, and 25 ml reservoir. The column was gently tapped to settle the silica gel and remove bubbles. One centimeter of sodium sulfate sandwiched the silica gel column. Any trace of residue was removed by eluting the column with 25 ml of 1% acetic acid/hexane rinse prior to addition of a sample. After the one ml extract was added to the top of the column, the stopcock was opened and the extract moved onto the column. The column was eluted at 1-2 ml/min with 1% acetic acid/hexane and the first 10 ml was discarded. The column was then eluted with 5% ethyl ether/hexane and 20 ml was collected in another concentrator tube. In the final step, the column was eluted with 10% ethyl ether/hexane and another 20 ml was collected. The two 20 ml fractions were combined and reduced under nitrogen to 1.0 ml and stored in GC vials until analysis. Just prior to GC-analysis an

internal standard solution was added, 5 ul of stock 1-Bromo-2-nitrobenzene (BNB).

The GC was a temperature programmed HP 5890, Series II equipped with a single heat injector, HP-1701 fused silica capillary column (30m X 0.32mm), and a Nitrogen-Phosphorus detector (NPD) connected via an A/D convertor to a HP 3550 LAS data system.

The analysis used multi-level calibration and a single internal standard peak for identification and quantification. Standards were prepared at levels expected to span the concentrations of the target compounds in the samples. A calibration curve was constructed for each compound and a relative response factor (RFF) based upon the response of the initial standard was calculated. Compounds were identified by retention time. The concentration for each compound detected in the sample was calculated from the general equation:

$$\frac{(\text{Target Comp Peak Area})(\text{Volume of Conc. Extract})(\text{Dilution Factor from Calib})}{(\text{Mean Calibrtation Factor})(\text{Volume Injected})(\text{Volume of Sample Extracted})}$$

Standards for chlorpyrifos were prepared at 0.2, 0.4, 2.0, and 4.0 μg/ml in hexane from concentrated stock (Chlorpyrifos 1000 μg/ml in Methyl tert-Butyl Ether, Lot No. LA52590 Supelco). Acceptable linearity of the standard curve

was achieved when the correlation coefficient was 0.990 or better for all necessary compounds (Avanti Corporation, 1995).

APPENDIX 3

Table A-1

Growth of *I. recurvum* in Synthetic Sea Water Fed *Isochrysus* for 21-Days

Acclimation temperature (°C), initial length (mm), final length (mm) and growth (mm) /mortality presented.

°C	Init Lth	Final Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr
35	11.55	11.43	-0.1	30	11.16	11.24	0.1	25	8.4	8.42	0	20	12.16	12.30	0.14
35	12.32	12.22	0	30	9.38	9.45	0.1	25	11.2	11.20	0	20	10.11	10.11	0
35	12.34	12.34	0	30	11.93	11.92	0	25	12.2	12.39	0.19	20	14.15	14.23	0.1
35	12.48	12.51	0	30	12.11	12.16	0.1	25	10.7	10.74	0	20	13.95	13.99	0
35	9.03	9.00	0	30	10.25	10.27	0	25	14.3	14.49	0.19	20	11.70	11.73	0
35	10.93	10.93	0	30	13.9	13.91	0	25	15.5	15.65	0.15	20	11.64	11.71	0.1
35	13.33	13.35	0	30	11.79	11.96	0.2	25	11.6	12.18	0.58	20	13.65	13.64	0
35	12.71	12.73	0	30	11.73	11.86	0.1	25	12.0	12.04	0	20	11.99	11.98	0
35	12.57	12.56	0	30	13.43	13.61	0.2	25	13.4	13.55	0.15	20	9.57	9.65	0.1
35	14.12	14.09	0	30	13.2	13.27	0.1	25	12.1	12.29	0.19	20	10.21	10.24	0
35	12.75	12.73	0	30	10.99	11.05	0.1	25	13.9	13.92	0	20	10.28	10.33	0.1
35	9.39	9.30	0	30	12.18	12.31	0.1	25	15.2	15.29	0.1	20	9.44	9.49	0.1
35	12.78	12.79	0	30	12.52	12.52	0	25	10.5	10.62	0.12	20	8.10	8.15	0.1
35	12.95	12.96	0	30	11.81	11.93	0.1	25	8.4	8.42	0	20	8.77	8.81	0
35	14.56	14.51	0	30	10.13	10.17	0	25	10.1	10.14	0	20	9.69	9.80	0.11
35	12.72	12.83	0.11	30	9.37	9.42	0.1	25	12.8	12.84	0	20	9.56	9.68	0.12
35	12.96	12.89	0	30	9.83	9.82	0	25	11.5	11.63	0.13	20	10.09	10.20	0.11
35	9.44	9.34	0	30	10.39	10.42	0	25	11.4	11.49	0.1	20	13.61	13.68	0.1
35	13.91	13.96	0.1	30	11.93	12.04	0.1	25	13.9	14.02	0.12	20	8.92	8.91	0
35	11.90	11.90	0	30	8.24	8.20	0	25	12.0	12.08	0.1	20	10.79	10.81	0
35	11.02	11.02	0	30	11.25	11.29	0	25	9.4	9.39	0	20	11.25	11.30	0.1
35	9.73	9.74	0	30	14.04	14.10	0.1	25	8.1	8.17	0.1	20	13.06	13.09	0
35	8.79	8.84	0.1	30	12.97	13.09	0.1	25	12.8	12.91	0.11	20	9.17	9.18	0
35	9.41	9.40	0	30	10.76	10.82	0.1	25	14.9	15.03	0.13	20	12.17	12.17	0
35	MORT			30	9.63	9.64	0	25	9.0	9.15	0.15	20	9.47	9.53	0.1
35	MORT			30	9.21	9.22	0	25	12.3	12.37	0.1	20	8.75	8.74	0
35	MORT			30	10.77	10.82	0.1	25	10.8	10.96	0.16	20	9.34	9.34	0
35	MORT			30	9.03	9.03	0	25	9.3	9.25	0	20	10.19	10.21	0
35	MORT			30	MORT			25	9.6	9.64	0	20	MORT		
35	MORT			30	MORT			25	10.1	10.09	0	20	MORT		
		MEAN	-0.01			MEAN	0.06			MEAN	0.10			MEAN	0.04

Table A-2

Growth of *I. recurvum* in Filtered Sea Water Fed *Isochrysus* for 21-Days

Acclimation temperature (°C), initial length (mm), final length (mm) and growth (mm) /mortality presented.

°C	Init Lth	Final Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr
35	27.0	27.0	0	30	23.5	23.8	0.3	25	26.0	26.1	0.1	20	24.0	24.2	0.2
35	27.5	27.5	0	30	25.0	25.0	0	25	23.5	23.5	0	20	25.2	25.3	0.1
35	27.2	27.2	0	30	27.5	27.5	0	25	21.0	21.0	0	20	24.8	24.8	0
35	27.8	28.4	0.6	30	21.5	22.0	0.5	25	26.8	26.8	0	20	25.0	25.7	0.7
35	22.0	22.2	0.2	30	26.0	26.6	0.6	25	28.0	28.3	0.3	20	23.0	23.1	0.1
35	26.0	26.5	0.5	30	24.0	24.4	0.4	25	27.0	27.0	0	20	22.0	22.0	0
35	26.0	26.0	0	30	23.0	24.0	1	25	26.0	26.2	0.2	20	21.0	21.0	0
35	23.0	23.0	0	30	26.0	26.5	0.5	25	25.5	25.5	0	20	26.5	26.5	0
35	25.0	25.0	0	30	23.5	23.5	0	25	22.5	22.5	0	20	28.0	28.0	0
35	25.0	25.5	0.5	30	27.5	27.7	0.2	25	27.5	27.6	0.1	20	27.0	27.6	0.6
35	24.0	24.3	0.3	30	24.0	24.5	0.5	25	23.0	22.7	0	20	26.3	26.3	0
35	26.0	25.5	-0.5	30	27.0	27.0	0	25	22.6	22.6	0	20	25.5	25.7	0.2
35	25.0	25.5	0.5	30	27.0	27.0	0	25	22.0	21.6	0	20	23.0	23.5	0.5
35	21.0	21.5	0.5	30	24.0	24.5	0.5	25	20.0	20.0	0	20	27.0	27.0	0
35	21.0	22.0	1	30	25.0	25.0	0	25	26.8	26.8	0	20	27.7	27.5	-0.2
35	23.0	23.0	0	30	24.8	24.8	0	25	25.8	25.8	0	20	24.0	24.0	0
35	22.0	22.4	0.4	30	25.0	25.0	0	25	26.5	26.9	0.4	20	25.0	26.0	1
35	28.0	28.0	0	30	23.0	23.8	0.8	25	27.0	27.2	0.2	20	24.0	24.2	0.2
35	20.0	20.2	0.2	30	23.0	23.0	0	25	26.5	26.5	0	20	25.3	26.0	0.7
35	23.0	23.0	0	30	22.8	23.8	1	25	23.0	23.2	0.2	20	23.9	23.5	-0.4
35	24.2	24.2	0	30	26.0	26.2	0.2	25	26.5	26.5	0	20	23.0	23.0	0
35	24.5	25.0	0.5	30	26.0	26.0	0	25	27.0	27.2	0.2	20	24.8	24.9	0.1
35	25.5	25.5	0	30	25.5	25.8	0.3	25	25.5	25.8	0.3	20	26.0	26.0	0
35	23.3	23.3	0	30	29.0	29.2	0.2	25	22.0	22.0	0	20	26.0	26.0	0
35	22.6	22.6	0	30	21.0	22.1	1.1	25	26.0	26.5	0.5	20	25.5	25.8	0.3
35	24.6	25.2	0.6	30	20.0	20.2	0.2	25	23.8	23.8	0	20	27.5	27.5	0
35	23.0	23.0	0	30	26.0	26.0	0	25	MORT			20	21.0	21.0	0
35	26.1	26.1	0	30	27.0	27.1	0.1	25	MORT			20	20.0	20.1	0.1
35	25.0	25.0	0	30	MORT			25	MORT				MORT		
35	24.0	24.0	0	30	MORT			25	MORT				MORT		
		MEAN	0.18			MEAN	0.30			MEAN	0.07			MEAN	0.15

Table A-3

Growth of *I. recurvum* in Filtered Sea Water Fed Roti-Rich for 21-Days

Acclimation temperature (°C), initial length (mm), final length (mm) and growth (mm) /mortality presented.

