TOXICITY TESTING WITH FISH, ZOOPLANKTON AND MUSSELS--A COMPARISON OF SENSITIVITIES

By

ANNE E. KELLER

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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To my parents,
Robert and Lucille Keller,
whose support and confidence in me
made all the difference

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ANNE E. KELLER
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Toxicity testing with aquatic organisms is commonplace today. Prior to their manufacture and use, the effects of pesticides, herbicides and toxic substances on biota and the environment must be assessed. However, the focus has been primarily on the fate of pelagic fauna, particularly fish and zooplankton. Little attention has been focussed on the responses of invertebrates, other than insects, to pollutants.

In recent years, there has been a significant decline in the once abundant freshwater mussel fauna, purportedly due to dam-building and pollution. Since little is known about the sensitivity of freshwater mussels to metals and pesticides, there has been no way to establish protective measures. Currently, the United States Environmental Protection Agency is using zooplankton as surrogates for freshwater mussels in

toxicity tests with no verification that the two are comparably sensitive.

This dissertation was designed (1) to determine how sensitive mussels are to metals and organics, (2) to compare the sensitivity to freshwater fish such as the fathead minnow and (3) to determine by comparison whether zooplankton are good substitutes for mussels in toxicity tests. Anodonta imbecilis was chosen as the test species because it was locally available, has a relatively long reproductive period and has been previously cultured in the laboratory.

Acute toxicity tests were performed with juvenile mussels in reconstituted freshwater. Copper, cadmium, chromium, mercury, zinc and nickel were the metals used. It was found that mussels were about as sensitive to metals as were zooplankton. Organic compounds assessed included lindane, toxaphene, chlordane, Hydrothol-191, PCP, carbaryl, atrazine, an unregistered pyrethroid pesticide, acetone, methanol and SDS. Anodonta imbecilis was not sensitive to any of these substances except PCP.

It appears that the use of zooplankton species, e.g. Daphnia magna or Ceriodaphnia dubia, as surrogates for freshwater mussels is appropriate in tests for metal toxicity, but may not be so for organic pollutants.

CHAPTER 1
INTRODUCTION

Although the use of aquatic organisms to test impacts of industrial, agricultural or wastewater effluents on the biota of streams and rivers is a common occurrence today, this is a relatively new development. Initial concern centered on the safety of chemicals to humans and domestic animals relative to their efficacy on target organisms (Casarett and Bruce 1980). However, as concern for the environment has increased, so have the number and uses of aquatic toxicity tests. There are now test methods for many vertebrate and invertebrate aquatic animals (Peltier and Weber 1985).

Aquatic toxicology arose as an outgrowth of the chemical revolution of the 1940s. Biologists, seeing adverse changes in the biota of streams receiving human and industrial wastes, advocated the use of fish or other aquatic species as a means of predicting the response of stream organisms to industrial wastes (Buikema et al. 1982). Regulatory control of water quality was established in the U.S. with the passage of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) in 1947 and the Federal Water Pollution Control Act (FWPCA)

in 1948. These pieces of legislation regulated the input of both conventional municipal and industrial waste into lakes, rivers and the ocean.

In 1970, when United States Environmental Protection Agency (USEPA) became the agency responsible for improving and protecting the nation's water resources, the major concern was still the impact on both drinking water supplies and harvestable aquatic species. While sewage treatment and receiving water quality improved over time, as recently as 1982 some 30 states were cited for water quality standards violations due to toxic pollutants (Wise 1985). Biomonitoring of effluents via acute toxicity tests became the standard method for assessing environmental impacts because such tests are inexpensive and directly measure biological response. Acute toxicity tests are also required for all new chemicals posing a potential risk to human health or the environment, and as supporting documentation for pesticide registration (Zucker 1985a, 1985b).

To date, over 165 species of aquatic organisms have been used in acute toxicity tests (Buikema et al. 1982). Rapid methods to assess chronic toxicity have recently been developed for several species, including Pimephales promelas, Ceriodaphnia dubia and Selenastrum capricornutum (Horning and Weber 1985). These new methods measure sublethal effects of toxicants on biota, but acute toxicity tests are still the more commonly

used. While it would be convenient to use only one or a few species to assess the impacts of various pollutants on aquatic organisms, such a practice would not be adequate. Species specific tests are necessary because of differences in biological sensitivity and because organisms from different habitats may be exposed to a wide range of pollutant concentrations. The need for a variety of test methods has been demonstrated by results of testing with many pollutants (Johnson and Finley 1980, Mayer and Ellersieck 1986).

Most of the accepted test organisms are pelagic species (fish and zooplankton) which are well-known, easy to culture, economically important or of public concern (Buikema et al. 1982). However, benthic organisms are more appropriate for use in tests assessing the impacts of pollution in flowing waters because they typify the fauna of lotic systems. Pelagic fauna are more representative of lacustrine habitats. To date, toxicity data for benthic organisms including aquatic oligochaetes, turbellarians, pelecypods and gastropods are still extremely limited, and only one toxicity test protocol for these organisms exists, i.e., for the exotic Corbicula fluminea (Foster 1979). Because its life cycle differs significantly from that of native North American freshwater clams (Unionidae),the use of C. fluminea as a model for other mollusk species is questionable.

The current dissertation research is divided into two large parts. The first part deals with the assessment of the chronic toxicity of Hydrothol-191, an aquatic herbicide, to Pimephales promelas (the fathead minnow) and Ceriodaphnia dubia, using recently developed EPA protocols. The Florida Department of Environmental Regulation foresaw an increased demand for Hydrothol use in Florida and wanted to know what impact this might have on nontarget organisms.

While both the fathead minnow and C. dubia are used to monitor the toxicity of wastewater effluents and assess the impact of pure compounds on aquatic organisms, neither of them is native to Florida (Lee et al. 1980). In addition, their responses have not been compared to those of benthos that inhabit canals, streams or rivers where Hydrothol is widely used for macrophyte control. Therefore, the second part of the dissertation contains results of test development work with a representative of native benthic fauna, the freshwater mussel, Anodonta imbecilis. The need for a toxicity test for native freshwater mollusks was apparent based on their importance in flowing waters, their taxonomic distinction from insects and other benthos that have been tested, and the need to corroborate the use by EPA of tests with Daphnia magna to estimate the sensitivity of mussels to pollutants.

My research with A. imbecilis was designed to (1) simplify the culture techniques permitting easier production of test organisms, (2) develop an acute toxicity test protocol for use in assessing the sensitivity of freshwater mussels to pesticides, metals and wastewater effluents, and (3) determine the toxicity of a number of pure compounds and effluents to A. imbecilis. Results from this work could then be used to determine whether it is appropriate for EPA to use D. magna in toxicity tests as a surrogate for freshwater mussels.

CHAPTER 2
LITERATURE REVIEW

Toxicity Testing With Pelagic Biota

The bulk of information on the toxicity of pollutants to aquatic biota was derived from tests with pelagic organisms. In particular, several species of economically important fish, e.g., Salmo gairdneri, Oncorhynchus tshawytscha, Lepomis macrochirus and Ictalurus punctatus, and a number of zooplankton species, e.g. Daphnia magna, Daphnia pulex, and Simocephalus spp. have been the most common test organisms (Johnson and Finley 1980, Mayer and Ellersieck 1986, Buikema et al. 1982). The latter group has been well studied because they are both easy to rear in the laboratory and important links in the aquatic food chain leading to fish.

Since the late 1970s there has been increasing interest in the development of short term chronic toxicity tests for fish that combine the simplicity of acute methods with the estimation of sublethal effects provided by lifecycle toxicity tests (Horning and Weber 1985). The latter can require months or years to complete, depending on the lifespan of individual

species. Chronic exposures to low concentrations of pollutants can affect reproduction, growth, behavior or species interactions, any of which may alter the structure of the aquatic community (Alabaster and Lloyd 1982, Rand 1985).

Studies by McKim (1977) and Macek and Sleight (1977) proved that exposure of critical life-stages of fish (embryos or larvae) to toxicants for 30-60 days provided toxicity estimates comparable to full life cycle tests. These early life stage (ELS) tests were soon adopted as the standards for estimating water quality criteria because they were faster and cheaper, as well as accurate (Horning and Weber 1985).

Further simplification followed as data from ELS tests showed that larval growth could be as sensitive a measure of sublethal toxicity as larval survival (Benoit et al. 1982, Woltering 1984, Birge et al. 1981). As a result, a seven-day fathead minnow larval survival and growth test was developed for effluent and single-compound toxicity evaluations (Norberg and Mount 1985). The method, published by EPA (Horning and Weber 1986), is described as a static-renewal subchronic toxicity test that uses larval growth as a measure of sublethal response.

Larval fathead minnows (< 24 h old) are exposed to a series of toxicant concentrations (usually 5) and a control comprised of dilution water. The test solutions

are changed daily, after a count of survivors has been made. Larvae are fed rinsed brine shrimp. At the end of the test, all surviving larvae are preserved in formalin until their growth can be assessed based on weight gain compared to that of controls. An LC50 is calculated using survival data.

The second subchronic toxicity test to be published by the EPA was the Ceriodaphnia dubia survival and reproduction test (Horning and Weber 1985). The impetus behind the development of the 7-day Ceriodaphnia survival and reproduction was somewhat different from that of the test with fathead minnows. While test duration was a factor, it was perhaps more related to the length of the work week than to expense since a cladoceran life cycle test may be completed in about 30 days (Mount and Norberg 1984). If the test is begun on a Friday, little maintenance time is required over the weekend. More intense effort is required as the test progresses.

Historically, Daphnia magna has been the most used species for the estimation of zooplankton acute sensitivity to pollutants (Mount and Norberg 1984, Buikema et al. 1982, Anderson 1980). Anderson (1980) described a series of 17 papers produced by Einar Naumann in 1933 and 1934 detailing various aspects of toxicity testing with D. magna. This was perhaps the real beginning of the use of D. magna in such tests.

With movement toward the use of chronic test methods in aquatic toxicology, both a lifetime (Buikema 1973, Winner and Farrell 1976) and 21-day chronic test (Biesinger and Christensen 1972) were developed for D. magna. As designed, these methods provided estimates of sublethal effects based on changes in fecundity (Buikema et al. 1980), but they were still too lengthy.

Under the auspices of the EPA, Mount and Norberg (1985) developed a 7-d subchronic toxicity test with a different species, Ceriodaphnia reticulata. C. reticulata was chosen (over D. magna) because it is widely distributed in North America, it was easier to culture than was D. magna and it produces three broods of young in seven days (Mount and Norberg 1984). These characteristics facilitate the performance of many tests in a short time, virtually anywhere. Since the developmental work by Mount and Norberg (1984), EPA has suggested the use of C. dubia in their protocol manual (Horning and Weber 1985).

A Ceriodaphnia dubia survival and reproduction test is begun with the collection of neonates (<24 h old) from the adult culture. Neonates are placed in individual test vessels consisting of 30 ml plastic cups containing 15 ml of solution. Isolation of individuals is necessary so that separate tallies of fecundity can be maintained for each animal. A daily count of survivors is made. Beginning on Day 3 or 4 of the test when the first brood

is produced, offspring are also counted. Adults are then moved to new test vessels and fed. This daily counting and transfer to new solutions continues until the test is terminated at seven days. A 7-d LC50 for adults is calculated, and their fecundity is used to measure sublethal effects.

Rationale For The Use of Freshwater Molluscs In Acute Toxicity Tests

To date, over 165 species of aquatic organisms have been used in acute toxicity tests (Buikema et al. 1982). Species specific tests are necessary because of differences in biological sensitivity and because organisms from different habitats may be exposed to a wide range of pollutant concentrations. The need for a variety of test methods has been demonstrated by results of testing with many pollutants (Johnson and Finley 1980, Mayer and Ellersieck 1986).

Most of the accepted test organisms are pelagic species (fish and zooplankton) which are well-known, easy to culture, economically important or of public concern. Little attention has been given the response of benthic macroinvertebrates, other than insects, to pollutants. Benthic invertebrates are more appropriate test organisms for flowing waters than are zooplankton because the latter are not typically found in such systems, and are therefore not good indicators of the impact of pollutants on lotic invertebrates. However, toxicity data for

benthic organisms, including aquatic oligochaetes, turbellarians, pelecypods and gastropods, are still extremely limited, and only one toxicity test protocol for these organisms exists, i.e., for the exotic clam Corbicula fluminea (Foster 1979). Non-insect benthos have been considered either unimportant or their responses have been extrapolated from those of the common test organisms. However, use of zooplankton or fish as surrogates for non-insect benthic fauna is questionable. Not only are conditions at the water-sediment interface different than those in the open water and difficult to assess with pelagic organisms, there ought to be specific information on the response of benthic organisms to pollutants since they represent distinctly different taxa (Buikema et al. 1982). One of the most widely distributed groups of macrobenthos native to streams in Florida is the unionid mussels. Little is known about their sensitivities to various pollutants entering their environments.

Distribution and Life History of Unionid Mussels

The vast majority (36 genera and 250 species) of bivalve mollusc species in North American continental waters belong to the family Unionidae (Burch 1973). The group as a whole is endemic to North America, but many species have limited ranges. Unionid mussels generally prefer lotic habitats with stable substrates and some

silt. They are distributed from southern Ontario to Florida and west to Washington and Oregon. However, the best studied and perhaps richest mussel (or clam) fauna is found in the eastern United States between the Appalachian Mountains and Mississippi River.

Several unique life history features were key to the evolutionary development of freshwater mussels from their marine ancestors (Stein 1971). These included the production of a parasitic glochidia larva rather than the free-living veliger, incubation of the larvae in the marsupia (gills) of the female and the requirement for a fish host during the 9-30 day parasitic phase. Reproduction begins when male mussels shed sperm into the water. Sperm cells are drawn into the incurrent siphon of the female mussel and become lodged in the gills on each side of her body that are specifically modified for incubation. Development to the bivalved glochidia occurs inside the female. During this time, the larvae may become infected by any number of bacterial, fungal, protozoan or water mite species, which may reside in the mussel permanently or temporarily.

When mature, glochidia are shed into the water via the excurrent siphon either directly onto fish hosts whose presence stimulates release or randomly broadcast into water currents (Buchanan 1980, Parmalee 1967). Complete development into juveniles requires a period of parasitism on fish during which the organ systems

develop. Distribution of unionid mussels is facilitated by their attachment to mobile hosts instead of by the production of mobile larvae as in marine bivalves (Fuller 1974). After encystment periods of varying times, glochidia drop off of their hosts and become free-living filter feeders (Arey 1932).

Habitat Destruction and Faunal Decline

Mussel fishing was a thriving business in the Illinois, Tennessee and Mississippi Rivers from the late 1800's to the mid-1960's (Isom 1969, van der Schalie and van der Schalie 1950, Starrett 1971). They were a source of freshwater pearls for jewelry, and their shells supplied the button industry with raw materials. Later, shell slugs were used as seeds in the Japanese cultured pearl industry (Parmalee 1967).

Ten thousand tons of mussel shells per year were harvested from the Tennessee River in the 1940's and 1950's, but harvests declined steadily during ensuing years (Starrett 1971, Parmalee 1967). Similar changes in abundance were observed in other eastern rivers during the same period (van der Schalie and van der Schalie 1971, Isom 1969, Starrett 1971). Several factors have been suggested as causes for the decline including overharvesting, habitat destruction by damming and pollution.

Overharvesting of mussels in the Tennessee, Illinois and Mississippi Rivers may have contributed significantly to the decreased abundance of some species (Starrett 1971, Forbes and Richardson 1919, Danglade 1914). In 1910, there were over 2,600 boats engaged in mussel fishing along the lower half of the Illinois River alone (Starrett 1971). Similar intensive harvests were made by the well-developed mussel industry of the Tennessee and Mississippi Rivers. Not only did such harvesting reduce populations directly, but it also may have reduced breeding stock below the replacement capacity of remaining stock, destroyed stream habitat and resulted in the death of disturbed but uncollected animals (Fuller 1974). While the button industry switched from pearl to plastic in the 1930's and 1940's, another use for mussel shells was found. Spheres of mussel nacre (pearlized shell) were used as nuclei by the Japanese cultured pearl industry beginning in the late 1950's. The use of SCUBA gear permitted the harvest of whole beds of mussels leading to localized extinction (Fuller 1974).

A second major contributor to the declining mussel fauna was the extensive habitat destruction resulting from damming activities of the Tennessee Valley Authority (TVA) beginning in the 1930's (Isom 1969). While adult mussels of some species prefer quiet water (Wilson and Clark 1912, Danglade 1914), juveniles and adults of many species need riffle water. Thus, damming may have

provided more habitat for some species, but reduced the area suitable for others. Decreased water flow also hinders reproduction by limiting dispersal of sperm and later, glochidia. Further, impoundment changes fish species distributions which may affect mussel recruitment since they are briefly parasitic on fish (Fuller 1974). Other consequences of damming include increased siltation which can lead to suffocation of mussels, as well as general loss of habitat and decreased recruitment success due to release of tailwaters into otherwise suitable stream reaches. The latter results from both low temperatures and low oxygen levels of tailwaters (Fuller 1974, Marking and Bills 1980, Ellis 1936).

Finally, the effects of water pollution by human waste, industry and agricultural activities have damaged the mussel fauna in many areas. Pulp and paper mills which release sawdust and process effluents destroyed mussel populations in Minnesota (Danglade 1974), the upper Tennessee River drainage (Ortman 1918), panhandle Florida (Heard 1970) and in areas around Ottawa, Canada (Mackie and Qadi 1973). As noted earlier, siltation is a problem for mussels and with its increase along with agricultural activities, the molluscs declined steadily.

The effects on mussel populations of pesticides and herbicides used in farming and aquatic weed control, and metals released in acid mine drainage and industrial effluents have only recently been examined. Little

conclusive evidence is available since the specific sensitivity of mussels to such pollutants is difficult to determine from field data, and laboratory exposures have been limited to a few studies of adults (Imlay 1971, Imlay 1974, Foster and Bates 1978). Development of better culture techniques and toxicity test procedures have begun to make experimental work possible.

Propagation Of Freshwater Mussels In Artificial Media

The unusual mode of reproduction of unionid molluscs makes their culture in the lab more difficult than it is for other molluscs that have free-living veliger larvae. The life cycle of unionid mussels includes a parasitic larva (glochidia) which normally attaches to fish gills or fins during early development. This stage must have fish to parasitize or a culture medium that would provide the necessary nutrients.

Earliest efforts to propagate freshwater mussels (LeFevre and Curtis 1912) in fish plasma were unsuccessful. Glochidia did not transform. By 1926, transformation of glochidia to juveniles had been accomplished with the use of an artificial medium (Ellis and Ellis 1926). However, glochidia were permitted to encyst on fish gills for 18-96 hours before being dissected out to incubate through transformation. The contents of their growth medium were described as including NaCl, KCl, $CaCl_2$, $NaHCO_3$, dextrose, a mixture

of amino acids, small quantities of phosphates and traces of magnesium salts (Ellis and Ellis 1926).

Research into mussel propagation lost its impetus as the button and mussel-fishing industries dwindled in the 1940's and 1950's. However, in the hope of replenishing declining natural populations, the Tennessee Valley Authority funded research to develop methods for in vitro propagation of these freshwater molluscs in the early 1980's (Isom and Hudson 1982, Hudson and Isom 1984). The goal was to develop a complete culture medium that eliminated the need for fish hosts during the larval stage and to produce a large number of juvenile mussels at one time. As a result, a culture medium containing a Ringers solution, vitamins, glucose, amino acids, antibiotics and fish plasma was developed as a substitute for live fish, (Isom and Hudson 1982). Glochidia were removed from the gills of ripe female mussels, rinsed several times in sterile water and put in the medium. Culture dishes were then placed in a temperature controlled CO_2 incubator ($23^{o} \pm 3^{o}$ C). The transformation of glochidia to juveniles takes 9-30 days ($23^{o} \pm 3^{o}$ C) depending on the species, culture temperature and degree of glochidia maturity at the start of incubation. While the Hudson and Isom method (1982, 1984) is far better than that of Ellis and Ellis (1926) which relied on the use of fish hosts for encystment of glochidia during transformation, further simplification

is desirable. The old method (Hudson and Isom 1982, 1984) is laborious, still requires the use of fish plasma which may not be readily available nor of consistent quality, and a CO_2 incubator. A simplified method for mussel culture is necessary before they can be available in the numbers needed for replenishment of declining wild stocks or for other purposes, e.g. toxicity tests.

Sensitivity of Unionid Molluscs to Environmental Pollutants

Interest in the effects of toxic pollutants on mussels directly and their use as environmental indicators in general has increased following the decrease in population sizes. Freshwater mussels have been suggested for use as biological monitors in lotic environments for many years. Biomonitors are important because analysis of water does not reflect biologically available concentrations of toxicants (Leard et al. 1980). Sometimes biota can be affected by concentrations below the detection limit of analytical instruments, and at other times, high ambient concentrations are benign because they are refractory or are adsorbed to particulate matter. The utility of freshwater mussels as biomonitors is enhanced by their sedentary lifestyle, their long lifespan compared to other invertebrate species and the fact that they live in the sediments while being filter-feeders. Thus, mussels are exposed to dissolved, particulate and sediment-sorbed contaminants

(Havlik and Marking 1987). However, current information on mussel sensitivities consists largely of species presence-absence data for locations impacted by toxics, and measurements of contaminants in shells or tissues (Havlik and Marking 1987). Experimental data on specific sensitivities are very limited.

Simmons and Reed (1973) used the presence and abundance of freshwater molluscs as indicators of biological recovery in the North Anna River, Virginia. The North Anna River was receiving acid mine drainage from a defunct coal mine. While aquatic insects re-established quickly below the confluence of the river and an unpolluted creek, molluscan species were absent for another 50 miles downstream. In this example, molluscs were more sensitive indicators of biological recovery than were insects, traditionally regarded as good biomonitors. Simmons and Reed (1973), however, suggested that the lack of mussel fauna in the acidified river reach may have been caused by siltation and loss of host fish for the glochidia.

Three species of mussels from the Illinois River near Peoria, Fusconaia flava, Amblema plicata and Quadrula quadrula, were analyzed for metals along with fish, tubificid worms, river sediment and water (Mathis and Cummings 1973). The portion of the river studied was highly industrialized and therefore received metallic effluents. The goal was to determine if there had been a

loss of species and if the loss could be related to metal concentrations. Mussels accumulated the metals to levels exceeding dissolved concentrations by 1-2 orders of magnitude, but had lower levels than those in the sediments. No attempt was made to determine species abundance, but the presence of a thriving mussel-fishing industry within the study area indicated that these three mussels were plentiful. Another species, Musculium transversum, the fingernail clam, was absent from polluted areas of the river where it had been common before 1954. While concentrations of metals in the tested adult clams may not have been lethal, this does not mean there was no impact on juveniles or on other species such as M. transversum which were absent.

Anderson (1977) analyzed shells and tissues of freshwater clams from the Fox River, Wisconsin for cadmium, copper, lead and zinc. He found that body burdens generally paralleled sediment concentrations while being much higher than were found in water. Metal concentrations were much lower in the shells (Cd<Cu<Zn<Pb) than in soft tissue (Cd<Cu<Pb<Zn). Of the latter, samples of gills had the highest levels of metals.

In one of the most important studies of mussel responses to dissolved metal pollution, Foster and Bates (1978) compared lethal concentrations of copper in Quadrula quadrula with state mandated water quality

criteria. Standing crops of mussels in the Muskingum River during a period prior to increased discharge of the metal effluent (1967-1970) were compared to a post-increase period (1972-1973). An 86% decrease in mussel standing crop occurred between the earlier and later study at a point 5 Km downstream from the effluent outfall. Body burdens of copper increased approximately 10-fold during 11-day laboratory exposures of Q. quadrula to the effluent. Mussels placed in in situ cages exhibited an even greater ability to concentrate copper. Their mean body burdens of copper, 20.64 ug Cu^{-1}/g after 14 days in the cages, were similar to that found after 11 day exposures to whole effluent in the laboratory. However, the toxic effluent comprised only 0.004% of the mean daily river flow at the point where the cages were suspended.

Juvenile mussels accumulated copper at a greater rate than adults. This finding was significant because it may be the result of increased metabolic rate associated with the sexual maturation process (Foster and Bates 1978). In that case, the reproductive capacity of the species may be compromised by the loss of juveniles, or by decreased recruitment success. Foster and Bates (1978) also emphasized that in developing water quality criteria, consideration should be given to the impact on a greater variety of stream fauna, including molluscs.

Metals such as manganese, zinc, cadmium, copper and lead may accumulate in mussels to levels higher than ambient concentrations depending on sediment conditions. Radioactive manganese (Mn^{54}) was concentrated at rates ranging from 11,000-40,000 times water and sediment levels in soft tissue and about 14,000 times in shells of Unio mancus var. elongatulus (Gaglione and Ravera 1964, Ravera 1964). These values were three times higher than in Anodonta cygnea from the same area. Levels were highest in U. mancus gills and lowest in the visceral sac. Mn^{54} was present at undetectable levels in water, sediment and other organisms. Leatherland and Burton (1974) measured cadmium levels in Anodonta cygnea in the Thames River, England. The mussels had concentrated Cd^{+2} from water with 0.49 mg/L to a tissue level of 9 mg/L. These studies demonstrate the validity of using molluscs as biomonitors for metals.

Reddy and Chari (1985) found increased production of enzymes involved in amino acid synthesis in the freshwater mussel Parreysia rugosa after exposure to mercury and copper. They attributed this increase to the higher demand for amino acids in metabolic processes and energy transfer in the stressed mussels.

One of the factors that can hinder the usefulness of molluscs as biomonitors is variability in accumulation rates of metals depending on tissue, age, sex or other unknown characteristics (Jones and Walker 1979, Bryan

1973, Ayling 1974). Selection of samples can obviate some of the variability but requires adequate field time and preliminary studies. However, in some cases, no explanation for the variability has been found so no blocking of samples or analyses can be used to overcome the problem. This may reduce the apparent responses of mussels to changes in ambient conditions and thus obscure determination of real responses (Jones and Walker 1979).

Using fractionation procedures that isolated trace metals from sediments, Tessier et al. (1984) determined that the accumulation of metals in tissues of Elliptio complanata was most strongly related to individual fractions rather than to total metals. Samples were extracted sequentially at decreasing pH with various acids to isolate metals bound to Fe-Mn oxides, carbonates, organic matter and those that were dissolved or residual. The mantle and gills of the molluscs contained the highest levels of metal (Cu, Zn, Mn, Fe) while the foot and adductor muscles had the lowest. This is a typical pattern observed in molluscs (Hobden 1970, Gaglione and Ravera 1964, Anderson 1977). The relationship between sediment levels and body burden depended on the metal species but were predictable from appropriate regression equations.

Pace and DiGuilio (1987) assayed the lead content of peat, sediment and clams (Rangia cuneata) from the Pungo River estuary in North Carolina. Using fractionation

techniques similar to those described in Tessier et al. (1984), they determined that lead levels in the clam tissues were very low (0.2-0.5 ug/g) compared to the peat (12.8 ug/g) reflecting the presence of lead in non-bioavailable forms. An analysis of heavy metals in three estuarine molluscs from the Spanish coast (Lopez-Artiguez et al. 1989) showed that the concentration of particular metals by molluscs is species-dependent. Oysters (Crassostrea angulata) accumulated very high levels of copper (180.45 ug/g) compared to Tapes decussatus and Cardium edule, while the latter had generally higher levels of As, Hg and Sn than did the other two species. Growth rates of mussels, as determined by changes in shell growth rings, were lower in rivers polluted with heavy metals such as silver, cadmium, iron, mercury and manganese (Imlay 1982) was determined by changes in shell growth rings.

A few laboratory studies have measured the response of mussels to specific metals. K^+ was toxic to four species of unionid clams at levels below those found in some rivers in the United States (Imlay 1974). The LC50 for Lampsilis radiata siliquoidea and Fusconaia flava was 15 mg/L after 36 hours of exposure. Amblema plicata was more sensitive, having an LC50 of 15 mg/L at 26 days 7, while 50% of the Actinonaias carinata died in 11 mg/L K^+ in eight days (Imlay 1971). Labos and Salanki (1964) recorded "abnormal" glochidial activity for Anodonta

cygnea in 3.91 mg/L K^+. Imlay (1971) used such data to successfully predict mussel distributions in rivers based on ambient K^+ levels (Imlay 1974). Anodonta cygnea was found to be more sensitive to K^+ than to any other cation in work by Lukacsovics and Salanki (1964). Since K^+ is a common effluent of paper mills, irrigation return water and petroleum brine, this sensitivity may have significance to the survival of mussel populations. Imlay (1971) showed that mussels were as sensitive to dissolved metals as other invertebrates and fish following laboratory exposure for several months. In particular, copper was lethal to the mussels at 25 ug/L, a value similar to that recorded for fish and non-molluscan invertebrates.