°C	Init Lth	Final Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr
35	27.0	27.0	0	30	23.8	23.7	0	25	26.1	26.4	0.3	20	24.2	24.2	0
35	27.5	28.0	0.5	30	25.0	25.3	0.3	25	23.5	23.5	0	20	25.3	25.9	0.6
35	27.2	27.2	0	30	27.5	27.5	0	25	21.0	21.0	0	20	24.8	24.8	0
35	28.4	28.4	0	30	22.0	22.0	0	25	26.8	27.3	0.5	20	25.7	25.7	0
35	22.2	22.3	0.1	30	26.6	27.2	0.6	25	28.3	28.5	0.2	20	23.1	23.1	0
35	26.5	26.5	0	30	24.4	24.4	0	25	27.0	27.1	0.1	20	22.0	22.0	0
35	26.0	26.4	0.4	30	24.0	24.0	0	25	26.2	26.2	0	20	21.0	21.0	0
35	23.0	23.0	0	30	26.5	26.8	0.3	25	25.5	25.5	0	20	26.5	27.0	0.5
35	25.0	25.0	0	30	23.5	24.2	0.7	25	22.5	22.5	0	20	28.0	28.0	0
35	25.5	26.0	0.5	30	27.7	27.7	0	25	27.6	27.6	0	20	27.6	27.5	-0.1
35	24.3	24.3	0	30	24.5	24.5	0	25	22.7	22.7	0	20	26.3	26.3	0
35	25.5	25.1	-0.4	30	27.0	27.3	0.3	25	22.6	22.6	0	20	25.7	25.7	0
35	25.5	25.5	0	30	27.0	27.0	0	25	21.6	21.6	0	20	23.5	23.7	0.2
35	21.5	21.9	0.4	30	24.5	24.5	0	25	20.0	20.0	0	20	27.0	27.0	0
35	22.0	22.0	0	30	25.0	25.0	0	25	26.8	26.8	0	20	27.5	27.5	0
35	23.0	23.0	0	30	24.8	25.2	0.4	25	25.8	25.6	-0.2	20	24.0	24.6	0.6
35	22.4	23.0	0.6	30	25.0	25.7	0.7	25	26.9	26.9	0	20	26.0	26.2	0.2
35	28.0	29.0	1	30	23.8	24.5	0.7	25	27.2	27.8	0.6	20	24.2	24.2	0
35	20.2	20.2	0	30	23.0	23.0	0	25	26.5	26.5	0	20	26.0	26.0	0
35	23.0	23.0	0	30	23.8	23.8	0	25	23.2	23.6	0.4	20	23.5	23.5	0
35	24.2	24.5	0.3	30	26.2	26.3	0.1	25	26.5	26.5	0	20	23.0	23.8	0.8
35	25.0	25.0	0	30	26.0	26.0	0	25	27.2	28.1	0.9	20	24.9	24.9	0
35	25.5	25.5	0	30	25.8	25.8	0	25	25.8	26.1	0.3	20	26.0	26.0	0
35	23.3	23.4	0.1	30	29.2	29.7	0.5	25	22.0	22.0	0	20	26.0	26.0	0
35	22.6	22.6	0	30	22.1	22.1	0	25	26.5	26.4	-0.1	20	25.8	25.8	0
35	25.2	26.1	0.9	30	20.2	20.5	0.3	25	23.8	23.8	0	20	27.5	27.5	0
35	23.0	23.0	0	30	26.0	26.0	0					20	21.0	21.5	0.5
35	26.1	26.3	0.2	30	27.1	27.1	0					20	MORT		
35	25.0	25.0	0												
35	MORT														
		MEAN	0.16			MEAN	0.17			MEAN	0.12			MEAN	0.12

Table A-4

Growth of *I. recurvum* in Filtered Sea Water Fed *Isochrysus* and Roti-Rich for 21-Days

Acclimation temperature (°C), initial length (mm), final length (mm) and growth (mm)/mortality presented.

°C	Init Lth	Final Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr	°C	Init Lth	Fin Lth	Gr
35	27.0	27.0	0	30	23.7	23.9	0.2	25	26.4	26.6	0.2	20	24.2	24.0	-0.2
35	28.0	28.2	0.2	30	25.3	25.7	0.4	25	23.5	23.5	0	20	25.9	26.0	0.1
35	27.2	27.2	0	30	27.5	27.3	-0.2	25	21.0	21.3	0.3	20	24.8	24.8	0
35	28.4	28.4	0	30	22.0	22.1	0.1	25	27.3	27.3	0	20	25.7	25.7	0
35	22.3	22.3	0	30	27.2	27.2	0	25	28.5	28.9	0.4	20	23.1	23.1	0
35	26.5	27.0	0.5	30	24.4	24.4	0	25	27.1	27.1	0	20	22.0	22.0	0
35	26.4	26.6	0.2	30	24.0	24.0	0	25	26.2	26.2	0	20	21.0	21.6	0.6
35	23.0	23.0	0	30	26.8	27.1	0.3	25	25.5	25.5	0	20	27.0	27.0	0
35	25.0	25.0	0	30	24.2	24.5	0.3	25	22.5	22.5	0	20	28.0	28.0	0
35	26.0	26.0	0	30	27.7	27.7	0	25	27.6	28.2	0.6	20	27.6	27.6	0
35	24.3	24.3	0	30	24.5	24.5	0	25	22.7	22.7	0	20	26.3	26.3	0
35	25.5	25.8	0.3	30	27.3	27.9	0.6	25	22.6	22.1	-0.5	20	25.7	25.7	0
35	25.5	25.5	0	30	27.0	27.0	0	25	21.6	21.6	0	20	23.7	24.6	0.9
35	21.9	21.9	0	30	24.5	25.0	0.5	25	20.0	20.0	0	20	27.0	27.0	0
35	22.0	22.0	0	30	25.0	25.0	0	25	26.8	27.4	0.6	20	27.5	27.5	0
35	23.0	23.0	0	30	25.2	25.2	0	25	25.8	25.8	0	20	24.6	25.2	0.6
35	23.0	23.0	0	30	25.7	25.7	0	25	26.9	26.9	0	20	26.2	26.2	0
35	29.0	29.3	0.3	30	24.5	25.6	1.1	25	27.8	27.8	0	20	24.2	24.4	0.2
35	20.2	20.2	0	30	23.0	24.2	1.2	25	26.5	26.5	0	20	26.0	26.0	0
35	23.0	23.0	0	30	23.8	23.8	0	25	23.6	23.6	0	20	23.5	23.5	0
35	24.5	24.7	0.2	30	26.3	26.3	0	25	26.5	26.8	0.3	20	23.8	23.8	0
35	25.0	25.5	0.5	30	26.0	26.0	0	25	28.1	28.1	0	20	24.9	24.9	0
35	25.5	26.0	0.5	30	25.8	25.8	0	25	26.1	26.1	0	20	26.0	26.2	0.2
35	23.4	23.4	0	30	29.7	29.7	0	25	22.0	22.3	0.3	20	26.0	26.0	0
35	22.6	22.9	0.3	30	22.1	22.1	0	25	26.5	26.5	0	20	25.8	25.8	0
35	26.1	26.1	0	30	20.5	20.6	0.1	25	23.8	23.8	0	20	27.5	28.2	0.7
35	23.0	23.0	0	30	26.0	26.7	0.7					20	21.5	21.5	0
35	26.3	26.3	0	30	MORT										
35	25.0	25.5	0.5												
		MEAN	0.12			MEAN	0.20			MEAN	0.08			MEAN	0.11

Table A-5

Growth of *I. recurvum* in Raw Sea Water for 21-Days

Initial length (mm), final length (mm) and growth (mm)/mortality presented.

	Init Lth	Final Lth	Gr		Init Lth	Fin Lth	Gr		Init Lth	Fin Lth	Gr
1	29.2	29.2	0	11	MORT			21	24.5	24.5	0
2	MORT			12	26.7	26.7	0	22	MORT		
3	MORT			13	MORT			23	MORT		
4	MORT			14	MORT			24	30.5	30.5	0
5	MORT			15	MORT			25	MORT		
6	MORT			16	MORT			26	MORT		
7	MORT			17	MORT			27	MORT		
8	27.2	27.2	0	18	25.2	25.2	0	28	MORT		
9	MORT			19	25.5	25.5	0	29	29.1	29.1	0
10	26.8	26.8	0	20	MORT			30	27.1	27.1	0
		MEAN	0			MEAN	0			MEAN	0

Table A-6

Growth of *I. recurvum* at Field Locations for 14-Days

Field Location, initial length (mm), final length (mm) and growth (mm)/removal presented.

BC	Init Lth	Final Lth	Gr	BC	Init Lth	Fin Lth	Gr	BC	Init Lth	Fin Lth	Gr	SRS	Init Lth	Fin Lth	Gr
1	20.0	22.1	2.1	2	19.3	20.0	0.7	3	16.7	17.9	1.2	SR	16.1	17.3	1.2
1	15.5	REM		2	17.0	19.1	2.1	3	15.6	16.4	0.8	SR	15.0	16.5	1.5
1	13.5	REM		2	19.2	20.1	0.9	3	12.5	14.8	2.3	SR	17.0	18.9	1.9
1	15.5	REM		2	18.5	19.5	1	3	12.5	REM		SR	15.2	17.8	2.6
1	14.0	REM		2	17.5	18.7	1.2	3	18.5	20.0	1.5	SR	15.5	17.3	1.8
1	15.1	REM		2	21.8	23.0	1.2	3	18.5	21.1	2.6	SR	18.0	19.1	1.1
1	13.5	REM		2	18.6	20.8	2.2	3	17.0	18.8	1.8	SR	16.0	17.9	1.9
1	15.5	REM		2	19.4	20.8	1.4	3	14.1	16.3	2.2	SR	19.5	20.5	1
1	17.5	REM		2	18.7	20.2	1.5	3	18.0	20.5	2.5	SR	17.0	18.2	1.2
1	16.5	REM		2	17.6	20.1	2.5	3	26.5	27.1	0.6	SR	14.0	16.0	2
1	13.5	REM		2	17.1	18.5	1.4	3	25.0	27.0	2				
1	17.1	REM		2	18.4	20.5	2.1	3	21.0	23.2	2.2				
1	20.5	REM		2	17.7	20.9	3.2	3	18.0	19.6	1.6				
1	22.2	REM		2	20.8	22.5	1.7	3	21.6	22.9	1.3				
1	22.8	REM		2	20.5	22.0	1.5	3	23.5	25.1	1.6				
1	20.5	REM		2	17.5	18.8	1.3	3	22.7	25.6	2.9				
1	19.6	REM		2	18.2	20.0	1.8	3	23.0	26.0	3				
1	19.5	REM		2	13.7	REM		3	18.3	20.3	2				
1	19.3	REM		2	15.6	16.8	1.2	3	22.5	23.5	1				
1	17.0	REM		2	17.7	19.5	1.8	3	17.0	19.3	2.3				
1	23.5	REM		2	19.3	20.8	1.5	3	12.9	13.7	0.8				
1	21.5	REM		2	15.3	16.2	0.9	3	14.5	15.9	1.4				
1	21.5	REM		2	20.6	23.5	2.9	3	13.7	18.9	5.2				
1	20.5	REM		2	17.3	21.2	3.9	3	12.3	14.0	1.7				
1	21.1	REM		2	16.6	18.9	2.3	3	20.0	22.0	2				
1	22.0	REM		2	18.2	20.0	1.8	3	19.7	REM					
1	18.7	REM		2	21.5	23.3	1.8	3	18.5	20.0	1.5				
1	23.7	REM		2	17.6	20.0	2.4	3	12.9	14.3	1.4				
1	23.0	REM		2	15.7	18.0	2.3	3	19.4	20.6	1.2				
1	21.5	REM		2	28.7	30.5	1.8	3	16.6	17.4	0.8				
						MEAN	1.8			MEAN	1.8			MEAN	1.62

Table A-7

Growth of juvenile *I. recurvum* at Field Locations for 14-Days

Field Location, initial length (mm), final length (mm) and growth (mm)/removal presented.

BC	Init Lth	Final Lth	Gr	BC	Init Lth	Fin Lth	Gr	BC	Init Lth	Fin Lth	Gr	SRS	Init Lth	Fin Lth	Gr
1	9.5	REM		2	6.5	7.0	0.5	3	8.7	9.8	1.1	SR	6.0	7.0	1
1	10.9	REM		2	5.0	5.3	0.3	3	7.1	8.0	0.9	SR	6.6	7.1	0.5
1	12.5	REM		2	5.1	6.5	1.4	3	8.2	8.7	0.5	SR	7.8	8.5	0.7
1	9.5	REM		2	6.2	7.5	1.3	3	7.6	8.9	1.3	SR	9.0	9.9	0.9
1	8.5	REM		2	4.7	5.2	0.5	3	7.5	9.0	1.5	SR	6.9	7.2	0.3
1	7.5	REM		2	6.2	7.2	1	3	6.7	8.0	1.3	SR	6.9	7.4	0.5
1	6.7	REM		2	4.6	5.2	0.6	3	8.1	8.8	0.7	SR	6.0	6.2	0.2
1	6.5	REM		2	5.5	6.0	0.5	3	6.5	7.2	0.7	SR	4.0	5.5	1.5
1	7.3	REM		2	5.0	REM		3	9.0	10.0	1	SR	5.0	5.2	0.2
1	5.3	REM		2	4.5	REM		3	7.3	8.0	0.7	SR	6.1	6.9	0.8
		MEAN	NA			MEAN	0.76			MEAN	0.97			MEAN	0.66

Table A-8

Diazinon 96-Hour LC50 Data for *Ischadium recurvum*

Diazinon concentration (ug/L), Exposure replicate number (10 per), Number dead, and Percent Mortality Reported.

Diaz Conc	Repl No.	No. Dead	% Mort.	Diaz Conc	Repl No.	No. Dead	% Mort.
1456	10	5	50	1715	10	6	60
	10	6	60		10	8	80
	10	8	80		10	10	100
	10	7	70		10	7	70
Total	40	26	65	Total	40	31	77.5
2300	10	10	100	3800	10	10	100
	10	10	100		10	10	100
	10	9	90		10	10	100
	10	10	100		10	10	100
Total	40	39	97.5	Total	40	40	100
5480	10	9	90	7040	10	10	100
	10	10	100		10	10	100
	10	9	90		10	10	100
	10	10	100		10	10	100
Total	40	38	95	Total	40	40	100
8950	10	10	100	Control	10	1	10
	10	10	100		10	1	10
	10	10	100		10	0	0
	10	10	100		10	1	10
Total	40	40	100	Total	40	3	7.5

Table A-9

Chlorpyrifos 96-Hour LC50 Data for *Ischadium recurvum*

Chlorpyrifos concentration (ug/L), Exposure replicate number (10 per), Number dead, and Percent Mortality Reported.

Chlo Conc	Repl No.	No. Dead	% Mort.	Chlo Conc	Repl No.	No. Dead	% Mort.
772	10	0	0	1134	10	7	70
	10	0	0		10	10	100
	10	4	40		10	8	80
	10	1	10		10	8	80
Total	40	5	12.5	Total	40	33	82.5
2550	10	10	100	4170	10	9	90
	10	10	100		10	9	90
	10	10	100		10	10	100
	10	8	80		10	10	100
Total	40	38	95	Total	40	38	95
7728	10	9	90	11528	10	10	100
	10	10	100		10	10	100
	10	10	100		10	10	100
	10	10	100		10	10	100
Total	40	39	97.5	Total	40	40	100
Control	10	2	20				
	10	0	0				
	10	1	10				
	10	0	0				
Total	40	3	7.5				

Table A-10

Mortality of *I. recurvum* in Diazinon Exposures

Diazinon concentration (μg/L), initial length (mm), and mortality (days) presented.

Diaz Conc	Init Lth	Mor	Diaz Conc	Init Lth	Mor	Diaz Conc	Init Lth	Mor	Diaz Conc	Init Lth	Mor	Diaz Conc	Init Lth	Mor
1.81	28.0		1281	26.5	7	1929	29.5	4	2600	25.7	5	5163	27.1	2
1.81	28.8	5	1281	30.3	9	1929	23.8	5	2600	30.5	2	5163	26.2	1
1.81	27.0		1281	31.5	9	1929	25.0	3	2600	28.0	2	5163	26.5	1
1.81	28.0		1281	30.0		1929	25.1	4	2600	31.3	4	5163	26.4	2
1.81	25.5	2	1281	27.5	5	1929	27.5		2600	26.6	4	5163	32.8	3
1.81	31.2		1281	27.5		1929	31.0	4	2600	31.2	4	5163	33.2	4
1.81	30.0	2	1281	25.9	4	1929	25.5	3	2600	27.5	1	5163	30.7	1
1.81	31.0	5	1281	29.1		1929	27.8	3	2600	26.9	1	5163	28.5	2
1.81	26.5		1281	25.0		1929	29.0	5	2600	31.0	1	5163	27.3	3
1.81	30.0	5	1281	28.0	9	1929	27.0	4	2600	30.0	2	5163	26.2	5
1.81	25.0		1281	28.0	4	1929	25.8	4	2600	30.4	4	5163	25.6	5
1.81	26.2	4	1281	26.5	3	1929	25.2	4	2600	25.9	4	5163	25.2	4
1.81	27.0	2	1281	32.2	9	1929	24.0	5	2600	30.0	3	5163	27.0	5
1.81	32.2	4	1281	29.9	10	1929	28.6	3	2600	25.8	4	5163	29.2	4
1.81	36.0		1281	28.0		1929	26.1	6	2600	27.0	2	5163	28.0	4
1.81	30.4	4	1281	29.3		1929	27.3	4	2600	30.0	5	5163	26.7	5
1.81	29.0	4	1281	25.0	6	1929	30.0	4	2600	24.5	3	5163	30.0	5
1.81	31.1	5	1281	26.0	4	1929	28.0	4	2600	26.0	5	5163	30.2	3
1.81	28.0	2	1281	25.1	3	1929	26.4	6	2600	28.0	2	5163	27.5	2
1.81	27.0	4	1281	25.0	5	1929	30.0	5	2600	24.5	5	5163	28.0	9
1.81	27.0		1281	26.0	6	1929	26.0	3	2600	28.0	3	5163	29.0	4
1.81	28.3	5	1281	27.8	3	1929	27.4	3	2600	24.8	4	5163	29.2	4
1.81	28.8	4	1281	28.6	4	1929	24.0	4	2600	31.8	1	5163	25.0	7
1.81	27.0	5	1281	29.6	5	1929	29.0	5	2600	26.2	5	5163	34.5	3
1.81	26.2		1281	25.8	5	1929	30.0	6	2600	28.5	3	5163	27.2	5
1.81	26.1	4	1281	30.0		1929	30.0	3	2600	28.0		5163	24.8	1
1.81	28.2	4	1281	26.2	8	1929	31.0	4	2600	22.8	1	5163	25.0	
1.81	28.0	5	1281	25.8	6	1929	27.0		2600	26.0	4	5163	28.0	4
1.81	26.9	4	1281	27.0	7	1929	28.0	5	2600	27.9	2	5163	28.9	4
1.81	30.8		1281	27.8		1929	27.5	6	2600	26.8	2	5163	31.1	4

Table A-11

Mortality of *I. recurvum* in Chlorpyrifos Exposures

Chlorpyrifos concentration (µg/L), initial length (mm), and mortality (days) presented.