In a field study of the effects of dissolved aluminum on Anodonta grandis grandis, mussels suffered no mortality and only transitory changes in blood ion composition (Malley et al. 1988). When the mussels were placed in an acidified lake to which alum was added, there was no change in Na^+, K^+, or SO^{-2} concentrations, a decline in Mg^{+2} and a slight increase in blood Cl^-. Increases in Ca^{+2} levels were attributed to compensatory mechanisms for maintenance of blood pH and were seen as more detrimental to mussels during chronic exposures to low pH than to aluminum.

As with metal toxicity data, information on mussel bioaccumulation or sensitivity to pesticides is largely

circumstantial with few experimental data being reported. Bedford et al. (1968) introduced specimens of two mussel species into the Red Cedar River, Michigan to determine whether they could be used to detect pesticides at low dissolved concentrations. Lampsilis siliquoidea and Anodonta grandis were found to concentrate DDT and its metabolites, methoxychlor and aldrin to levels many times greater than were present in the dissolved or particulate fractions. However, the mussels contained lower levels of pesticides than were detected in the sediments. These results indicated the feasibility of using freshwater mussels for detecting the presence of pesticides in running water. Leard et al. (1980) found the insecticides parathion, DDT, chlordane, toxaphene and their metabolites in seven mussel species from streams draining agricultural areas where these pesticides were in use. Pesticide accumulation varied with species, but there was a decrease in DDT body burden during the period after DDT use was limited. During that time, an increase in toxaphene and parathion levels was measured in the clams. This was a reflection of their increased use in place of DDT (Leard et al. 1980). Sphaerium corneum concentrated dieldrin to 1000 times ambient levels in laboratory experiments and field studies (Boryslawskyj et al. 1987). Diazinon and parathion are also taken up by mussels at high rates (Miller et al. 1966). None of

these studies gave data on lethality of the compounds to mussels.

Laboratory toxicity experiments determined the 96-h LC50 of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) for Ligumia sp. to be 8.3 mg/L for small individuals (< 9 cm long) and 11.7 mg/L for larger specimens (> 16 cm). This is 1.5-4 times the normal stream water concentration in treated areas and similar to the sensitivities of the crayfish Orconectes (17.8 mg/L) and Gammarus pseudolimnaeus (22.3 mg/L) (Johnson and Finley 1980). The lampricide Bayer 73 was lethal to 50 per cent of adult Elliptio dilatatus at 382 ug/L concentration (Rye and King 1976). The LC50 for rotenone was 2.7 mg/L in soft water for adult Lampsilis sp. (Farringer 1972).

The response of adductor muscle activity in glochidia of Anodonta cygnea was used as a measure of toxicity by Varanka (1977, 1978). Spontaneous adductor muscle activity is crucial if glochidia are to attach to their hosts. In a series of experiments, Varanka applied the muscle-contraction inducer tryptamine to glochidia and recorded baseline contraction rates. He then exposed larvae to tryptamine + pesticide to determine the effect of the pesticide on this activity. Results of 30-minute exposures to malathion, 2,4-D and Shell-DD indicate that the EC50s (effective concentration for 50% of the sample) based on adductor muscle activity were considerably

higher than the usual environmental levels of these pesticides. However, the fact that muscle activity responded to such short exposures suggests that further study with longer exposure times may be worthwhile.

Though in these and other studies the mussel bioaccumulation capacity for metals and pesticides is great, we have very few measures of the lethal or sub-lethal effects of such exposures. With such a limited database, it is premature to draw conclusions about the sensitivity of mussels to environmental exposures to metals or pesticides. The majority of studies to date consist of species surveys and measurements of tissue or shell toxicant levels. How the presence of these contaminants may affect mussel survival, growth or reproduction remains to be determined. Laboratory exposures of mussels of various species and age groups to pesticides, metals or organic pollutants would provide extremely valuable measures of direct effects. Such data would allow us to separate the effects of habitat destruction, siltation and competition with Corbicula sp. from response to pollutants.

CHAPTER 3
THE SENSITIVITY OF THE FATHEAD MINNOW (PIMEPHALES PROMELAS) TO HYDROTHOL-191 AT 15° AND 25° C

Introduction

The United States Environmental Protection Agency (EPA) has recently advocated the use of short-term chronic toxicity tests for biological monitoring of water and wastewater (Horning and Weber 1985). Such tests were developed by EPA on the basis of their cost-effectiveness, rapidity and requirement for low sample volumes over the course of the test. These features allow the methods to be implemented in on-site effluent toxicity evaluations, as well as in toxicity assessments of pure compounds and samples shipped to central laboratories. Implementation of short-term chronic toxicity methods was justified by evidence that these early life cycle tests reasonably approximated more complete, full life cycle chronic toxicity tests that had been the standard for many years (Norberg and Mount 1985).

One of these tests employs fathead minnow larvae (Pimephales promelas) in a seven-day, static renewal, survival and growth test. Test results are based on the

survival and growth (weight gain) of larval fathead minnows over seven days in the presence of a range of toxicant concentrations. It is a new evaluative method for which few test results are available. Norberg and Mount (1985) determined the chronic toxicity of several industrial effluents and receiving waters, as well as zinc, copper and Dursban during their test development work. The 7-d test with fathead minnow larvae gave results similar to those from much longer (3-6 months) early life stage (ELS) tests.

The current study was designed to evaluate the toxicity of the herbicide Hydrothol-191 to fathead minnows. Hydrothol (endothall) acts to decrease photosynthesis and cellular respiration in turfgrass and to decrease the production of amylase in germinating barley seeds (Ashton and Crafts 1981). It has a half-life of 10 days, biodegradation by bacteria being the primary fate process leading to the decline of Hydrothol concentrations (Reinert and Rodgers 1987). Its effects, similar to those of Actinomycin D, are not reversible with benzyladenine (Penner and Ashton 1968). Since actinomycin D selectively inhibits the synthesis of m-RNA, the translator of DNA messages during protein (enzymes) production, it is hypothesized that Hydrothol also interferes with m-RNA production (Ashton and Crafts 1981).

Support for this assertion was given by the effect of Aquathol-K (dipotassium endothall, Pennwalt Corp.) on the smoltification of juvenile Chinook salmon (Oncorhynchus tshawytscha). When juvenile salmon were exposed to water with endothall levels as low as 3 mg/L for four or fourteen days prior to their transfer from freshwater to artificial seawater, they were unable to survive (Liguori et al. 1983). Fish that were allowed to recover in freshwater for 10 days prior to their transfer to seawater survived well. Histopathologic analyses indicated that hypertrophy of branchial epithelium occurred in fish exposed to 10 mg/L or more of endothall.

The process of smoltification involves changes in plasma levels of thyroxine, triiodothryonine, and gill ATPase activity. Both triiodothryonine and ATPase are found in gill tissues. Liguori et al. (1983) suggested that in endothall damaged gill tissue, the levels of triiodothryonine and ATPase may be depressed, although neither was measured in their study. Since Hydrothol interferes with smoltification possibly related to levels of ATPAse, it may be the result of its inhibitory effects on m-RNA synthesis (Liguori et al. 1983). Therefore, the impact of Hydrothol on organisms should be temperature dependent as are most chemical reactions (Wilson 1972).

In Florida, Hydrothol is registered for use in the control of algae, Hydrilla verticillata, Myriophyllum spicatum, Utricularia spp., Valisneria spp. and other

submersed macrophytes, as well as for several agricultural purposes (Dupes and Mahler 1982, Blackburn and Weldon 1963). Although there are some data on the acute toxicity of Hydrothol to non-target aquatic organisms, only limited data are available on its chronic toxicity to aquatic biota. The Florida Department of Environmental Regulation (FDER), seeing the likelihood of increased Hydrothol use because of its efficacy in the long-term control of aquatic macrophytes, requested the evaluation of its potential impact on non-target organisms.

FDER was also interested in knowing what if any effect water temperature might have on the toxicity of Hydrothol to freshwater fish. There were almost no data indicating the effect of temperature on Hydrothol toxicity (Walker 1963). Since many physiological processes are affected by temperature because of the pivotal role of enzymes in cellular respiration and the synthesis or degradation of organic compounds (Wilson 1972), it was important to determine whether water temperature would alter the toxicity of Hydrothol to organisms.

The goals of this investigation were: (1) to determine the chronic toxicity of Hydrothol to the fathead minnow and (2) evaluate the impact of temperature on its toxicity. These data would be useful

in the development of better guidelines for the use of this herbicide in Florida.

Materials and Methods

Test Organism

Fathead minnows (Pimephales promelas) were acquired from the EPA-Newton Laboratory in Cincinnati, Ohio. Fish embryos were sent in insulated containers by express mail and hatched in transit. Newly hatched fathead minnow larvae preferably less than 24 hours old were used to initiate a test.

Test Organism Food

Fathead minnow larvae were fed live brine shrimp nauplii (Artemia salina) raised from eggs in the laboratory. Brine shrimp nauplii were incubated at 25° C and harvested when nauplii were less than 24 h old (Peltier and Weber 1985, Horning and Weber 1985). Fathead minnow larvae (10/500 ml test vessel) were fed 0.1 ml harvested brine shrimp (approximately 1000 nauplii) three times daily at 4-hour intervals.

Dilution Water

Moderately hard reconstituted freshwater was used as diluent throughout the test. It was prepared by adding the following constituents to 1 l of deionized

water: 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4$ and 8.0 mg KCl. This produced water with a pH of 7.4-7.8, a hardness of 80-100 mg/L $CaCO_3$ and an alkalinity of 60-70 mg/L $CaCO_3$ (Horning and Weber 1986). Dilution water was made in bulk and stored in a plastic carboy at 15° or 25° C for the duration of each seven day test.

Test Chemical

The toxicity of Hydrothol-191 (Pennwalt Corp., Philadelphia, PA), an agricultural and aquatic herbicide was evaluated in this study. Hydrothol, the trade name for the alkylamine salt formulation of endothall (7-oxabicyclo [2,2,1] heptane-2,3-dicarboxylic acid), is used extensively to control Hydrilla verticillata and Myriophyllum spicatum. Test solutions were made fresh daily by dilution of a stock solution of Hydrothol with moderately hard reconstituted freshwater (v/v). All Hydrothol concentrations given are nominal.

Reference Toxicant

Cadmium chloride, obtained from EPA (Quality Assurance Branch, EMSL, United States Environmental Protection Agency, Cincinnati, OH 45268) was the reference toxicant for fathead minnow tests. Three tests were performed with $CdCl_2$ to evaluate the consistency of test results. Later, $CdCl_2$ was used as a check on test organism quality in each definitive test with Hydrothol.

Range Finding Test

In order to obtain the appropriate range of Hydrothol concentrations to be used in 7-day tests an acute toxicity range finding test was conducted. It was determined that 0% larval mortality occurred at 100 ug/L and that 100% mortality occurred at 1000 ug/L. Consequently, the definitive 7-day Hydrothol concentration range was 50-1000 ug/L.

Seven-day Survival and Growth Toxicity Test

Toxicity tests were initiated by placing larvae in 1 liter borosilicate beakers (test chambers) containing 500 ml control or test water. Larvae were transferred into duplicate test chambers by a large-bore pipet until each test chamber contained 10 larvae, for a total of 20 larvae at each Hydrothol test concentration.

Definitive tests were conducted at 15^{o} C and 25^{o} C in constant temperature rooms, with a photoperiod of 16 hours light and 8 hours of darkness. The test chambers were randomized at the beginning of the test. Ninety per cent of the test solution was renewed every day after a large-bore pipet was used to siphon dead brine shrimp and other debris from the bottom of the test chambers.

Chemical and physical analyses of test water were conducted according to standard EPA methods (EPA 1983). Dissolved oxygen, temperature, pH, conductivity, alkalinity and hardness were measured at the beginning of

each 24 hour exposure at all test concentrations and in the control.

The numbers of live and dead larvae in each test chamber were recorded daily, and the dead larvae were removed. After seven days of exposure the test was terminated. The surviving larvae were removed and preserved as a group in 4% formalin. At a later date, the groups of preserved larvae were rinsed in distilled water and dried at 105^{o} C for a minimum of 2 hours. Dry weights of each group of larvae were measured to the nearest 0.001 g.

Statistical Analysis

All LC50 and 95% confidence intervals were calculated using the TOX-Dat multimethod computer program (Peltier and Weber 1985, Horning and Weber 1985). Survival data were arcsine-transformed and analyzed by Dunnett's Procedure which includes an analysis of variance (ANOVA), followed by a comparison of each toxicant concentration with the control. From this analysis a No Observed Effect Concentration (NOEC) and a Lowest Observable Effect Concentration (LOEC) were calculated. In addition, the Chronic Value (ChV) was determined by calculating the geometric mean of the NOEC and LOEC.

Growth (dry weight) data were also analyzed by Dunnett's Procedure (SAS 1986). The average dry weight

of larvae from each replicate test chamber was entered into the program. The results of the analysis of variance (ANOVA) and regression analysis were used to determine statistically significant effects of the various concentrations of Hydrothol and temperature (15° C and 25° C) on larval growth and survival.

Results

Dilution Water Quality

Water quality (Table 3-1) during the 7-day tests with fathead minnow larvae was within the range expected for moderately hard reconstituted freshwater (Horning and Weber 1986). Test temperature, regulated in a constant temperature room, was maintained at 15° or 25° C (± 1° C).

Reference Toxicant Tests

Results of reference toxicity tests with $CdCl_2$ indicated that methodology was of acceptable quality and that larvae used in the tests were healthy (Table 3-2). No trends of increasing or decreasing sensitivity of test organisms to Cd^{2+} were noted.