Chlor Conc	Init Lth	Mor	Chlor Conc	Init Lth	Mor	Chlor Conc	Init Lth	Mor	Chlor Conc	Init Lth	Mor	Chlor Conc	Init Lth	Mor
0.557	25.0	9	345	26.4	3	1059	27.0	10	2400	25.6	4	5495	21.0	3
0.557	28.9		345	27.8	3	1059	25.8	11	2400	27.8	11	5495	25.0	3
0.557	23.2	13	345	28.2	5	1059	25.2		2400	27.2	10	5495	24.0	3
0.557	28.9	9	345	26.0	5	1059	21.4	7	2400	27.0	10	5495	27.5	4
0.557	25.0		345	22.0	5	1059	27.8	10	2400	23.6	4	5495	21.0	5
0.557	23.0	11	345	26.2	8	1059	26.5	6	2400	26.0	12	5495	27.8	3
0.557	28.0	8	345	22.2	6	1059	27.0	8	2400	24.2	11	5495	23.5	3
0.557	26.0	7	345	25.0	7	1059	24.0		2400	24.8	4	5495	25.0	4
0.557	23.2	9	345	26.1		1059	27.9	7	2400	27.5	3	5495	25.0	4
0.557	27.2	5	345	27.2	4	1059	29.0		2400	21.0	9	5495	25.0	4
0.557	25.6	5	345	26.5	5	1059	23.0	10	2400	20.8	10	5495	27.0	3
0.557	29.0	14	345	26.2		1059	26.0	7	2400	22.3	9	5495	27.0	4
0.557	26.5		345	29.0	5	1059	29.3	8	2400	20.5	5	5495	25.0	5
0.557	23.5	9	345	23.8	5	1059	26.6		2400	21.5	12	5495	20.0	4
0.557	25.0		345	26.2	5	1059	21.9		2400	23.1	10	5495	20.0	
0.557	26.0	5	345	24.5	8	1059	22.6	8	2400	24.0	4	5495	24.8	5
0.557	24.6	4	345	26.8	6	1059	25.7	3	2400	28.8		5495	26.5	4
0.557	24.8	5	345	29.9	5	1059	20.2		2400	21.5	3	5495	26.2	4
0.557	23.9		345	26.1	3	1059	25.0	3	2400	25.7	7	5495	25.2	5
0.557	25.6	5	345	24.0	11	1059	28.0		2400	25.0	9	5495	21.9	5
0.557	27.2	4	345	26.0	10	1059	24.0	6	2400	22.5	8	5495	24.2	3
0.557	27.0		345	27.2	8	1059	23.0		2400	21.9	7	5495	20.1	4
0.557	26.5		345	27.9	6	1059	21.2	8	2400	22.0	9	5495	23.4	3
0.557	27.5		345	25.2	3	1059	26.2	12	2400	25.5	7	5495	22.1	5
0.557	24.3	11	345	27.0		1059	27.0	10	2400	20.4	10	5495	21.9	7
0.557	21.8	5	345	24.2		1059	25.0	8	2400	26.3	4	5495	25.0	3
0.557	24.1		345	30.0	11	1059	26.2	8	2400	21.0	7	5495	22.1	3
0.557	26.0	5	345	27.1	10	1059	23.5		2400	27.0	7	5495	23.0	4
0.557	24.5	7	345	26.0	8	1059	25.8		2400	21.2	7	5495	21.8	4
0.557	23.0		345	30.8		1059	25.0	7	2400	22.9	4	5495	28.0	4

Table A-12

Upper Temperature Tolerance of *Ischadium recurvum* at Four Acclimation Temperatures

Acclimation Temperature (Ac Tp), Heat Coma Temperature (HCT), Length (Lth), and Mean Heat Coma Temperature ($\bar{x}$) Reported.

Ac Tp	Lth	HCT	$\bar{x}$	Ac Tp	Lth	HCT	$\bar{x}$	Ac Tp	Lth	HCT	$\bar{x}$	Ac Tp	Lth	HCT	$\bar{x}$
35	27.0	44.4	45.7	30	23.5	44.5	45.4	25	26.0	42.9	43.4	20	24.0	42.8	43.7
35	27.5	46.3		30	25.0	44.1		25	23.5	43.2		20	25.2	44.0	
35	27.2	45.3		30	27.5	45.5		25	21.0	42.9		20	24.8	42.7	
35	27.8	44.4		30	21.5	44.0		25	26.8	43.0		20	25.0	43.6	
35	22.0	46.3		30	26.0	45.1		25	28.0	42.8		20	23.0	43.6	
35	26.0	45.3		30	24.0	45.8		25	27.0	45.0		20	22.0	42.9	
35	26.0	44.4		30	23.0	46.4		25	26.0	44.2		20	21.0	43.8	
35	23.0	46.3		30	26.0	45.0		25	25.5	43.3		20	26.5	44.9	
35	25.0	45.3		30	23.5	46.3		25	22.5	44.9		20	28.0	44.9	
35	25.0	44.4		30	27.5	45.1		25	27.5	42.8		20	27.0	44.2	
35	24.0	46.3		30	24.0	46.8		25	23.0	43.5		20	26.3	43.7	
35	26.0	45.3		30	27.0	46.0		25	22.6	42.8		20	25.5	44.0	
35	25.0	44.4		30	27.0	46.5		25	22.0	44.5		20	23.0	44.0	
35	21.0	46.3		30	24.0	45.1		25	20.0	45.0		20	27.0	42.8	
35	21.0	45.3		30	25.0	46.3		25	26.8	43.9		20	27.7	44.6	
35	23.0	46.5		30	24.8	46.0		25	25.8	44.8		20	24.0	43.5	
35	22.0	46.6		30	25.0	46.5		25	26.5	45.1		20	25.0	43.2	
35	28.0	46.2		30	23.0	45.8		25	27.0	43.6		20	24.0	43.7	
35	20.0	46.2		30	23.0	44.4		25	26.5	43.3		20	25.3	43.1	
35	23.0	46.1		30	22.8	45.6		25	23.0	45.7		20	23.9	43.8	
35	24.2	45.8		30	26.0	45.1		25	26.5	44.5		20	23.0	43.2	
35	24.5	45.9		30	26.0	45.1		25	27.0	44.1		20	24.8	43.8	
35	25.5	46.6		30	25.5	45.5		25	25.5	44.5					
35	23.3	45.9		30	29.0	43.6									
35	22.6	45.5		30	21.0	44.6									
35	24.6	46.2		30	20.0	46.0									
35	23.0	46.2		30	26.0	45.4									

Table A-13 (A)

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Chlorpyrifos Exposures

Chlorpyrifos Concentration ([Chlor] in ug/L), Heat Coma Temperature (HCT), Length (Lth in mm), Weight (Wt in g)and Means Reported.

[Chlor]	HCT	Lth	Wt	[Chlor]	HCT	Lth	Wt
0.6	44.0	37.0	5.5	78	43.8	37	5.5
0.6	43.3	26.5	2.4	78	41.1	29	3.6
0.6	44.3	31.0	3.4	78	43.9	29	3.8
0.6	43.7	25.0	1.9	78	44.1	29	2.8
0.6	44.2	30.0	3.3	78	43.3	34	5.5
0.6	43.8	28.0	2.2	78	43.4	30	3.4
0.6	43.2	34	4.6	78	44.9	35	5.6
0.6	44.2	31	3.2	78	43.4	30	3.7
0.6	43.5	30	5.0	78	43.6	34	3.3
0.6	42.5	28	2.0	78	42.5	33	5.6
0.6	45.0	34	6.2	78	37.8	35	3.5
0.6	43.7	29	3.2	78	41.5	29	2.7
0.6	43.9	32	5.5	78	38.0	31	3.3
0.6	43.7	28	3.4	78	44.2	32	3.8
0.6	43.9	26	2.2	78	43.9	34	5.5
0.6	43.9	32	3.7	78	40.9	28	3.2
0.6	43.9	27	2.9	78	40.9	27	2.4
0.6	39.0	27	2.2	78	41.7	27	2.5
Mean	**43.37**	**29.83**	**3.68**	**Mean**	**41.94**	**31.25**	**3.76**

Table A-13 (B)

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Chlorpyrifos Exposures

Chlorpyrifos Concentration ([Chlor] in ug/L), Heat Coma Temperature (HCT), Length (Lth), Weight (Wt) and Means reported.

[Chlor]	HCT	Lth	Wt	[Chlor]	HCT	Lth	Wt
86	42.4	28	2.8	165	40.9	32	
86	42.1	28	2.5	165	43.4	29	2.9
86	43.9	31	2.6	165	43.7	28	2.9
86	42.2	35	4.5	165	43.7	31	3.4
86	42.0	34	2.5	165	43.1	29	3.2
86	39.6	33	2.9	165	40.9	29	4.4
86	40.2	31	3.4	165	42.9	30	4.6
86	40.2	30.5	2.6	165	43.9	29	3.2
86	42.8	31	4.5	165	37.8	26	2.4
86	41.2	34	4.9	165	39.6	32	3.1
86	43.3	31	3.1	165	43.9	34	6.1
86	43.5	32	3.9	165	41.7	27	2.6
86	42.1	30	3.2	165	43.7	32	3.1
86	43.4	27.5	1.8	165	43.0	28	3.4
86	44.9	26	3.4	165	44.0	32	3.6
86	42.2	37	3.5	165	45.7	24	3.7
86	43.0	28	3.2	165	41.4	25	3.7
86	42.5	34	3.5	165	Mort		
Mean	**42.53**	**30.96**	**3.03**	**Mean**	**42.69**	**28.94**	**3.46**

Table A-13 (C)

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Chlorpyrifos Exposures

Chlorpyrifos Concentration ([Chlor] in ug/L), Heat Coma Temperature (HCT) Length (Lth), Weight (Wt) and Means reported.

[Chlor]	HCT	Lth	Wt	[Chlor]	HCT	Lth	Wt
344	39.9	31	3.0	727	39.8	32	3.5
344	41.1	27	2.5	727	42.8	32	3.9
344	44.9	25	2.1	727	42.8	35	5.5
344	42.1	30	2.5	727	39.2	28	2.3
344	43.4	29	3.3	727	40.8	31	3.9
344	43.4	37	5.8	727	36.1	37	5.8
344	42.4	28	2.4	727	42.0	29	3.4
344	42.4	27	2.0	727	37.1	35	6.3
344	42.4	32	3.7	727	38.8	35	4.8
344	42.5	36	4.5	727	43.4	32	6.7
344	42.6	35	3.8	727	41.6	27	2.3
344	42.9	30	3.2	727	39.3	31	3.3
344	42.0	28	2.6	727	41.4	30	3.4
344	40.1	43	5.8	727	38.4	25	2.9
344	43.3	31	4.0	727	35.4	31	3.6
344	42.3	29	4.2	727	36.0	27	2.7
344	43.8	30	3.6	727	41.4	26	2.7
344	43.8	27	2.7	727	43.0	28	2.7
Mean	**42.52**	**30.83**	**3.43**	**Mean**	**39.82**	**29.67**	**3.73**

Table A-14

Upper Temperature Tolerance of *Ischadium recurvum* After 24-Hour Rinse Following Chlorpyrifos Exposures

Chlorpyrifos Concentration ([Chlor] in ug/L), Heat Coma Temperature (HCT), Length (Lth), Weight (Wt) and Means reported.

[Chlor]	HCT	Lth	Wt	[Chlor]	HCT	Lth	Wt
0.6	43.7	29	3.2	78	43.4	29	
0.6	43.7	32	5.5	78	43.3	37	4.8
0.6	43.7	28	3.4	78	39.6	34	4.6
0.6	43.9	26	2.2	78	43.8	32	5.5
0.6	43.9	32	3.7	78	43.8	31	3.8
0.6	43.9	27	2.9	78	43.1	29	2.6
0.6	44.6	34	4.6	78	43.1	30	2.4
0.6	43.6	30	4.2	78	45.3	27	2.8
0.6	45.1	32	5.0	78	42.9	26	2.3
0.6	44.4	31	4.9	78	39.7	37	4.4
0.6	45.1	31	3.1	78	38.3	26	2.8
0.6	44.4	34	5.9	78	44.7	29	2.7
Mean	**44.17**	**30.5**	**4.05**	**Mean**	**42.51**	**30.7**	**3.5**
86	42.7	34	4.6	344	43.8	30	3.4
86	44.5	37	7.9	344	38.5	35	4.5
86	43.2	27	2.6	344	40.2	28	2.6
86	41.4	35	3.9	344	42.1	36	17
86	41.9	29	2.4	344	39.1	39	4.5
86	39.3	30	2.5	344	44.7	31	3.3
86	43.9	30	3.5	344	36.9	25	2.0
86	44.3	27	3.0	344	45.5	29	3.6
86	44.7	36	4.3	344	45.5	25	1.9
86	38.5	34	4.1	344	40.3	26	2.3
86	40.4	34	5.7	344	39.0	23	1.7
86	44.5	32	3.5	344	40.9	27	2.5
Mean	**42.44**	**32.1**	**4.0**	**Mean**	**41.38**	**29.5**	**3.15**

Table A-15 (A)

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Diazinon Exposures

Diazinon Concentration ([Diaz] in ug/L), Heat Coma Temperature (HCT), Length (Lth), Weight (Wt) and Means Reported.

[Diaz]	HCT	Lth	Wt	[Diaz]	HCT	Lth	Wt
0.024	42.8	33	3.2	100	45.0	33	4.5
0.024	44.2	31	4.0	100	45.0	35	6.0
0.024	45.3	37	5.6	100	41.5	28	2.6
0.024	43.7	42	7.7	100	45.1	29	3.2
0.024	45.3	31	3.0	100	39.2	30	3.7
0.024	45.3	28	3.6	100	44.9	28	3.1
0.024	43.9	31	3.9	100	44.1	32	4.6
0.024	43.0	30	2.8	100	45.0	33	3.8
0.024	43.9	32	3.6	100	44.9	35	5.3
0.024	42.8	35	5.7	100	44.9	29	3.7
0.024	43.9	33	4.7	100	44.9	29	2.9
0.024	42.6	26	2.3	100	44.8	32	3.1
0.024	44.0	30	3.0	100	44.2	31	3.8
0.024	44.0	30	3.8	100	38.4	35	4.9
0.024	43.8	30	2.8	100	43.7	33	4.0
0.024	44.2	31	3.5	100	45.3	25	2.5
0.024	44.4	30	3.9	100	43.4	27	3.6
0.024	44.0	23	4.7	100	Mort		
Mean	**43.95**	**31.3**	**4.0**	**Mean**	**43.78**	**30.8**	**3.8**

Table A-15 (B)

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Diazinon Exposures

Diazinon Concentration ([Diaz] in ug/L), Heat Coma Temperature (HCT), Length (Lth), Weight (Wt) and Means Reported.

[Diaz]	HCT	Lth	Wt	[Diaz]	HCT	Lth	Wt
136	44.7	36	4.4	258	43.8	31	5.0
136	44.8	27	2.2	258	45.4	28	3.4
136	44.1	32	3.1	258	42.0	37	4.9
136	43.8	30	3.5	258	43.8	28	4.0
136	44.5	38	6.2	258	43.8	33	2.7
136	42.2	28	3.0	258	44.7	26	2.0
136	45.3	30	3.5	258	39.7	34	3.3
136	43.2	31	3.2	258	42.6	28	2.7
136	44.9	32	4.0	258	43.1	30	3.0
136	43.3	27	2.8	258	41.3	30	2.4
136	43.9	32	3.7	258	43.2	32	3.3
136	43.3	31	3.9	258	40.4	28	3.6
136	44.4	36	4.7	258	43.3	28	3.4
136	44.4	34	5.6	258	42.9	31	3.8
136	44.6	26	2.9	258	44.6	28	2.6
136	44.6	32	4.2	258	45.0	31	3.0
136	38.6	28	4.2	258	44.1	32	3.5
136	41.2	33	4.3	258	44.4	36	6.4
Mean	**43.66**	**31.3**	**3.9**	**Mean**	**43.23**	**30.6**	**3.5**

Table A-15 (C)

Upper Temperature Tolerance of *Ischadium recurvum* for 72-Hour Diazinon Exposures

Diazinon Concentration ([Diaz] in ug/L), Heat Coma Temperature (HCT), Length (Lth), Weight (Wt) and Means Reported.

[Diaz]	HCT	Lth	Wt	[Diaz]	HCT	Lth	Wt
496	42.3	31	4.2	1314	40.1	26	2.4
496	42.3	30	2.8	1314	40.1	28	2.8
496	43.0	29	4.7	1314	39.2	32	3.6
496	45.2	34	3.3	1314	40.8	34	3.9
496	43.9	27	2.8	1314	40.2	32	3.7
496	42.4	32	3.6	1314	42.0	34	4.3
496	42.3	33	4.1	1314	40.9	30	3.5
496	39.6	30	2.7	1314	38.4	41	8.0
496	42.3	30	3.6	1314	36.7	31	2.7
496	44.9	28	3.3	1314	40.0	26	2.3
496	43.5	30	3.5	1314	35.1	31	3.6
496	43.5	31	3.3	1314	41.3	32	3.5
496	38.8	32	3.5	1314	39.3	34	4.4
496	35.7	28	2.8	1314	35.5	34	4.6
496	42.9	29	3.1	1314	39.5	31	3.9
496	41.4	34	3.4	1314	41.6	33	4.1
496	41.8	33	5.0	1314	36.1	36	5.0
496	44.6	31	3.5	1314	37.2	27	2.5
Mean	**42.24**	**30.7**	**3.5**	**Mean**	**39.11**	**31.8**	**3.8**

Table A-16

Upper Temperature Tolerance of *Ischadium recurvum* After 24-Hour Rinse Following Diazinon Exposures

Diazinon Concentration ([Diaz] in ug/L), Heat Coma Temperature (HCT), Length (Lth), Weight (Wt) and Means Reported.