Survival and Growth of Fathead Minnow Larvae

Survival. Comparative data for survival at 15° and 25° C indicated that Hydrothol toxicity was not changed by test temperature (Tables 3-3 and 3-4). At 96-h, the LC50 was

Table 3-1. Range of water quality characterstics of moderately hard reconstituted freshwater used in replicate fathead minnow tests at 15° and 25° C.

Test	pH	D.O. (mg/L)	Alkalinity (as mg/L $CaCO_3$)	Hardness
15° C				
1	7.8-8.0	9.0-9.1	52-56	66-68
2	7.6-7.8	9.1-9.4	44-62	72-92
3	7.8-7.9	8.5-9.1	59-62	87-90
25° C				
1	7.7-8.0	6.9-7.1	66-69	80-86
2	7.8-7.9	7.1-7.3	62-63	77-79
3	7.7-7.8	7.2-7.4	58-61	73-82

Table 3-2. Results of reference toxicant tests with $CdCl_2$ for fathead minnow larvae.

Test No.	LC50 (ug/L as Cd^{2+})	95% Confidence Interval
1	30.2	25.2-37.9
2	20.9	17.7-25.2
3	14.0	10.3-18.8

393 ± 198 ug/L at 15° C and 468 ± 44.4 ug/L at 25° C. The values were not different ($p \leq 0.05$). The 7-d LC50s were lower at both temperatures than after four days, but again, the effect of temperature itself was not significant. The 7- LC50 was 233 ± 57.3 ug/L at 15° C and 304 ± 46.4 ug/L at 25° C.

It may be suggested that the temperature increase from 15° C to 25° C was not sufficient to produce a change in toxicity. However, chemical reactions generally double with a 10° C increase in temperature (Wilson 1972). Furthermore, a measurable increase in the toxicity of the inorganic salt of endothall (Aquathol-K) was seen for five species of fish tested by Johnson and Finley (1980). Although the test species was not given, Aquathol toxicity increased approximately fourfold in 96-h tests at 22° C vs 7° C. The LC50 at 7° C was 1740 mg/L, while at 22° C it was only 323 mg/L. In another study, Walker (1963) found a 13% to 43% increase in the toxicity of Hydrothol to bluegill sunfish, redear sunfish, largemouth bass and yellow bullhead with only a 5° C increase in test temperature. Since endothall compounds are contact type membrane-active herbicide and affect protein synthesis (Ashton and Crafts 1981), they should be more biologically active at elevated temperatures.

The fact that the toxicity of Hydrothol to fathead minnows did not change with a 10° C increase in

Table 3-3. Ninety-six hour and 7-day LC50s for larval fathead minnow survival at 15° C.

Test No.	96-hour (95% C.I.) LC50 (ug/L)	7-day (95% C.I.) LC50 (ug/L)
1	310 (132-530)	203 (164-240)
2	250 (189-32)7	197 (155-253)
3	619 (265-1060)	299 (237-382)
MEAN (± SD)	393 (± 198)	233 (± 57.2)

Table 3-4. Ninety-six hour and 7-day LC 50's for larval fathead minnow survival at 25° C.

Test No.	96-hour (95% C.I.) LC50 (ug/L)	7-day (95% C.I.) LC50 (ug/L)
1	519 (441-634)	251 (132-530)
2	447 (389-523)	324 (256-400)
3	438 (367-531)	337 (267-417)
MEAN (± SD)	468 (± 44.4)	304 (± 46.4)

temperature may be attributed to species differences. Interspecific responses to pollutants can differ remarkably even at a set temperature (Johnson and Finley 1980, Mayer and Ellersieck 1986).

Growth. There was no statistically significant weight gain in controls at 15° C compared to larvae in any of the Hydrothol concentrations (Table 3-5). Since fathead minnows spawn at temperatures above 17° C (Peltier and Mount 1985), their larvae may simply not be adapted to grow at temperatures lower than 17° C. Therefore, at 15° C survival rather than growth data were used to determine the chronic impact of Hydrothol on fish larvae.

Survival was significantly inhibited at Hydrothol concentrations $\geq$265 ug/L (the lowest observed effect concentration) at 15° C, while no effects were seen at concentrations $\leq$132 ug/L (the no observed effect concentration) (Table 3-6). Based on these data, the chronic value (ChV) for survival was calculated as 186 ug/L. The ChV is the geometric mean of the NOEC and LOEC and is equivalent to the maximum allowable toxicant concentration (MATC) used by regulatory agencies. Growth was a more sensitive measure of toxicity at 25° C than was survival (Table 3-7). Growth was significantly lower ($p \leq 0.05$) at concentrations $\geq$132 ug/L while survival was not significantly affected until Hydrothol concentrations were $\geq$ 265 ug/L. Using an LOEC of 265

Table 3-5. Seven-day growth and survival of fathead minnow larvae exposed to Hydrothol at 15°

Hydrothol percent survival[a]	Mean concentration (ug/L)	Mean weight[a] (± SD) (mg)
Control	0.107 (± 0.024)	95
50	0.106 (± 0.023)	98
132	0.087 (± 0.012)	93
265	0.109 (± 0.038)	55[b]
530	-[c]	0[b]

[a] Mean values ± standard deviation (SD) from three independent seven-day tests.
[b] Significantly different from control at the 0.05 level.
[c] Fish all died before seven days, no weight determined.

Table 3-6. Seven-day growth and survival of fathead minnow larvae exposed to Hydrothol at 25° C.

Hydrothol concentration (ug/L)	Mean weight[a] (mg)	(± S.D.)	Mean percent survival[a]
Control	0.420	(± 0.074)	100
50	0.348	(± 0.049)	90
132	0.300[b]	(± 0.016)	100
200	0.318[b]	(± 0.059)	90
265	0.198[b]	(± 0.084)	70[b]
530	0.255[b]	(± 0.078)	5[b]
1060	-[c]		0[b]

[a] Mean values ± standard deviation (SD) from three independent seven-day tests.
[b] Significantly different from control at the 0.05 level.
[c] Fish all died before seven days, no weight determined.

ug/L and an NOEC of 265 ug/L, the chronic value (ChV) for survival of fathead minnow larvae in Hydrothol was calculated to be 230 ug/L at 25° C. This means that survival of fathead minnow larvae is threatened by long-term exposures to Hydrothol concentrations that exceed 230 ug/L. The ChV for growth was 81 ug/L. At concentrations higher than 81 ug/L, growth impairment could result from chronic exposure to Hydrothol.

The relationship between growth and temperature was examined further with regression analysis (Table 3-8). While there was no significant ($p \leq 0.05$) relationship between the concentration of Hydrothol and growth at 15° C ($R^2=0.0002$), there was a significant relationship ($R^2=0.3940$) at 25° C. In addition, a two-way ANOVA (with factor interaction) indicated that when 15° and 25° C data were combined, temperature was more important than Hydrothol concentrations in determining larval weight gain ($p \leq 0.05$). Once again, temperature was an important factor in determining the chronic effect of Hydrothol on growth of fathead minnow larvae, even though survival was not significantly affected by temperature. It has been proposed that survival may be a satisfactory endpoint for chronic toxicity tests because in many cases it has proven to be an adequate indicator of long-term impacts (Mayer et al. 1986). However, at 25° C fathead minnow larval survival was substantially less sensitive than growth as an endpoint measurement.

Table 3-7. No effect, lowest effect, and chronic value concentrations (ug/L) of Hydrothol for fathead minnow larvae at 15° and 25° C.

Parameter	Survival 15° C	Growth 15° C	Survival 25° C	Growth 25° C
No Effect Concentration (NOEC)	132	-	200	50
Lowest Effect Concentration (LOEC)	265	-	265	132
Chronic Value (ChV)	186	-	230	81

Table 3-8. Regression analysis of the relationship between Hydrothol concentration (X) and growth (Y) at 15° and 25° C.

15° C	25° C
$Y = 0.0000032\ X + 0.102$	$Y = -0.0004\ X + 0.379$
$R^2 = 0.0002$[a]	$R^2 = 0.3940$[b]
$F = 0.003$	$F = 15.604$
$Pr > F = 0.9545$	$Pr > F = 0.0006$

[a]Not significant at $p \leq 0.05$. [b]Significant at $p \leq 0.05$.

Discussion

The results of several acute toxicity studies (Table 3-9) with fish species using Hydrothol are available for comparison with LC50 values calculated from the current study. Johnson and Finley (1980) determined the 96-h LC50 of 0.75 mg/L for fathead minnows at 18^{o} C. This value is close to my 96-h LC50 for the same species. Rainbow trout (Salmo gairdneri) and golden shiner (Notemigonus crysoleucas) were much less sensitive to Hydrothol as evidenced by their four day LC50s of 1.7 mg/L and 1.6 mg/L, respectively (Mudge et al. 1986, Finlayson 1980). Other workers reported a Hydrothol no mortality range of from 3.0-55.0 mg/L (Holmberg and Lee 1976, Liguori et al. 1983, Berry 1984). Since Hydrothol-191 is applied at a concentration of 1-5 ppmw (part per million water) and has a half-life of 10 d (Blackburn et al. 1971, Reinert et al. 1985), it poses a potential threat to fish. Its concentration can remain above the 96-h LC50 for 10-20 days.

Only limited chronic toxicity data are available from other studies with endothall products (e.g. Hydrothol and Aquathol-K) (Liguori et al. 1983, Eller 1973). In a study of the impact of 10 mg/L Hydrothol on juvenile Chinook salmon (Oncorhynchus tshawytscha), Liguori et al. (1983) observed marked changes in branchial tissues. Effects included epithelial

Table 3-9. Comparison of literature LC50 values for Hydrothol to various freshwater fish.

Test Organism	Stage or Wet Wt. (g)	Temp (C)	96-h LC50 (mg/L)	Reference
Golden shiner[a]	0.62	18	1.6	1
Rainbow trout	25	15	1.7	2
Rainbow trout	1.2	13	0.56	3
Rainbow trout	-	-	1.3	4
Cutthroat trout	1.0	10	0.18	4
Channel catfish	0.3	18	0.49	4
Bluegill	0.5	24	0.94	4
Bluegill	-	-	1.2	4
Fathead Minnow	0.6	18	0.75	3
Fathead minnow[b]	larvae	25	0.39	5
Fathead minnow[b]	larvae	15	0.47	5

[1]Finlayson (1980). [2]Mudge et al. (1983). [3]Johnson and Finley (1980). [4]Pennwalt Corp. (1980). [5]Current study.

hyperplasia and lamellar fusion. At lower exposure levels (<10 mg/L), no histopathologic effects were detected. The 14-d LC50 was 62.5 mg/L of endothall.

The most sensitive measure of Aquathol-K toxicity was the seawater test (Liguori et al. 1983). In this experiment, survival of juvenile Chinook salmon after placement in seawater was measured following their exposure to endothall concentrations of 10.1-105.7 mg/L for 14 days. Transfer to seawater simulated the migration of this species to the ocean during the smoltification process, a critical stage in their development. All fish died within three days of entry into seawater. Even at sublethal concentrations endothall exerts an effect that impairs important physiological processes related to osmoregulation (Liguori et L. 1983).

Eller (1973) followed histopathological changes in bluegill exposed to Hydrothol-191 for up to 112 days. He found significant but transitory changes in gill epithelium in fish exposed to 0.3 mg/L of the herbicide. Epithelial hyperplasia and lamellar fusion were noted in bluegill during the first 14 days of exposure. After that time, gill damage gradually reversed and gills were normal by the end of the study. Some abnormalities were noted in hepatic and testicular cells, but they were not conclusively related to Hydrothol concentration (Eller 1973).

The limited data relating test temperature to the toxicity of Hydrothol to fish comes from a study by Walker (1963). He found that 96-h LC50s for bluegill sunfish (0.05 mg/L), redear sunfish (0.10 mg/L), largemouth bass (0.14 mg/L) and yellow bullhead (0.31 mg/L) at 23.8^{o} C were reduced by 43%, 44%, 36% and 13%, respectively, at 18.3^{o} C. Reductions in Hydrothol toxicity due to test temperature were greater for three of these four species than was measured for the fathead minnow in the current study. The only exception was the yellow bullhead. Interspecific differences in sensitivity to herbicides is seen throughout the literature (Johnson and Finley 1980). The greater effect of test temperature on Hydrothol toxicity to species tested by Walker (1963) than for the fathead minnow may be due simply to species differences. No other explanations are readily apparent.

Hydrothol is relatively toxic to fish in comparison with other herbicides such as 2,4-D, dichlobenil, diquat, and PCP (Table 3-10) that may enter the aquatic environment. 2,4-D is applied to ponds and lakes for control of water hyacinth (Ag Consultant 1988) and to agricultural fields for control of broadleaf weeds (Ware 1978). Its mode of action via a complex mixture of effects on cell division and nucleic acid metabolism is somewhat different than the impairment of m-RNA production caused by Hydrothol (Ware 1978, Ashton and

Table 3-10. Summary of acute toxicity data for selected herbicides that may enter the aquatic environment in Florida[a].

Herbicide and test organism	Animal wt. (g)	Temp. (°C)	96-h LC50 (mg/L)
2,4-D			
fathead minnow	0.5	17	18.0
bluegill	1.4	17	7.5
Dichlobenil			
fathead minnow	0.8	18	6.0
bluegill	1.5	18	8.3
Diquat			
bluegill	1.3	12	245
PCP			
fathead minnow	1.1	20	0.21
bluegill	0.4	15	0.03
channel catfish	0.3	20	0.07
Aquathol			
bluegill	1.3	22	343
channel catfish	0.4	12	>150
Hydrothol			
fathead minnow	0.6	18	0.75
fathead minnow[b]	0.4	15	0.39
fathead minnow[b]	0.4	25	0.47

[a]Data from Johnson and Finley (1980). [b]Data from the current study.

Crafts 1981). The 96-h LC50 for 2,4-D was 18.0 mg/L for fathead minnow and 7.5 mg/L for bluegill (Johnson and Finley 1980). These levels are far below both the application rates for aquatic environments or the expected levels entering water from treated agricultural areas (Ag Consultant 1988).

Dichlobenil, an inhibitor of CO_2 fixation and oxidative phosphorylation (Ware 1978), is used to eliminate Chara, Potamogeton spp. and Myriophyllum spp. in marshes. Its 96-h LC50 for fathead minnows is 6.0 mg/L and 8.3 mg/L for bluegill (Johnson and Finley 1980). In normal use, dichlobenil is not toxic to fish in treated areas (Ag Consultant 1988).