[Chlor]	HCT	Lth	Wt	[Chlor]	HCT	Lth	Wt
0.024	44.6	29	3.8	100	44.6	30	3.1
0.024	44.2	35	4.0	100	45.0	31	3.0
0.024	44.2	33	3.7	100	43.5	26	
0.024	44.4	36	4.4	100	39.2	27	2.1
0.024	44.2	29	3.5	100	45.0	27	2.8
0.024	44.0	27	2.1	100	39.0	27	2.2
0.024	44.0	32	3.8	100	39.1	32	2.8
0.024	43.6	29		100	43.5	28	2.8
0.024	43.6	35	4.4	100	43.8	34	3.1
0.024	43.6	34	3.4	100	45.0	32	4.9
0.024	42.0	30	2.7	100	43.5	35	5.1
0.024	40.8	31	3.0	100	43.5	35	5.2
Mean	**43.55**	**31.9**	**3.5**	**Mean**	**42.84**	**30.7**	**3.4**
136	38.7	27	2.7	496	45.0	28	3.4
136	38.8	29	2.7	496	36.8	28	2.7
136	44.6	31	3.4	496	45.0	27	2.5
136	42.7	29	2.0	496	40.0	29	1.8
136	43.8	34	3.3	496	44.5	28	3.1
136	40.9	27	2.6	496	45.1	30	3.7
136	42.2	35	4.7	496	42.0	25	2.0
136	42.2	27	2.7	496	42.0	28	2.5
136	42.8	28	3.0	496	43.0	30	3.1
136	43.8	37	4.8	496	44.3	29	3.6
136	42.6	38	4.2	496	38.9	32	3.1
136	40.2	31	2.8	496	40.2	35	2.4
Mean	**41.94**	**31.1**	**3.2**	**Mean**	**42.23**	**29.1**	**2.8**

Table A-17

Diazinon 96-Hour LC50 Data for *Corbicula fluminea*

Diazinon concentration (ug/L), Exposure replicate number (10 per), Number dead, and Percent Mortality Reported.

Diaz Conc	Repl No.	No. Dead	% Mort.	Diaz Conc	Repl No.	No. Dead	% Mort.
919.64	6	0	0	1,875.04	6	1	17
	6	0	0		6	1	17
	6	0	0		6	1	17
	6	0	0		6	1	17
Total	24	0	0	Total	24	4	17
3,785.84	6	4	67	7,607.46	6	4	67
	6	3	50		6	3	50
	6	5	83		6	4	67
	6	3	50		6	4	67
Total	24	15	63	Total	24	15	63
15,250.68	6	4	67	Control	6	0	0
	6	6	100		6	0	0
	6	4	67		6	0	0
	6	5	83		6	0	0
Total	24	19	79	Total	24	0	0

Table A-18

Diazinon 21-Day LC50 Data for *Corbicula fluminea*

Diazinon concentration (ug/L), Exposure replicate number (10 per), Number dead, and Percent Mortality Reported.

Diaz Conc	Repl No.	No. Dead	% Mort.	Diaz Conc	Repl No.	No. Dead	% Mort.
59.0	6	0	0	155.32	6	1	17
	6	0	0		6	0	0
	6	0	0		6	0	0
Total	18	0	0	Total	18	1	6
346.40	6	1	17	728.56	6	6	100
	6	0	0		6	2	33
	6	0	0		6	6	100
Total	18	1	6	Total	18	14	78
1,492.9	6	6	100	3021.52	6	6	100
	6	6	100		6	6	100
	6	6	100		6	6	100
Total	18	18	100	Total	18	18	100
Control	6	* 1	17				
	6	* 3	50				
	6	* 6	100				
Total	18	* 10	56				

* All mortality occurred after day 10.

Table A-19

Upper Temperature Tolerance of *Corbicula fluminea* at Three Acclimation Temperatures

Acclimation Temperature (°C), Heat Coma Temperature (HCT; °C), Length (Lth; mm), and Means Reported.

Accl Temp	HCT	Lth	Accl Temp	HCT	Lth	Accl Temp	HCT	Lth
10	32.1	16.05	20	43.9	19.24	30	42.5	16.6
10	27.4	20.04	20	43.5	17.6	30	43.9	19.5
10	32.1	18.38	20	44.5	17.46	30	44.6	18.2
10	28.1	19.84	20	43.5	16.95	30	45.1	17.2
10	32.2	20.24	20	42.8	16.43	30	46.2	20.4
10	29.9	17.98	20	45.4	17.35	30	44.2	17.7
10	29.0	17.64	20	47.5	16.55	30	44.5	20.9
10	28.1	19.62	20	44.9	15.85	30	45.1	18.4
10	26.0	17.64	20	44.4	14.13	30	43.3	20.2
10	25.8	21.05	20	45.0	16.15	30	44.2	18.6
10	26.2	18.18	20	43.9	16.84	30	44.9	17.4
10	28.1	18.36	20	45.2	13.66	30	44.9	15.4
10	27.1	16.78	20	44.6	18.36	30	43.2	19.3
10	27.2	20.47	20	43.4	16.85	30	43.9	18.5
10	40.2	19.66	20	44.1	17.15	30	43.6	17.6
10	28.1	16.81	20	44.3	15.58	30	43.5	17.8
10	26.5	18.47	20	44.1	17.38	30	43.6	19.4
10	27.1	17.31	20	44.1	17.87	30	42.7	18.7
10	26.4	20.16	20	43.5	17.53	30	44.6	17.8
10	28.1	18.50	20	44.6	18.67	30	44.9	16.9
10	38.1	17.41	20	44.6	17.35	30	44.2	19.3
10	28.6	19.14	20	44.4	18.46	30	44.6	17.2
10	26.6	16.88	20	44.4	18.51	30	45.8	19.1
10	26.5	18.20	20	44.4	17.3	30	44.7	18.2
Mean	**28.9**	**18.5**	**Mean**	**44.3**	**17.1**	**Mean**	**44.2**	**18.4**

Table A-20 (A)

Upper Temperature Tolerance of *Corbicula fluminea* for Diazinon Exposures at 10°C

Diazinon Concentration (ug/L), Heat Coma Temperature (HCT; °C), Length (Lth;mm), and Means Reported.

[Diaz]	HCT	Lth	[Diaz]	HCT	Lth	[Diaz]	HCT	Lth	[Diaz]	HCT	Lth
771.5	42.2	16.09	250.75	33.2	17.57	177.45	30.5	18.06	54.55	43.9	20.39
771.5	28.3	17.87	250.75	40.9	18.75	177.45	29.2	18.23	54.55	41.7	19.91
771.5	32.2	17.98	250.75	35.7	18.73	177.45	28.1	18.34	54.55	40.5	18.48
771.5	29.3	16.52	250.75	31.4	17.19	177.45	33.1	17.57	54.55	31.4	18.50
771.5	27.8	17.13	250.75	28.1	19.2	177.45	30.4	18.70	54.55	40.0	19.38
771.5	30.8	16.74	250.75	42.1	19.46	177.45	38.0	16.48	54.55	27.2	18.37
771.5	38.2	18.72	250.75	38.1	20.04	177.45	39.9	16.52	54.55	43.2	18.27
771.5	36.2	17.49	250.75	39.8	19.41	177.45	41.8	17.89	54.55	41.9	19.08
771.5	31.4	19.46	250.75	30.3	20.16	177.45	39.1	16.21	54.55	40.5	17.62
771.5	44.7	19.19	250.75	38.8	17.17	177.45	38.3	17.84	54.55	42.0	19.12
771.5	43.1	18.09	250.75	29.2	16.78	177.45	41.7	19.30	54.55	40.7	17.57
771.5			250.75	29.1	17.97	177.45	29.1	18.23	54.55		
Mean	**34.9**	**17.8**	**Mean**	**34.7**	**18.5**	**Mean**	**34.9**	**17.8**	**Mean**	**39.4**	**18.8**

Table A-20 (B)

Upper Temperature Tolerance of *Corbicula fluminea* for Diazinon Exposures at 20°C

Diazinon Concentration (ug/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Diaz]	HCT	Lth	[Diaz]	HCT	Lth	[Diaz]	HCT	Lth	[Diaz]	HCT	Lth
771.5	44.0	16.62	250.75	45.0	17.73	177.45	42.8	15.1	54.55	46.3	17.17
771.5	44.0	17.38	250.75	44.7	17.80	177.45	43.9	16.61	54.55	44.2	17.45
771.5	45.5	13.84	250.75	44.9	16.50	177.45	43.2	19.57	54.55	44.2	16.41
771.5	45.0	18.68	250.75	45.0	14.08	177.45	43.6	18.00	54.55	44.4	19.52
771.5	45.5	18.31	250.75	44.2	18.50	177.45	43.2	14.51	54.55	43.8	15.70
771.5	45.5	19.79	250.75	45.1	17.39	177.45	43.0	21.28	54.55	43.6	18.85
771.5	43.1	17.51	250.75	45.0	18.07	177.45	45.1	14.22	54.55	45.0	18.02
771.5	44.9	20.0	250.75	46.8	17.91	177.45	44.9	17.99	54.55	44.5	16.77
771.5	42.9	18.3	250.75	44.9	15.89	177.45	43.6	17.18	54.55	43.3	14.13
771.5	43.8	14.09	250.75	44.9	18.96	177.45	44.0	16.62	54.55	44.2	18.51
771.5	42.9	17.09	250.75	44.4	18.47	177.45	45.1	16.58	54.55	45.5	16.92
771.5	44.2	18.55	250.75	45.0	17.4	177.45			54.55	45.3	18.88
Mean	**44.3**	**17.5**	**Mean**	**45.0**	**17.4**	**Mean**	**43.9**	**17.1**	**Mean**	**44.5**	**17.4**