Diquat is the most widely used herbicide for control of broadleaf weeds along ditchbanks and irrigation canals (Gangstad 1986). In lakes and slow-moving waters, Diquat use controls coontail (Ceratophyllum demersum), bladderwort (Utricularia spp.) and pondweed (Potamogeton spp.). It is a contact herbicide that reduces photosynthetic activity (Ware 1978). Treatment of canal banks with 2,4-D at recommended doses results in a water concentration of only 0.025-0.061 mg/L (Gangstad 1986), far below the 96-h LC50 of 245 mg/L for bluegill.

PCP (pentachlorophenol), on the other hand, is not used much anymore primarily because of its extreme toxicity to biota (Ware 1978). PCP is a non-selective herbicide and preharvest defoliant. It has multiple

routes of action including plasmolysis and protein precipitation and is destructive to all cells (Ware 1978). Its toxicity to fish is evident from the 96-h LC50 of 0.21 mg/L for fathead minnow, 0.03 mg/L for bluegill and 0.07 mg/L for channel catfish (Johnson and Finley 1980).

Results of the current study using fathead minnow larvae indicate that the use of Hydrothol in the aquatic environment should be limited to properly trained professionals. It is a highly toxic herbicide for which there are a number of substitutes. The effect of temperature on the toxicity of Hydrothol can be substantial. It should be applied at the lowest water temperature at which it will control the particular macrophyte of interest. This temperature will vary based on the species of plant because Hydrothol is most effective when applied early in the growing season (Ag Consultant 1988).

CHAPTER 4
AN ASSESSMENT OF THE CHRONIC TOXICITY OF HYDROTHOL-191 TO THE ZOOPLANKTER CERIODAPHNIA DUBIA USING A 7-DAY SURVIVAL AND REPRODUCTION TEST

Introduction

Since zooplankton are an extremely important part of most aquatic ecosystems and contribute substantially to the food supply of fish (Horning and Weber 1985, Mount and Norberg 1984), these organisms have been used extensively in toxicity tests. Early studies used either Daphnia magna or Daphnia pulex as test organisms. However, each of these species had their shortcomings. D. magna has a limited distribution in aquatic systems and neither animal is easy to culture in the laboratory.

Ceriodaphnia was chosen for use in a new subchronic toxicity test for several reasons (Horning and Weber 1985, Mount and Norberg 1984). Ceriodaphnia reproduce more rapidly (3 broods in a week) than Daphnia, are ubiquitous, and are somewhat easier to culture under laboratory conditions (Horning and Weber 1985). The static renewal Ceriodaphnia dubia survival and reproduction test (Horning and Weber 1985) was developed as a substitute for the 21- to 28-day Daphnia chronic toxicity test. Toxicity is based on survival and reproduction over a 7-day period in the newer

test. Thus, the toxic effects of chronic exposure to a substance may be more easily and rapidly assessed than methods using D. magna.

The Florida Department of Environmental Regulation (FDER) requested the determination of the chronic effects of Hydrothol-191 on C. dubia using this new test method. Their concern stemmed from the growing use of Hydrothol in Florida aquatic systems for control of several species of macrophytes. Specifically, too little was known about its long-term impacts on non-target organisms. Since this herbicide has a half-life of 10 days (Reinert and Rodgers 1987), it can remain at potentially toxic levels in the environment for 10 days or more. During that time, zooplankton biomass could be seriously lowered if Hydrothol affected both survival of adults and their reproductive capacity. In that case, their role as a food source for fish would be impaired.

The C. dubia survival and reproduction test was designed to measure the effects of toxicants on survival of adults and production of young (Mount and Norberg 1984, Horning and Weber 1985). Tests were performed at 15° and 25° C to see if temperature at the time of Hydrothol application would affect its impact on zooplankton. If so, field use could be limited to times when water temperature and plant growth activities were compatible.

Materials and Methods

Test Organism

Ceriodaphnia dubia stock obtained from EPA-Newtown, Ohio was used to start a laboratory culture. The animals were maintained in 1 L beakers in a 25° C environmental chamber, with 16 hours of light and 8 hours of dark.

Test Chemical

Several formulations of endothall (7-oxabicyclo [2,2,1] heptane-2,3-dicarboxylic acid) are used in Florida for control of aquatic weeds and algae. However, the chronic toxicity of the alkylamine form of endothall, i.e. Hydrothol-191 (Pennwalt Corp., Philadelphia, PA), was assessed in this project based on the response of Ceriodaphnia dubia during a 7-day test. Hydrothol concentrations were not measured, but were calculated based on volume/volume dilutions of the 53% active ingredient (the alkylamine salt of endothall) indicated on the product label.

Dilution Water

Moderately hard reconstituted freshwater (Horning and Weber 1985) inoculated with bacteria-rich aerobically digested trout chow and aged for one week, was used as the culture medium (Table 4-1). The addition of bacteria and aging of the dilution water has been suggested (De Graeve and Cooney 1987, FDER 1986, Mount and Norberg 1984) to stabilize water quality and increase ambient food levels.

Table 4-1. Dilution water quality parameters for *Ceriodaphnia dubia* Survival and Reproduction Tests.

Parameter	Mean	S.D.
pH	6.84	0.08
Alkalinity (as mg/L $CaCO_3$)	53.21	1.55
Hardness (as mg/L $CaCO_3$)	86.83	1.56
Conductivity (umhos/cm)	349.3	3.4

Bacteria are a major food source for Ceriodaphnia (Norberg and Mount 1985). Thus, while cultures were fed daily, the presence of a high background bacterial population assured that food density was adequate to support high reproduction. Aeration was provided by a small air pump set at minimum output to prevent oxygen depletion by bacterial respiration.

Test Organism Food

Ceriodaphnia were fed a mixture of digested trout chow, Cerophyll, and yeast (Horning and Weber 1985) provided at a rate of 3 ml/L of water per day. Most cultures developed a lush algal growth which was allowed to remain even though water in the culture chambers was replaced weekly. The algae provided an extra food source.

Reference Toxicant Tests

At least once a month, a reference toxicant test using sodium dodecyl sulfate (SDS) was performed to verify that the in-house Ceriodaphnia cultures were healthy and nominally sensitive. That is, LC50s for SDS were compared to those in the literature to ensure that their responses to the test chemical were not due to an inherent sensitivity. The SDS was obtained from EPA-Cincinnati specifically for use as a reference toxicant.

Several toxicity tests were also performed using $CuSO_4$. The results of tests with $CuSO_2$ proved to provide more consistent results.

Range-Finding Test

A 48-hour range finding test was performed at the two test temperatures (15° and 25° C) before definitive testing began. Hydrothol concentrations ranged from 100-3200 ug/L based on percent active ingredient (ai) as indicated on the product label. Dilution and control water were moderately hard reconstituted freshwater "conditioned" with a bacterial inoculum and aerated for a week.

Preparation For Chronic Toxicity Tests

Approximately one week prior to the start of a test, 20 brood animals were obtained as neonates and placed in separate 30 ml plastic cups containing 15 ml of culture medium. They were fed 0.2 ml of the TCY mixture and 0.2 ml of an algal mixed culture (Chlamydomonas, Klebsomidium and Euglena) each day. Algal supplements have been suggested for use in C. dubia toxicity tests to promote high fecundity (Cowgill et al. 1985). Water was changed every other day. Neonates to be used as test organisms were harvested from these brood chambers during a 4- hour period on about the seventh day. They were held 12-24 hours prior to the start of each test.

Chronic Toxicity Tests

Toxicity tests were performed at 15° and 25° C in a constant temperature room with a 16L:8D lighting regime. To begin each test, five toxicant solutions were prepared from

a concentrated stock diluted with moderately hard reconstituted freshwater. Hydrothol concentrations of 25 ug/L to 400 ug/L were used. Test chambers were filled with 15 ml of toxicant or control water and the neonates were randomly distributed among them, one to each chamber. Each treatment consisted of 10 replicate chambers placed in a plywood rack. Test solutions were prepared and renewed daily. The presence and number of young were recorded for each chamber daily before transferring the adult organisms to fresh test solutions.

Ceriodaphnia were fed 0.2 ml of TCY and 0.2 ml of algal culture following transfer to clean vessels. Temperature, pH, alkalinity, hardness and conductivity of the dilution water were measured each day. Since the dilution water was saturated with oxygen by aeration, dissolved oxygen measurements were not made. Each test was terminated after 7 days, and the mean young production per adult was calculated for each treatment and the control.

Statistical Analysis

Statistical analysis followed standard EPA protocol based on the original number of adult animals used per test chamber, i.e. if one died, it was still included in the calculation of mean brood number and size (Horning and Weber 1985). LC50s for Hydrothol were calculated with the TOX-DAT Multi-method (Peltier and Weber 1985). This series of computer programs calculates the LC50 and 95% confidence

intervals by 3 methods: moving-average angle, binomial and probit.

Fisher's Exact Test was used to identify treatments in which adult survival was significantly different from the control. No further analysis was performed on such treatments. However, reproduction data were analyzed for toxicant levels in which adult survival was not significantly different from the control using ANOVA and Dunnett's Procedure. This differentiation between treatments with and without significant adult mortality was necessary because average reproductive capacity would have been affected by the number of live adults. Based on the results of the Dunnett's Procedure, the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) were determined and the Chronic Value (ChV) was calculated (Horning and Weber 1985). The ChV is the geometric mean of the NOEC and LOEC.

Results

Reference Toxicant

Results of reference toxicant tests using sodium dodecyl sulfate (SDS) indicated that the test organisms were nominally sensitive (Table 4-2). LC50s varied from 2.8-5.5 mg/L. Literature values for SDS 48-hour LC50s are 1.5-8.2 mg/L for Ceriodaphnia dubia (FDER 1986) and 7-13 mg/L for

Table 4-2. Results of the Ceriodaphnia dubia 48-hour reference toxicant tests using sodium dodecyl sulfate (SDS) calculated by the moving average angle.

LC50 (mg/L SDS)	95% Confidence Interval
2.83	2.01-3.62
5.53	4.66-6.74
4.61	3.80-5.41

for Daphnia magna, substantially lower Daphnia magna. Reference toxicity tests with $CuSO_4$ produced a 48-hour LC50 of 92.7 ± 36 ug/L.

Acute Toxicity

A 48-hour range-finding test was used to delineate the appropriate Hydrothol concentrations for the chronic toxicity tests (Table 4-3). The Ceriodaphnia dubia 48-hour LC50 was 0.49 ± 0.03 mg/L Hydrothol at 25° C. This agrees well with the published LC50 for Daphnia sp. at 21° C, 0.36 mg/L (Pennwalt Corp. 1980), but is substantially lower than values recorded for several algal species. Mudge et al. (1986) found 1.5 mg/L Hydrothol to be the LC50 for an algal mix (Cyclotella, Euglena, Fragilaria, Nitzschia and Pediastrum) after five days of exposure. No other acute data on plankton responses to Hydrothol are available.

At 15° C, the C. dubia 48-hour LC50 was 1.43 ± 0.32 mg/L Hydrothol. This increased tolerance of C. dubia to Hydrothol compared to results at 25° C may be attributable to a lower metabolic rate at the lower temperature (Gophen 1976). Since Hydrothol is membrane-active and apparently affects m-RNA production (Ashton and Crafts 1981), its impacts may be dampened with decreased temperature because processes involved in protein synthesis would be slower. Such a response would be typical of chemical reactions in general, as well as those mediated by enzymes (Wilson 1972).

Table 4-3. Acute toxicity of Hydrothol to various aquatic organisms.

Organism	Temp. °C	LC50 mg/L (95 % C.I.)	Duration of Test (h)
C. dubia*	25	0.495[a] (0.363-0.7.65)	48
Daphnia sp.	25	0.360[b]	48
algal mix	20.5	1.50[c]	120
C. dubia	15	1.43[a] (1.09-2.00)	48

* Ceriodaphnia dubia.
[a] Results of the current experiments.
[b] Pennwalt Corp. 1980.
[c] Mudge et al. 1986.

Chronic Toxicity

Survival. The Ceriodaphnia dubia survival and reproduction test permits the calculation of a 7-day LC50 and uses changes in reproductive capacity over a 7-day period as a measure of sub-lethal chronic toxicity (Horning and Weber 1985).

At 25° C, the 7-day LC50 was 190 ± 6.2 ug/L (Table 4-4). This value is lower than the suggested Hydrothol field application rate of 1-5 mg/L (Pennwalt Corp. 1980) by over an order of magnitude and points to the potential hazards of Hydrothol use in aquatic systems. Since its half-life is approximately 10 days (Blackburn et al. 1971, Reinert et al. 1985), the impact of normal field application on the food chain could be devastating. While fish may escape the treated areas providing there are refugia, widespread use of Hydrothol in a lake could markedly reduce the zooplankton populations, which are less mobile, for 1-2 weeks after its application. Consequently, during periods of high fish reproduction, fry could be adversely affected by low zooplankton availability.

Based on the results of the 48-hour tests in which C. dubia had a higher LC50 at 15° C than at 25°, it was expected that the LC50 at seven days would also be higher for the 15° C test. However, this was not the case. The LC50 was significantly ($p \leq 0.05$) lower at 15° C (143 ± 4.6 ug/L), than at 25° C (190 ± 6.2 ug/L) (Table 4-5). Why this reversal in sensitivity occurred is not clear. It is

Table 4-4. LC50s from three replicate Ceriodaphnia dubia 7-day Hydrothol toxicity tests at 15° and 25° C based on the moving average angle method.

	LC50 ug/L at 15° C (95 % C.I.)	LC50 ug/L at 25° C (95 % C.I.)
	149 (114-199)	192 (134-326)
	141 (103-210)	195 (142-306)
	141 (103-210)	183 (141-255)
MEAN (s.d.)	143.7 (4.6)	190 (6.2)

Table 4-5. Reproduction data for replicate tests of Ceriodaphnia dubia exposed to various concentrations of Hydrothol at 25° C for 7 days.