Table A-20 (C)

Upper Temperature Tolerance of *Corbicula fluminea* for Diazinon Exposures at 30°C

Diazinon Concentration (ug/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Diaz]	HCT	Lth	[Diaz]	HCT	Lth	[Diaz]	HCT	Lth	[Diaz]	HCT	Lth
771.5	44.9	19.5	250.75	46.1	20.9	177.45	46.0	17.5	54.55	45.0	17.8
771.5	46.2	18.9	250.75	41.7	17.7	177.45	44.5	20.3	54.55	44.7	17.4
771.5	46.3	18.3	250.75	42.8	16.2	177.45	43.4	15.8	54.55	44.9	16.7
771.5	44.0	17.0	250.75	45.8	21.4	177.45	46.1	21.2	54.55	43.9	19.2
771.5	45.9	17.3	250.75	43.1	18.0	177.45	46.1	18.2	54.55	45.2	19.9
771.5	43.1	16.8	250.75	43.1	16.5	177.45	44.8	19.4	54.55	42.3	17.9
771.5	44.8	18.9	250.75	48.4	18.4	177.45	44.9	17.1	54.55	42.9	18.1
771.5	43.8	15.9	250.75	45.3	17.2	177.45	44.4	17.8	54.55	46.3	18.3
771.5	40.8	21.8	250.75	44.2	15.5	177.45	44.5	18.2	54.55	44.3	18.4
771.5	46.3	19.0	250.75	44.4	18.4	177.45	43.3	17.2	54.55	49.4	21.2
771.5	49.5	18.6	250.75	43.7	16.1	177.45	45.4	18.7	54.55	45.5	17.0
771.5	44.5	17.6	250.75	47.3	17.3	177.45			54.55	45.4	17.8
Mean	**45.0**	**18.3**	**Mean**	**44.7**	**17.8**	**Mean**	**44.9**	**18.3**	**Mean**	**45.0**	**18.3**

Table A-21 (A)

Upper Temperature Tolerance of *Corbicula fluminea* for Copper Exposures at 10°C

Copper Concentration (ug/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Cu]	HCT	Lth	[Cu]	HCT	Lth	[Cu]	HCT	Lth	[Cu]	HCT	Lth
1311	26.3	18.53	710	25.5	15.87	350	26.0	18.62	205	24.5	20.3
1311	26.5	20.02	710	26.0	19.46	350	25.9	18.49	205	30.1	20.10
1311	26.9	19.45	710	44.1	20.39	350	34.6	19.84	205	24.5	17.2
1311	25.1	18.91	710	26.4	18.01	350	29.3	19.12	205	43.2	18.9
1311	26.9	19.50	710	25.6	17.81	350	25.9	17.16	205	25.9	18.9
1311	42.0	18.24	710	25.5	19.58	350	26.0	18.4	205	43.3	17.7
1311	25.1	19.5	710	26.6	17.0	350	39.9	21.3	205	26.3	18.0
1311	25.3	17.3	710	24.6	17.3	350	25.9	18.1	205	25.6	18.1
1311	41.5	18.0	710	25.2	18.8	350	40.9	19.1	205	38.6	20.0
1311	23.5	19.5	710	25.4	19.2	350	28.9	21.0	205	26.0	17.4
1311	26.6	18.0	710	24.5	18.4	350	26.2	19.4	205	25.5	20.8
1311	24.8	17.3	710	25.4	19.10				205	28.8	17.5
Mean	**28.4**	**18.7**	**Mean**	**27.1**	**18.4**	**Mean**	**30.0**	**19.1**	**Mean**	**30.2**	**18.7**
179	25.0	17.99	111	24.0	20.1						
179	24.4	17.72	111	25.1	17.4						
179	24.5	17.66	111	42.9	19.7						
179	26.3	16.60	111	24.3	19.6						
179	24.9	17.91	111	26.1	18.8						
179	41.9	19.81	111	24.0	17.5						
179	24.7	18.8	111	25.4	19.3						
179	26.8	21.6	111	25.3	18.9						
179	25.5	17.9	111	25.3	16.8						
179	25.5	18.1	111	32.2	18.8						
179	25.5	17.8	111	29.2	18.7						
179	25.6	19.3	111	25.1	17.0						
Mean	**26.7**	**18.4**	**Mean**	**27.4**	**18.6**						

Table A-21 (B)

Upper Temperature Tolerance of *Corbicula fluminea* for Copper Exposures at 20°C

Copper Concentration (ug/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Cu]	HCT	Lth	[Cu]	HCT	Lth	[Cu]	HCT	Lth	[Cu]	HCT	Lth
1311	45.1	13.56	710	44.6	12.07	350	44.3	17.01	205	40.1	13.33
1311	44.5	17.46	710	44.6	18.15	350	44.5	12.94	205	44.0	12.89
1311	42.9	15.99	710	43.9	13.18	350	44.6	12.55	205	45.4	13.02
1311	44.9	11.48	710	40.4	12.95	350	42.3	14.34	205	45.0	12.13
1311	44.4	15.53	710	42.2	14.98	350	44.9	17.03	205	44.0	12.51
1311	42.5	15.82	710	41.9	16.6	350	43.8	17.35	205	44.0	18.91
1311	43.9	18.47	710	43.3	12.91	350	44.5	13.73	205	42.4	12.46
1311	44.2	19.08	710	44.4	15.72	350	43.8	13.5	205	44.7	12.78
1311	44.0	19.68	710	42.4	18.46	350	42.5	17.22	205	43.8	13.06
1311	43.8	16.39	710	44.3	15.4	350	44.2	12.85	205	44.5	17.28
			710	43.4	13.55	350	44.5	12.59	205	42.4	17.45
			710	44.1	13.09	350	43.7	13.91	205	44.9	12.65
Mean	**44.0**	**16.3**	**Mean**	**43.3**	**14.8**	**Mean**	**44.0**	**14.6**	**Mean**	**43.8**	**14.0**
179	42.9	16.6	111	41.0	14.04						
179	43.3	12.03	111	42.4	17.71						
179	41.1	15.21	111	44.4	13.97						
179	43.3	13.19	111	44.4	17.59						
179	42.5	14.64	111	44.0	16.39						
179	42.4	17.02	111	41.0	17.54						
179	42.5	13.45	111	43.4	13.9						
179	42.5	14.48	111	44.0	17.78						
179	42.4	13.31	111	42.5	15.95						
179	42.5	20.57	111	43.7	19.98						
179	44.8	17.29	111	43.3	13.36						
179	41.4	17.47	111	41.7	19.01						
Mean	**42.6**	**15.4**	**Mean**	**43.0**	**16.4**						

Table A-21 (C)

Upper Temperature Tolerance of *Corbicula fluminea* for Copper Exposures at 30°C

Copper Concentration (ug/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Cu]	HCT	Lth	[Cu]	HCT	Lth	[Cu]	HCT	Lth	[Cu]	HCT	Lth
1311	42.8	18.6	710	43.0	19.6	350	42.6	16.2	205	42.7	17.5
1311	40.5	19.7	710	40.5	19.6	350	39.9	17.0	205	41.8	20.4
1311	42.8	20.3	710	40.0	18.9	350	43.3	20.2	205	42.2	19.8
1311	43.0	18.4	710	42.6	20.8	350	42.6	17.7	205	40.5	16.6
1311	39.1	18.8	710	40.0	19.6	350	41.6	20.8	205	41.6	18.0
1311	40.8	18.7	710	39.9	19.9	350	40.3	19.2	205	45.5	20.0
1311	40.8	16.8	710	41.3	17.6	350	40.2	17.3	205	39.7	18.5
1311	40.8	18.5	710	40.8	18.6	350	40.2	17.6	205	42.5	17.8
1311	43.1	19.6	710	41.2	20.6	350	40.7	16.9	205	41.5	19.0
1311	45.0	17.7	710	39.6	18.2	350	39.9	18.7	205	40.2	17.0
1311	40.8	19.4	710	42.7	20.2	350	40.3	17.1	205	42.0	16.8
			710	40.1	19.6	350	44.6	18.9	205	41.4	19.9
Mean	**41.8**	**18.8**	**Mean**	**41.0**	**19.4**	**Mean**	**41.4**	**18.1**	**Mean**	**41.8**	**18.4**
179	41.2	18.4	111	43.2	16.6						
179	42.8	17.7	111	43.8	17.1						
179	42.8	17.1	111	45.1	21.6						
179	43.1	19.5	111	46.1	17.23						
179	43.6	16.9	111	43.4	21.5						
179	44.2	19.8	111	42.2	17.4						
179	42.5	14.8	111	43.9	17.8						
179	42.8	20.2	111	43.1	18.1						
179	42.8	17.4	111	45.2	18.1						
179	41.6	19.5	111	42.5	17.6						
179	41.3	19.0	111	42.9	20.1						
			111	43.1	15.4						
Mean	**42.6**	**18.2**	**Mean**	**43.7**	**18.2**						

Table A-22 (A)

Upper Temperature Tolerance of *Corbicula fluminea* for Chlorine Exposures at 10°C

Chlorine Concentration (mg/L) Coma Temperature (HCT), Length (Lth), and Means Reported.