[Hydrothol] (ug/L)	Final Survival %	Mean (S.D.) young/female	Mean No. broods/female
0	100	11.6(3.2)	2.50
25	90	5.0(2.8)	1.33 *
50	90	4.1(2.6)	0.80 *
100	90	2.9(2.6)	0.80 *
200	70	0	0
400	0	0	0
0	100	11.8(3.7)	2.50
25	100	5.9(3.4)	1.10 *
50	80	4.3(4.1)	0.90 *
100	80	1.8(1.5)	0.70 *
200	80	0	0
400	0	0	0
0	100	23.6(4.4)	2.9
25	100	5.3(2.8)	1.1 *
50	100	0.2	0.2 *
100	80	0	0
200	70	0	0
400	0	0	0

* Indicates a significant difference from control at $p \leq 0.05$.

opposite to the response of several fish species tested at 18.3° and 23.3° C by Walker (1963), while fathead minnows (Chapter 3) showed no significant change in sensitivity to Hydrothol with a 10° C increase in temperature (15°-25° C). Over time, mortality at the two temperatures became equal. Such results demonstrate the utility of chronic studies in assessing the impact of a toxicant on aquatic biota.

Chronic effects of Hydrothol on reproduction. Chronic toxicity tests are designed to measure more subtle (sublethal) responses of organisms to toxicants than are acute tests. The Ceriodaphnia dubia survival and reproduction test (Horning and Weber 1985) uses changes in reproductive rate over a 7-day period as a measure of sublethal toxicity. The effects of a toxicant on zooplankton reproduction is more subtle but no less significant than its lethality. Even if a population of Ceriodaphnia dubia survives the initial stress of toxicant input it is still possible that fecundity may decline or cease. The impact of such an occurrence could seriously alter trophic level interactions in the ecosystem.

Seven-day reproduction data for C. dubia exposed to Hydrothol at 25° C indicated that even at concentrations as low as 25 ug/L (the lowest test concentration), Hydrothol affected fecundity (Table 4-6). Control animals produced 2.5-2.9 broods of young each and an average of 11.6-23.6 young during the tests. At a Hydrothol concentration of 25 ug/L, fecundity was significantly ($p \leq 0.05$) reduced to 1.10-

Table 4-6. Summary of the chronic toxicity of Hydrothol at 25° C to Ceriodaphnia dubia based on reproduction.

		[Hydrothol] 10 ug/L		
	Control	Rep. 1	Rep. 2	Rep. 3
Final Survival %	90	60	100	100
Mean No. (S.D.) Young/ female	25.4(6.9)	14.8(9.9)*	20.7(4.7)	18.3(4.2)*
Mean No. (S.D.) Broods/ female	2.9(0.32)	2.7(0.67)	2.4(1.07)	2.4(0.52)
NOEC (ug/L)	N/A	<10	10	<10
LOEC (ug/L)	N/A	25	25	25
ChV (ug/L)	N/A	<15.8	15.8	<15.8

* Denotes significant difference from control at $p \leq 0.05$.

1.33 broods and an average of 5.0-5.9 offspring per adult female. This lowest observed effect concentration (LOEC) was almost eight times lower than the LC50 at 25° C, 190 ± 6.2 ug/L Hydrothol.

At higher toxicant concentrations, reproduction was reduced even more. Since Hydrothol affects the production of m-RNA (Ashton and Crafts 1981), its impact on C. dubia reproduction is not surprising. Normal production and development of eggs is a process requiring adults to have healthy metabolic capacity. If protein synthesis is impaired by the limited availability of m-RNA, enzymes would become limiting factors.

To determine the NOEC (no observed effect concentration) used to calculate a chronic value (ChV), I ran 3 additional 7-day tests with controls and 10 ug/L Hydrothol test concentrations (Table 4-7). At 25° C, the chronic value (ChV) for Hydrothol is less than or equal to 15 ug/L based on reproduction.

Since there was no reproduction in seven days in the tests performed at 15° C, no statistical analysis of toxicant effects was possible. The fact that no reproduction occurred at this low temperature is no surprise. McNaught and Mount (1985) found that the 7-day C. dubia reproduction test became a 28-day test at 18° C. Even at 20° C it took nine days for C. dubia to produce three broods of offspring (Cowgill et al. 1985). At 15° C, the

Table 4-7. Acute toxicities to zooplankton of several herbicides used to control submergent macrophytes in Florida lakes.

Herbicide	Organism	48-hour LC50 mg/L	Temperature °C
Hydrothol-191	C. dubia	0.49	25
Aquathol-K	D. magna	316[a]	25
diquat	D. magna	7.1*[d]	21
dichlobenil	D. pulex	3.7[b]	15
	Simocephalus	5.8[b]	15
	D. magna	9.8*[d]	21
2,4-D	D. magna	100*[d]	21
diuron	Simocephalus	2.0[b]	15
	D. pulex	1.4[b]	15
	D. magna	47.0[d]	21

[a] Pennwalt Corp. 1980.
[b] Johnson and Finley 1980.
[c] Water hardness 272 ppm $CaCO_3$.
* IC50 at 26-h. [d]Crosby and Tucker 1966.

metabolic rate decreases significantly from that at 22° C (Gophen 1976), and was reflected in a much slower reproductive rate.

Discussion

The acute toxicity of Hydrothol to Ceriodaphnia dubia has been determined based on survival at 48-h. My findings confirm previous conclusions that Hydrothol is considerably more toxic to zooplankton than some alternative compounds (Table 3-4). For example Aquathol-K, an inorganic salt of endothall, has a 48-h LC50 of 316 mg/L. That level is far above field use levels (5-10 mg/L) and should not pose a threat to zooplankton (Pennwalt Corp. 1980). Hydrothol is often chosen over Aquathol because the former is better for control of algae and remains effective longer.

Dichlobenil is another effective herbicide that is non-toxic to aquatic fauna at normal use concentration (Ag Consultant 1989). It acts to inhibit CO_2 fixation and oxidative phosphorylation in plants. Johnson and Finley (1980) determined that the 48-h LC50 for D. pulex was 3.7 mg/L and for Simocephalus spp. it was 5.8 mg/L, both at 15° C.

A common herbicide used in water hyacinth (Eichhornia crassipes) control programs is 2,4-D (Ag Consultant 1989). It is also used to eliminate broadleaf weeds in sorghum, sugar cane and alfalfa fields. By a complex mixture of effects at the cellular level, this herbicide inhibits cell

division and impairs nucleic acid metabolism. At 21° C, the 48-h EC50 of 2,4-D was >100 mg/L for D. magna (Crosby and Tucker 1966).

Various algae, water hyacinth, coontail (Ceratophyllum demersum), hydrilla (Hydrilla verticllata), pondweeds (Potamogeton spp.) and several broadleaf weeds in agricultural fields can be controlled with the use of diuron. Diuron is a substituted urea herbicide (Weed Society of America 1979) that inhibits photosynthesis by blocking electron transport (Ashton and Crafts 1981). The 48-h LC50 was 2.0 mg/L for Simocephalus and 1.4 mg/L diuron for D. pulex. These values represent levels exceeding those produced by proper weed control programs (Ware 1979).

Because of their important role in the aquatic food web, the response of zooplankton to long-term treatments with a pesticide is important to know before it is widely used. However, until recently there were no accepted methods to assess impacts on zooplankton reproduction. Even now, only acute toxicity test (24-48 hours) data are required by EPA for pesticide registration (Zucker 1985a). Only two other studies have provided information on the chronic effects of endothall herbicides on zooplankton.

Serns (1975) followed the response of zooplankton to a 5 mg/L Aquathol-K exposure from June through October. Plant control was effective, resulting in the increased presence of Chara, but no significant change in the structure or composition of the zooplankton community was noted.

Cladocerans and copepods exhibited their usual seasonal changes in density.

Results from a field study of the efficacy and impacts of Aquathol-K and Hydout, a pelletized amine formulation used to control Hydrilla, found little effect on zooplankton populations (Westerdahl 1983). A movement of zooplankton into the water column as plant height decreased and an increase in naupliar size 49 days after treatment were noted. However, zooplankton community structure and composition remained constant throughout the post-treatment period.

Results from the aforementioned studies contradict those of my laboratory study with Hydrothol-191. I found a significant reduction in reproduction by Ceriodaphnia dubia in concentrations as low as 0.016 mg/L. There are several factors that may explain these differences. First of all, neither the study by Serns (1963) nor the work by Westerdahl (1983) used the same formulation as I did. Serns (1963) tested Aquathol-K, while Westerdahl (1983) used both Aquathol-K and Hydout. The toxicity of Aquathol-K to aquatic organisms is several orders of magnitude lower than that of Hydrothol-191 (Pennwalt Corp. 1980, Johnson and Finley 1980). Hydout is an amine formulation, as is Hydrothol-191, but the former is a slow-release granular product, while Hydrothol 191 is a liquid. This difference may affect the amount of herbicide in solution at any time.

Second, in the laboratory study I renewed the test solutions each day, thereby maintaining a constant exposure level. The studies by both Serns (1963) and Westerdahl (1983) were conducted outdoors using one application of the herbicide. Therefore, concentrations of endothall began to decrease immediately due to microbial degradation and biotransformation (Reinert and Rodgers 1987).

Finally, adsorption of a herbicide may remove significant amounts from the pool of biologically active compound in waters containing macrophytes, algae and sediments. Both of the field studies were performed in the presence of natural flora and sediment (Serns 1963, Westerdahl 1983). Thus, effective concentrations of endothall were likely reduced compared to those in the laboratory test vessels. The latter contained only the test organism and solution.

This study showed that temperature had a measurable effect on the toxicity of Hydrothol to C. dubia. However, the relationship between temperature and survival after 2-d exposures was inverse to that noted in 7-d tests. At 48-h, the LC50 at 15^{o} C was 1.43 mg/L Hydrothol, while at 25^{o} C it was 0.49 mg/L. With 7-d exposures, the LC50s decreased at both test temperatures, but the survival rate was lower at 15^{o} C than at the higher temperature. The reasons for this contradiction are unclear. A lower metabolic rate may have initially protected the animals in 15^{o} C tests from the impact of Hydrothol on m-RNA production. However, it

appears that with longer exposure low temperature compounded the toxicity of Hydrothol. Because this herbicide is applied in late spring or early summer, zooplankton in Florida should not be concurrently exposed to both low temperature and Hydrothol toxicity.

CHAPTER 5
SIMPLIFICATION OF *IN VITRO* CULTURE TECHNIQUES FOR FRESHWATER MUSSELS

Introduction

Recently, there has been growing concern over the loss of freshwater mussel species (Unionidae) and their declining densities in areas perturbed by pollution and installation of dams. These molluscs, historically abundant in most North American waters, inhabit both lakes and streams. The area with the greatest number of species and individuals was the Mississippi River and its tributaries, notably the Cumberland, Tennessee and Ohio rivers (Burch 1973).

The unusual mode of reproduction of unionid molluscs makes their culture in the laboratory more difficult than other groups that have free-living veliger larvae. The life cycle of unionid mussels includes a parasitic larva (glochidium) that normally attaches to fish gills or fins during early development. To propagate these molluscs *in vitro*, a suitable culture medium is necessary to provide the nourishment usually obtained from the host fish. With the hope of replenishing declining natural populations, the Tennessee Valley Authority began funding research to develop methods for *in vitro* propagation of these freshwater molluscs in the early 1980s (1982,

1984). The goal was to eliminate the need for fish hosts during the larval stage so that laboratory culture of mussels would be practical. In turn, such artificial propagation would produce a large number of juvenile mussels for use in restoration of lost natural populations.

As a result, a culture medium containing vitamins, glucose, amino acids, antibiotics and fish plasma in place of live fish was developed (Isom and Hudson 1982, 1984; Isom 1986). The transformation in culture of glochidia to juveniles takes 9-30 days ($23^{o} \pm 3^{o}$ C) depending on the species, culture temperature and glochidia maturity at the start of incubation (Isom and Hudson 1982). While the Hudson and Isom method (1982) is far better than that of Ellis and Ellis (1926) which relied on the use of fish hosts for encystment of glochidia during transformation, further simplification is desirable. The old method (Isom and Hudson 1982, 1984) is laborious, still requires the use of fish plasma which may not be readily available nor of consistent quality, and a CO_2 incubator.

A simplified method for mussel culture is necessary before juveniles can be available in the numbers needed for replenishment of declining wild stocks or for other purposes, e.g. toxicity tests. The objectives of the work described here were: (1) to use standard tissue culture media and plasma available from commercial

suppliers to propagate unionid mussels *in vitro* as a means of simplifying the culture technique, (2) to determine if the use of non-bicarbonate organic buffers, i.e. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) or 3-[N-morpholino] propanesulfonic acid (MOPS), would circumvent the need for a CO_2 incubator to maintain pH, and (3) to test the efficacy of these methods in the culture of several species of mussels.

Materials and Methods

Test Organisms

Glochidia of *Anodonta imbecilis*, the feeble mussel, was used in culture experiments. Since longterm propagation and culture of unionid mussels has not been achieved to date, gravid females must be collected when they are naturally available. In northern Florida, females carrying mature glochidia can be found from April through June. Most of the mussels used in the development of these culture techniques were collected from the Suwannee River, Florida. Several specimens of *A. imbecilis* were obtained from Dr. Paul Yokely, of the University of Northern Alabama.

Anodonta imbecilis was chosen because it had been successfully cultured by Isom and Hudson (1982), it is a widely distributed mussel and has a relatively long reproductive period (two to three months depending on the location). These characteristics are important when

choosing an organism as a potential bioassay animal, one of the proposed uses of juvenile mussels produced by these in vitro techniques.

Plasma substitutes

Culture techniques developed by Isom (1986) and Isom and Hudson (1982) were used as the starting point for simplification. Their culture medium, modified from Ellis and Ellis (1926) and Eagle (1959), uses vacuum sterilized fish plasma as a nutrient source during culturing in place of the fish themselves. It contains a mixture of amino acids, salts, glucose, vitamins, antibiotics (carbenicillin, rifampin, gentamycin, amphotericin B) and phenol red as a pH indicator. A typical 15 X 60 mm culture dish would contain 2 ml of medium, 1 ml of serum and 0.5 ml of the antibiotic/antimycotic agents as described in Isom (1986). Glochidia are removed from the gills of a female mussel, washed and added (500-1000) to the culture medium under a sterile hood. The plates are placed in a CO_2 incubator (5% CO_2) at $23^{o} \pm 3^{o}$ C. Isom and Hudson (1982) found 23^{o} C to be the incubation temperature that allowed transformation but kept bacterial and fungal growth to a minimum. Cultures are monitored daily under a microscope to follow the process of organogenesis. When the foot becomes active and other parts are developed, juveniles are said to be transformed. They are then placed in

water where they can begin siphoning water for oxygen and food.