[Cl]	HCT	Lth	[Cl]	HCT	Lth	[Cl]	HCT	Lth	[Cl]	HCT	Lth
5.7	27.4	18.9	3.1	26.1	19.5	1.41	25.9	17.6	0.6	25.4	18.2
5.7	40.3	17.4	3.1	26.1	18.9	1.41	25.2	20.4	0.6	26.7	20.3
5.7	36.3	21.0	3.1	24.7	18.6	1.41	27.9	20.6	0.6	39.1	20.9
5.7	25.3	18.8	3.1	26.0	18.4	1.41	25.1	18.4	0.6	25.1	20.5
5.7	32.4	17.0	3.1	25.2	17.8	1.41	25.1	18.2	0.6	25.8	20.1
5.7	25.9	18.0	3.1	26.5	18.5	1.41	27.4	18.8	0.6	26.8	19.1
5.7	26.6	19.2	3.1	24.8	17.3	1.41	37.9	19.8	0.6	26.9	21.2
5.7	25.0	18.4	3.1	25.1	17.1	1.41	25.0	21.0	0.6	24.9	19.3
5.7	42.9	19.4	3.1	25.1	18.9	1.41	25.6	19.9	0.6	41.7	17.7
5.7	25.5	20.7	3.1	25.1	18.1	1.41	32.2	18.0	0.6	24.9	18.3
5.7	24.7	18.8	3.1	25.5	20.4	1.41	25.6	18.0	0.6	28.0	18.2
5.7	24.6	17.4	3.1	25.8	19.0	1.41	25.1	18.4	0.6	25.2	20.5
Mean	**29.7**	**18.8**	**Mean**	**25.5**	**18.5**	**Mean**	**27.3**	**19.1**	**Mean**	**28.4**	**19.5**
0.19	26.3	20.2	0.09	44.6	23.3						
0.19	27.6	16.6	0.09	25.0	20.5						
0.19	25.3	16.7	0.09	25.0	18.1						
0.19	38.1	18.0	0.09	39.2	22.2						
0.19	31.8	19.9	0.09	33.7	18.7						
0.19	25.3	20.7	0.09	26.2	19.5						
0.19	25.2	19.7	0.09	26.4	20.4						
0.19	25.3	19.1	0.09	25.0	19.9						
0.19	25.3	17.7	0.09	25.4	18.1						
0.19	27.0	18.2	0.09	25.5	18.6						
0.19	28.0	21.2	0.09	25.0	19.0						
0.19	25.2	19.1	0.09	25.1	22.5						
Mean	**27.5**	**18.9**	**Mean**	**28.8**	**20.1**						

Table A-22 (B)

Upper Temperature Tolerance of *Corbicula fluminea* for Chlorine Exposures at 20°C

Chlorine Concentration (mg/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Cl]	HCT	Lth	[Cl]	HCT	Lth	[Cl]	HCT	Lth	[Cl]	HCT	Lth
5.7	42.4	13.97	3.1	43.8	12.69	1.41	44.2	13.69	0.6	43.6	13.34
5.7	44.0	13.63	3.1	42.9	14.41	1.41	45.2	17.41	0.6	44.6	18.09
5.7	42.0	13.01	3.1	44.8	13.64	1.41	44.4	13.37	0.6	44.7	13.05
5.7	44.7	12.67	3.1	44.2	12.55	1.41	44.2	17.24	0.6	45.7	12.83
5.7	44.7	17.57	3.1	43.9	14.47	1.41	44.9	13.9	0.6	44.3	17.42
5.7	45.5	12.74	3.1	45.9	13.51	1.41	45.6	13.85	0.6	44.1	18.01
5.7	45.8	12.76	3.1	44.9	14.24	1.41	45.0	15.9	0.6	44.1	13.72
5.7	44.7	15.2	3.1	45.0	13.44	1.41	42.5	14.73	0.6	44.9	13.47
5.7	45.4	14.6	3.1	44.2	13.63	1.41	44.3	14.02	0.6	45.6	13.89
5.7	45.3	16.1	3.1	44.2	13.79	1.41	44.7	12.32	0.6	45.4	12.97
5.7	45.1	18.72	3.1	43.3	19.45	1.41	44.8	13.16	0.6	43.5	12.9
5.7	45.1	13.03	3.1	45.0	16.67	1.41	44.5	11.87	0.6	44.0	13.52
Mean	**44.6**	**14.5**	**Mean**	**44.3**	**14.4**	**Mean**	**44.5**	**14.3**	**Mean**	**44.5**	**14.4**
0.19	45.1	12.82	0.09	44.7	13.9						
0.19	45.2	16.93	0.09	43.8	12.43						
0.19	45.5	13.57	0.09	42.8	12.04						
0.19	43.5	18.64	0.09	44.1	16.7						
0.19	44.9	17.88	0.09	44.7	12.37						
0.19	45.7	15.57	0.09	44.6	18.05						
0.19	43.2	14.91	0.09	43.5	15.83						
0.19	42.5	12.92	0.09	45.0	13.84						
0.19	45.1	12.3	0.09	42.2	12.67						
0.19	44.3	13.55	0.09	44.1	13.13						
0.19	44.5	11.07	0.09	44.5	17.27						
0.19			0.09	43.2	13.44						
Mean	**44.5**	**14.6**	**Mean**	**43.9**	**14.31**						

Table A-22 (C)

Upper Temperature Tolerance of *Corbicula fluminea* for Chlorine Exposures at 30°C

Chlorine Concentration (mg/L), Heat Coma Temperature (HCT), Length (Lth), and Means Reported.

[Cl]	HCT	Lth	[Cl]	HCT	Lth	[Cl]	HCT	Lth	[Cl]	HCT	Lth
5.7	45.0	18.9	3.1	46.8	20.0	1.4	45.4	16.5	0.6	44.0	16.9
5.7	43.3	16.7	3.1	42.3	18.6	1.4	44.9	17.8	0.6	45.7	19.6
5.7	43.3	18.5	3.1	45.3	16.8	1.4	43.5	17.1	0.6	43.8	17.1
5.7	44.4	16.7	3.1	44.4	18.5	1.4	40.8	16.9	0.6	42.8	19.3
5.7	44.4	18.7	3.1	44.4	20.0	1.4	45.4	18.3	0.6	44.8	17.7
5.7	44.4	18.4	3.1	45.5	17.3	1.4	43.6	18.3	0.6	42.5	17.7
5.7	45.3	17.5	3.1	44.9	17.0	1.4	43.6	19.2	0.6	46.4	18.3
5.7	44.1	18.6	3.1	44.4	18.4	1.4	44.2	21.0	0.6	45.6	18.9
5.7	42.8	17.4	3.1	44.5	17.5	1.4	44.3	19.2	0.6	44.1	16.9
5.7	44.6	17.2	3.1	43.1	18.1	1.4	46.6	17.3	0.6	43.5	19.6
5.7	43.7	17.7	3.1	43.7	18.3	1.4	45.7	17.7	0.6	42.8	18.2
5.7	46.6	19.1	3.1	44.4	17.4	1.4	42.9	17.3	0.6	45.7	17.2
Mean	**44.3**	**18.0**	**Mean**	**44.5**	**18.2**	**Mean**	**44.2**	**18.1**	**Mean**	**44.3**	**18.0**
0.19	42.8	16.8	0.09	43.9	17.3						
0.19	45.8	20.7	0.09	44.4	18.3						
0.19	45.4	17.8	0.09	44.4	16.6						
0.19	45.5	19.8	0.09	43.9	20.0						
0.19	44.6	19.0	0.09	45.9	19.0						
0.19	44.3	19.1	0.09	46.6	18.4						
0.19	45.7	18.5	0.09	45.7	17.9						
0.19	44.7	19.2	0.09	42.9	17.9						
0.19	43.9	17.4	0.09	44.7	16.7						
0.19	45.3	17.8	0.09	44.0	21.3						
0.19	44.4	18.9	0.09	43.1	19.2						
0.19	43.5	19.4	0.09	44.2	20.6						
Mean	**44.7**	**18.7**	**Mean**	**44.5**	**18.6**						

Table A-23

Water Chemistry for Exposures of *Corbicula fluminea* to Toxicants

Dissolved Oxygen (mg/L), pH [H+], Hardness (mg CaCO3), and Alkalinity (mg CaCO3) for Controls and Each Toxicant at the Highest Concentration Before and After the 24-hour Incubator Exposure Reported.

Toxicant	Accl Temp	Time	DO	pH	Hardness	Alkalinity	Conductivity
Control	10	T0	11.1	7.54	176	117.5	347
Control	10	T24	10.1	7.71	176	125	430
Control	20	T0	9	7.23	284	150	----
Control	20	T24	4.1	7.5	321	150	----
Control	30	T0	6.8	7.55	156	77.5	500+
Control	30	T24	3.2	8.0	208	125	500+
Diazinon	10	T0	11.1	7.54	176	117.5	347
Diazinon	10	T24	10.0	7.66	156	125	410
Diazinon	20	T0	9	7.23	284	150	----
Diazinon	20	T24	3.41	7.73	356	175	----
Diazinon	30	T0	6.8	7.55	156	77.5	500+
Diazinon	30	T24	2.8	8.1	216	150	500+
Copper	10	T0	11.1	7.54	176	117.5	347
Copper	10	T24	10.7	7.96	180	115	428
Copper	20	T0	9	7.23	284	150	----
Copper	20	T24	6.6	7.83	1080	133.75	----
Copper	30	T0	6.8	7.55	156	77.5	500+
Copper	30	T24	2.8	8.33	204	135	498
Chlorine	10	T0	11.1	7.54	176	117.5	347
Chlorine	10	T24	10.7	7.71	92	90.5	310
Chlorine	20	T0	9	7.23	284	150	----
Chlorine	20	T24	4.4	7.66	288	137.5	----
Chlorine	30	T0	6.8	7.55	156	77.5	500+
Chlorine	30	T24	3.7	7.7	164	112.5	470

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