While standard tissue culture methods require the use of plasma or serum (Ham and McKeehan 1979) because they contain essential proteins, growth factors and hormones that enhance cell division, there has been no indication of their specific role in glochidia transformation. Isom and Hudson (1982) determined that fish plasma was an absolute necessity for successful transformation of glochidia for all mussel species they cultured. However, verification of their findings was desirable since the simplification of procedures afforded by the substitution of a more readily available protein source would be substantial. Therefore, two modifications of the culture medium studied were first, the substitution of other protein sources for fish plasma at 5% w/v and second, the use of other sera (33 % v/v) readily available from tissue culture supply houses in place of fish plasma.

Protein sources were acetone precipitates of trout liver, salmon liver and rabbit pancreas, and bovine casein (Sigma Chemical Co.). For each protein, 3 g of powdered extract were mixed in 60 ml of distilled water for three minutes by vortexing. The resulting slurry was centrifuged at 1500 g for 5 minutes to remove undissolved materials. One ml of the supernatant was used per three ml of culture medium. Alternate sera used were bovine,

neonatal calf and horse. These sera were used at the same final culture concentration as was fish plasma (1 ml/2ml growth medium). Glochidia were also cultured in medium with no protein source or plasma. Culture medium with fish plasma was used as the control.

Per cent transformation of glochidia in each culture medium was used as the measure of success of the modification. ANOVA and Duncan's Procedure were used to analyze results from a total of 6 trials with 2 plates per treatment. Three microscope fields (40X) were counted per treatment to determine the number of glochidia that had transformed vs those that had not. The untransformed glochidia included those that did not begin to develop at all due to lack of maturity, those that did not complete transformation and those that were non-viable after 24 h. In cases where glochidia transformed but the juveniles were lethargic and survived only 24 hours, such a response was taken as an indication of morbidity and the medium judged unsuccessful in producing juveniles for field or laboratory use.

CO_2_Incubator

Once the necessity for plasma was tested, the use of organic buffers in place of the CO_2 incubator as a pH-stat was investigated. In the Isom and Hudson (1982) method, culture pH is maintained in the optimal range (7.3-7.4) by a HCO_3-CO_3 buffer system based on $NaHCO_3$ and

CO_2. The CO_2 atmosphere is provided by a CO_2 incubator. MOPS (3-[N-morpholino] propanesulfonic acid) and HEPES (hydroxyethylpiperazine-N'-2-ethanesulfonic acid), non-bicarbonate organic buffers, are widely used in tissue culture methods for many cell lines (Ham and McKeehan 1979).

To test their efficacy in pH maintenance in a non-CO_2 environment, either MOPS or HEPES were added to the complete medium (0.22 %) in addition to $NaHCO_3$, and the pH was adjusted to 7.3-7.4 by titration with NaOH. Five hundred to a thousand glochidia were cultured at $23^{o} \pm 3^{o}$ C in pairs of culture dishes containing standard medium with bicarbonate, or medium fortified with MOPS or HEPES (0.22% w/v) in addition to $NaHCO_3$. Incubation temperature was set at $23^{o} \pm 3^{o}$ C based on results of Isom and Hudson's work. One dish was then placed in an incubator with 5% CO_2 at 100% relative humidity, while its duplicate was incubated in ambient air at $24^{o} \pm 3^{o}$ C. Again, per cent transformation was the parameter used for statistical analysis.

Use of Commercial Media

A third series of experiments was designed to see if standard tissue culture media could be used in place of the medium developed by Isom and Hudson (1982). Their medium must be made from many separate reagents that are components of commercial media, e.g. Medium 199 (M199)

and Dulbecco's Modified Eagle's Medium with high glucose (DME). The advantages of using commercial media are that they are: (1) easier to use, (2) readily available, and (3) manufactured under consistent conditions with quality control that may not be possible in all research laboratories.

Glochidia were cultured in the Isom and Hudson (1982) medium, M199 or DME (with added antibiotics), and horse serum (1 ml/2ml medium). DME and M199 were hydrated in distilled water, adjusted to pH 7.3-7.4 with NaOH and filter-sterilized prior to their use, just as was the Isom and Hudson medium. Per cent transformation was compared among the three media as a measure of media suitability for mussel culture.

Species Cultured

Finally, Anodonta imbecilis, Lampsilis teres and Villosa lienosa were cultured using M199, DME or Isom and Hudson's (1982) medium and horse serum. As mentioned before, A. imbecilis has been cultured in vitro for several years by Hudson and Isom (1982). Hudson and Isom (1982) have also had success culturing V. lienosa and L. teres using fish plasma and their own culture medium. These species are less widely distributed than A. imbecilis but are common in northern Florida and were collected in the Suwannee River (Burch 1973). The usefulness of simpler culture methods would be greatly

enhanced if a number of species could be transformed using them. Transformation of Villosa lienosa and Lampsilis teres was also attempted using horse serum and the commercial media.

Results

In the first group of tests, transformation success ranged from 0% with casein to a mean of 95.5% for neonatal calf serum based on six trials (Table 5-1). While they did develop, juvenile mussels transformed in the salmon and trout media were not as healthy (inactive, lethargic) as those from the neonatal calf and horse media although the transformation success for these four groups were not significantly different based on ANOVA and Duncan's procedure ($p \leq 0.05$).

Since in vitro propagation of mussels is designed to provide stock either for replenishment of declining wild populations or for toxicity testing, survivability past transformation is important. Therefore, salmon and trout acetone precipitates of liver were judged inadequate serum substitutes. While transformation success was as good in neonatal calf serum ($95.5 \pm 1.9\%$) as it was in horse serum ($94.7 \pm 4.0\ \%$), horse serum was selected over neonatal calf serum because the latter is more expensive. In all cases, the use of sterile serum eliminated or markedly decreased bacterial growth common

Table 5-1. Per cent transformation of Anodonta imbecilis glochidia in media with various protein sources or sera for 6 trials with 2 plates counted per treatment.

Serum or Protein Source	Mean %	(s.d.)
Neonatal calf serum	95.5[a]	(1.87)
Horse serum	94.7[a]	(3.98)
Salmon liver	91.5[a]	(5.39)
Trout liver	83.0[ab]	(13.83)
Fish plasma	81.8[b]	(7.47)
Rabbit pancreas	67.5[b]	(20.17)

[abc]Treatments with the same letters were not significantly different from each other ($p \leq 0.01$).

in cultures with fish plasma. This was a major problem in earlier work (Isom and Hudson 1982).

Results from the second group of experiments testing transformation success for Anodonta imbecilis cultures incubated either in a CO_2 (5%) atmosphere or ambient air indicated that there was significantly ($p \leq 0.05$) more transformation in CO_2-incubated cultures (Table 5-2). This was true whether fish plasma, horse serum or neonatal calf serum was used. Neither Villosa lienosa nor Lampsilis teres transformed to the juvenile stage in any of the media in numbers that were useful.

It was hypothesized that increased pH, apparent from color changes of the phenol red indicator, might be the cause of the lower success of glochidia cultured in ambient air. So, L-15 of Liebovitz (Ham and McKeehan 1979), a medium designed for incubation of cell cultures without a CO_2 environment, was used in numerous culture tests. None of the cultures were successful. It appears that mussel glochidia require CO_2 to transform.

Another simplification in the in vitro culture of freshwater mussels was achieved by the substitution of commercial powdered tissue culture media for the idiosyncratic medium of Isom and Hudson (1982). M199 and DME contain many of the same components found in Isom and Hudson's medium plus 4-5 times the glucose (Table 5-3), but can be made by simply hydrating a powder and

Table 5-2. Transformation success for Anodonta imbecilis glochidia incubated in Isom and Hudson's (1984) basal salt medium in CO_2 (5%), in ambient air and different buffer systems. Values are based on three trials using two plates per treatment.

Growth medium	Mean % Transformation (s.d.)	
	CO_2 incubated	Ambient air
Fish plasma, $NaHCO_3$	86.8^{a} (6.4)	42.0^{de} (2.0)
Horse serum, $NaHCO_3$	76.0^{ab} (9.6)	35.7^{e} (8.5)
Neonatal calf, $NaHCO_3$	60.0^{bcd} (40.1)	8.5^{fg} (11.4)
Fish plasma, MOPS[1]	78.5^{ab} (15.6)	74.1^{ab} (22.6)
Horse serum, MOPS	48.2^{cde} (8.7)	24.5^{ef} (12.8)
Neonatal calf, MOPS	76.7^{ab} (9.5)	0
Fish plasma, HEPES[2]	73.2^{ab} (25.0)	75.7^{ab} (23.6)
Horse serum, HEPES	77.7^{ab} (14.1)	43.2^{cde} (20.7)
Neonatal calf, HEPES	68.7^{abc} (10.4)	0

[a-g] Treatments with the same letters were not significantly different from each ($p \leq 0.01$).

1 MOPS=3-[N-morpholino] propanesulfonic acid.

2 HEPES=N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

Table 5-3. Components of growth media from Isom and Hudson (1982), DME[a] and M199[a]. Concentrations are in mg/L.

Compound	Isom and Hudson	DME	M199
$CaCl_2$	1200.00	265.00	265.00
$MgCl_2 \cdot 6H_2O$	1000.00	--	--
NaCl	1530.00	4400.00	4500.00
KCl	99.00	400.00	400.00
$NaHCO_3$	2200.00	--	--
arginine	1.05	84.00	70.00
cystine	0.24	63.00	26.00
histidine	0.31	42.00	21.90
isoleucine	0.52	105.00	40.00[b]
leucine	0.52	105.00	120.00[b]
lysine	0.58	146.00	70.00
methionine	0.15	30.00	30.00[b]
phenylalanine	0.32	66.00	50.00[b]
threonine	0.48	95.00	60.00[b]
tryptophan	0.10	16.00	20.00[b]
tyrosine	0.36	104.00	57.70
valine	0.46	94.00	50.00[b]
alanine	0.089	--	50.00[b]
asparagine	0.132	--	--
aspartate	0.133	--	60.00[b]
arginine	--	--	70.00
glycine	0.075	30.00	50.00
glutamine	0.147	584.00	100.00
proline	0.115	--	40.00
serine	0.105	42.00	50.00[b]
taurine	0.31	--	--
ornithine	0.10	--	--
choline chloride	0.01	4.00	0.50
folic acid	0.01	4.00	0.01
biotin	--	--	0.01
nicotinic acid	--	--	0.025
pyridoxine-HCl	--	--	0.025
pantothenic acid	--	--	0.01
myo-inositol	0.02	7.2	0.05
nicotinamide	0.01	--	0.025
niacinamide	--	4.00	0.025
calcium pantothenate	0.01	4.00	0.01
pyridoxal	0.01	4.00	0.025
riboflavin	0.001	0.40	0.01
thiamine	0.01	4.00	0.01
p-amino benzoic acid	--	--	0.05
dl-a-tocopherol	--	--	0.01
glucose	1000.00	4500.00	1000.00
Ferric nitrate	--	0.10	0.72
$MgSO_4$	--	100.00	100.00
$NaHPO_4$	--	109.00	125.00

Table 5-3. Continued.

Compound	Isom and Hudson	DME	M199
HEPES	--	5959.00	5959.00
sodium acetate	--	--	50.00
cysteine	--	--	0.11
glutamic acid	--	--	150.00[b]
hydroxyproline	--	--	10.00
vitamin A acetate	--	--	0.14
ascorbic acid	--	--	0.05
calciferol	--	--	0.10
menadione	--	--	0.016
ATP	--	--	1.00
adenylic acid	--	--	0.20
cholesterol	--	--	0.20
deoxyribose	--	--	0.50
glutathione	--	--	0.50
guanine	--	--	0.30
hypoxanthine	--	--	0.30
PSM[c]	--	--	20.00
ribose	--	--	0.50
uracil	--	--	0.30
xanthine	--	--	0.34
thymine	--	--	0.30
adenine sulfate	--	--	10.00

[a]Sigma Chemical Co. [b]DL-isomers; all others are the L-isomer.
[c]Polyoxyethylene sorbitan monooleate.

adjusting the pH to that appropriate for mussel cultivation (pH 7.3-7.4).

Transformation success was significantly lower ($p \leq 0.05$) in the Isom and Hudson (1984) medium than in either DME or M199 based on results from five tests with four replicates per medium (Table 5-4). Horse serum was used in all three media. Not only are these commercial media easier to use, they are also more than adequate for culturing Anodonta imbecilis.

With some 68 different components in the three media, it is impossible to attribute the greater transformation success in the commercial media to a particular factor. The basic requirements for glochidial transformation are unknown (Isom and Hudson 1982). There is an enormous number of combinations of media components that could have been important in promoting transformation including: (1) the presence of HEPES in both commercial media, (2) the presence of $MgSO_4$ and $NaPO_4$ in the commercial media, (3) a higher concentration of glycine in DME and M199 than is in the Isom and Hudson (1982) medium, (4) a higher concentration of NaCl and less $CaCl_2$ in the commercial media, or any number of other differences. The main concern of this research was to simplify the culture technique. It was not a primary goal to define the nutritional requirements of glochidia.

Table 5-4. Transformation success for glochidia of Anodonta imbecilis cultured in the Isom and Hudson (1982) basic salt medium, DME[a] and M199[a]. Means with the same letter are not significantly different ($p \leq 0.05$).

Medium	% Transformation (s.d.)
DME	65.8[a] (16.7)
M199	65.4[a] (12.9)
Hudson and Isom	51.2[b] (11.6)

[ab]Values with the same letters were not significantly different from one another ($p \leq 0.05$).

Discussion

The culture of unionid mussels has been significantly simplified. The successful substitution of a commercially available medium (M199 or DME) for the complex culture medium developed by Isom and Hudson (1986) was accomplished in the culture of three species of mussels. Transformation success was similar to that obtained by Isom and Hudson (1982). These dehydrated media are inexpensive, readily available and easy to use. The finding that horse serum is an adequate replacement for fish plasma is important. While the latter can be purchased inexpensively as sterile, certified disease-free culture medium, fish plasma must be collected and sterilized by the researcher. I am not aware of any commercial source for disease-free, sterile fish plasma.

Bacterial and fungal contamination, cited as major problems by Isom and Hudson (1982), have been virtually eliminated even without sterilizing instruments or glochidia rinse water. Apparently, fish plasma was the main source of microbes since glochidia would still have microbial contaminants acquired while in their mother's gills whether culture materials were sterile or not.

Further work to determine the minimal and optimal nutrient requirements of these molluscs may be useful. The components of Isom and Hudson's (1982) medium were arrived at by inclusion of all amino acids found in fish plasma. Perhaps another medium would suffice or may

eliminate the necessity for the CO_2 incubator during culture.

The commercial culture medium L-15 of Leibovitz (Ham and McKeehan 1979), which is designed for use without CO_2 was tested. In this medium, galactose is substituted for glucose as the energy source. Numerous attempts to culture glochidia of A. imbecilis, Lampsilis teres and Villosa lienosa in L-15 of Liebovitz without CO_2 were unsuccessful.

Using the methods developed here, the in vitro propagation of freshwater mussels can be performed in any facility that has a CO_2 incubator and funds sufficient to purchase the inexpensive media and horse serum. The substitution of commercial media and horse serum for that suggested by Isom and Hudson (1982) simplifies the culture process and could lead to standardization of techniques.

Hundreds of juveniles can be produced from only a few gravid females. The harvest of artificially propagated juveniles is easy and predictable, and produces thousands of same-age organisms. In vitro culture is preferable to the alternative--infection of fish with glochidia and subsequent collection as the mussels drop from their cysts--because the mussels are kept in small culture dishes. Thus, juveniles do not have to be located from amongst aquaria debris and fish feces. In addition, the transformation process can be

observed microscopically via the culture dishes. This can facilitate studies of glochidium development and more accurate timing of laboratory experiments.

One of the main factors hampering broader research with mussels is that we do not have basic knowledge of their physiology and reproductive cycles. Because of its economic importance, much effort has been expended over the years in the development of propagation techniques for the oyster (Crassostrea virginica). To date, similar interest in freshwater mussel propagation is lacking. Therefore, we have no methods to regulate mussel reproduction. Research into the sensitivity of mussels to aquatic pollutants is currently limited by the availability of naturally gravid adults.

CHAPTER 6
A TEST PROTOCOL FOR DETERMINING THE ACUTE TOXICITY OF POLLUTANTS TO JUVENILE FRESHWATER MUSSELS

Introduction

To date, over 15 toxicity tests using 80 species of freshwater organisms have been developed (Buikema et al. 1978, Peltier and Weber 1985, Horning and Weber 1985) to assess the impact of pesticides and hazardous chemicals on non-target organisms. Results from these tests provide oversight and enforcement capabilities for state and federal agencies involved in the preservation of water quality as stipulated in the Clean Water Act of 1977 (PL 95-217). Both vertebrates, such as fish, and many invertebrate species, e.g., oligochaetes, zooplankton and insects, are currently used as test organisms (Peltier and Weber 1984, Bailey and Lin 1978). Therefore, several questions must be considered before yet another toxicity test protocol is developed. These include whether: (1) the organism represents a group whose sensitivity to pollutants has not been assessed to date and for which there are no substitutes (2) the organism is on the endangered or threatened species list and therefore deserves special attention (3) a simple,

precise test endpoint (e.g. death, loss of activity, cessation of growth) can be devised (4) currently available test and analytical methods can be adapted for use with this organism. Each of these items is important in determining whether there is a need for the test and how the results can be used.

Since acute toxicity test (48-h) results are still required for pesticide registration (Zucker 1985a and 1985b) and for pre-manufacture tests for new chemicals under the Toxic Substances Control Act (Foster 1985), and are preferable in many other cases because of their precision and ease of performance, development of an acute toxicity test should precede that of a chronic method. Chronic test methods require more knowledge of the organism's life history, physiology and sensitivity to toxicants, and are generally more difficult to develop and validate (Buikema et al. 1982).

The development of a toxicity test protocol for the freshwater mussel Anodonta imbecilis was undertaken with EPA funding after consideration of many criteria. Early aquatic toxicity test methods assessed the impact of pollutants on fish and their food sources (Buikema et al. 1982). These methods were standardized for use in virtually any laboratory under the guidelines of the American Society for Testing and Materials (ASTM 1980) and the EPA (Peltier and Weber 1985, Zucker 1985a and 1985b).

Requirements for standardization stem from the desire to compare results among both test species and toxicants to determine relative sensitivities used in setting exposure limits. Chief among the requirements for test species were that they: (1) were widely available and ecologically important (2) could be reared in the laboratory (3) were important in the food chain leading to man (4) were sensitive to pollutants and (5) were well known physiologically, genetically and taxonomically (Rosenberger et al. 1978, USEPA 1976, Buikema et al. 1982). Such criteria were more easily satisfied by fish and their zooplankton food sources because these groups were of use to man and had been studied for years already. However, there are a number of reasons to use invertebrates other than zooplankton in toxicity tests.

The most important reason to use invertebrates other than zooplankton in toxicity assessments is that they are an integral and basic part of the ecosystem (Maciorowski and Clarke 1980). Toxic pollutants not only affect invertebrates directly, but through impacts on invertebrates can affect other organisms both higher and lower in the food chain. Thus, the structure and function of the entire ecosystem can be altered by the loss of a species of invertebrates.

For example, in the mid 1950's the fingernail clam (Musculium transversum) virtually disappeared from a 140-

mile stretch of the Illinois River, probably due to metal pollution (Mathis and Cummings 1973, Palompis and Starrett 1960). As a result, a number of waterfowl that depended on this clam as a food source declined dramatically in the river and fish that preyed on the clam were found to have slower growth rates (Mills et al. 1966, Starrett 1972). Repercussions were surely experienced at other trophic levels.

There is a great deal of diversity in morphology, physiology and ecological role among invertebrates. These differences lead to variable and often unpredictable responses to toxicants that cannot be assessed using vertebrate animals (Maciorowski and Clarke 1980). Diverse habitat and food source requirements of invertebrates provide for the use of test endpoints not possible in fish toxicity tests. These include mollusc shell growth (U.S.P.H.A. 1976), ciliary beat (Paparo and Sparks 1977, Price and Schiebe 1978) and burrowing (Stirling 1975), insect gill beats (Maki et al. 1973) and the drift behavior of Gammarus pseudolimnaeus (Fahmy and Lush 1975). In addition, most of the endpoints used in fish tests can also be used.

Finally, invertebrates are easily adaptable to laboratory conditions. Most of them are macroscopic but still small enough to rear and maintain in limited volumes of water. Test solutions can therefore be used sparingly (Maciorowski and Clarke 1980). Their short

life cycles and high fecundity result in the availability of large numbers of test animals, and the potential for chronic exposure tests if desired. The parthenogenic reproductive strategies of many invertebrate species (rotifers, ostracods, daphnids, mayflies) ensure genetic homogeneity (Maciorowski and Clarke 1980). Freshwater mussels, though not parthenogenic, produce thousands of glochidia per individual female (Barnes 1963).

Mussels are broadly distributed throughout North America. While the use of freshwater mussels by humans for food or in industry has declined in the last 20 years, their role in the aquatic ecosystem remains unchanged. Mussels, which feed on plankton and detritus (Fuller 1974), convert this energy to biomass that is available to waterfowl, fish and mammals such as raccoons or muskrats. We do not know the extent of mussel sensitivity to pollutants, largely because laboratory culture techniques have been lacking and a toxicity test protocol has not been developed.

Over 70 species of freshwater mussels are endangered or threatened (USFWS 1989). Although mussels are sediment infauna like many aquatic insects for which test methods already exist, they represent a group that is taxonomically distinct from aquatic insects. In addition, mussels are permanent residents of the aquatic environment, while insects are not. Therefore, the response of mussels to toxicants cannot be extrapolated

from that of insects (or any other organism) until test results support such an assumption. An examination of the literature on the responses of various species to toxicants substantiates this statement.

While fish and invertebrates have similar sensitivities to DDT (LC50 1-10 ug/L), toxaphene (1-26 ug/L), chlordane (3-115 ug/L) and cadmium (50-900 ug/L) for example, there are large differences in the sensitivities of these groups to other toxicants (Johnson and Finley 1980, Giesy et al. 1977). Rainbow trout had a 96-h LC50 of 340 ug/L for the molluscicide Bayluscide, while the 48-h LC50 was 2,000 ug/L for Gammarus pseudolimnaeus, 25,000 ug/L for Orconectes sp.and 200 ug/L for Pteronarcys (Johnson and Finley 1980). The fish was more sensitive to Bayluscide than were most of the invertebrates.

But the reverse was true in tests with the insecticide carbaryl. Daphnia pulex had a 48-h LC50 of 6.4 ug/L for the insecticide carbaryl, while the crayfish Procambarus had a 96-h LC50 of 1,900 ug/L, the stonefly Claassenia a 96-h LC50 of 5.6 ug/L and the fathead minnow a 96-h LC50 of 14,600 ug/L (Johnson and Finley 1980). Experimental data provide information on toxicity trends for species and can lead to generalizations about the toxicity of types of compounds such as in QSAR (quantitative structure activity relationships) analyses. Without data, however, no extrapolation or generalization

is safe to make. We cannot predict the sensitivity of freshwater mussels from data on fish, daphnids, insects, or even from marine molluscs.

Anodonta imbecilis was chosen as the test species because it is widely distributed, has a long reproductive period and has been reared in the laboratory previously. The remaining considerations regarding the choice of test endpoints, water source, food source and presence or absence of particulates were addressed individually. The goal of this work was to develop a simple and inexpensive method to assess the acute toxicity of various compounds or effluents to freshwater mussels.

Materials and Methods

General Conditions

A typical acute toxicity test performed as part of pesticide registration documentation is a 24- to 48-h static test using soft reconstituted freshwater at 12-22 $\pm$ 2^{o} C depending on the test animal used (ASTM 1978, Zucker 1985a and 1985b). Animals are not fed during testing.

Soft water is required as the diluent in pesticide registration toxicity tests because water hardness can decrease the solubility, and therefore, the toxicity of pesticides to aquatic organisms (Sprague 1985, Hellawell 1986). Tests conducted in hard water would not give information on susceptibility of organisms to a compound

in our many soft water lakes and streams. Similar protection is afforded animals when hard water is used in metal toxicity tests. Animals are not fed during such a test. The suggested photoperiod is 16 hours of light followed by 8 hours of darkness (Zucker 1985a, 1985b).

Death or immobilization are the usual endpoints in acute toxicity tests because they are decisive (Peltier and Weber 1985, Buikema et al. 1982, Zucker 1985a, ASTM 1978). In A. imbecilis, death is indicated by the cessation of heartbeat. The beating heart can been seen at low magnification (20-40X) through the shell valves.

Physical conditions. Test chambers initially consisted of 200 ml crystallizing dishes containing 150 ml of dilution water and loosely covered with plastic wrap. Later, 15 X 60 mm covered pyrex Petri dishes with 15 ml water were substituted for the larger chambers. Petri dishes provided enough sample volume for chemical analyses, and were easier to work with than crystallizing dishes. It was convenient to have a lid for test chambers that permitted some exchange of air and still protected test animals from dehydration and airborne particulates. Petri dishes are also inexpensive, widely available, easy to transport for microscopic examination of test animals, and provide for the use of replicates that are physically separate.

All tests were static. That is, test solutions were not renewed during the exposure period except in cases where toxicants were known to have half-lives of less than 4 days.

Water quality. Two water types were tested as diluents--soft reconstituted freshwater (Peltier and Weber 1986) and diluted well water. Soft reconstituted freshwater (Table 6-1) was chosen for two reasons. First of all, tests conducted under EPA pesticide registration guidelines require the use of soft water (Zucker 1985a and 1985b), the constituents of this water (deionized water and several salts) are widely available and easy to use, and thousands of toxicity tests have already been conducted using this water.

Second, many evaluations of metal toxicity have shown that toxicity can be dramatically reduced in hard water due to the chelation of metal ions from solution (Sprague 1985). A major goal of laboratory toxicity tests is to provide "worst-case" data that may err on the side of conservatism rather than minimizing the projected effect of toxicants on aquatic organisms. Whether this is ecologically sound is impossible to assess adequately in the laboratory but since environmental conditions vary considerably among regions, a standard condition using reconstituted soft freshwater was established. Well water diluted to a hardness similar to soft reconstituted

Table 6-1. Summary of characteristics (mean ± s.d.) of soft reconstituted freshwater and diluted well water used as diluent and control water in toxicity tests with *Anodonta imbecilis*.

PARAMETER	RECONSTITUTED FRESHWATER	WELL WATER
pH	7.54 ± 0.07	7.61 ± 0.15
Hardness (mg/L)	39.03 ± 0.07	51 ± 0.85
Temperature (^{o}C)	23.5 ± 0.03	23.5 ± 0.03
Dissolved oxygen (mg/L)	8.51 ± 0.17	8.43 ± 0.31

water (Table 6-1) was used to determine whether the reconstituted water provided adequate water quality for the mussels. If so, the latter is preferable because it is standard and easy to make.

Water was not aerated in the test chambers since that disturbed the very small juvenile mussels (0.33 mm) and was not necessary due to the large surface to volume ratio between the test solutions and mussels. Survival in the two types of control water was determined during an 8-day test period. A series of replicate test chambers with each water type were used to measure survival of mussels. Replicates contained 10 juveniles each which were not fed during the test.

Feeding Tests

The withholding of food is a common practice in acute toxicity tests (ASTM 1978, Peltier and Weber 1985). It was important, however, to determine whether survival success in control water alone was adequate to conduct tests with unfed mussels. Therefore, duplicate tests were performed with 1-day and 10-day post-transformation juveniles using either no food, Chlorella, or YTC--a mixture of yeast, trout chow and pulverized grass (Cerophyll).

Chlorella was reared in the laboratory, concentrated by centrifugation and added to test chambers to make a final concentration of 5×10^5 cells/ml. YTC

was added at a rate of 0.5 ml/50 ml of dilution water. Food was provided every other day for the 8-day test period. This test length was chosen because it was twice as long as a 96-h test and thus would conservatively indicate the adequacy of the feeding regime.

Evidence from earlier mussel culture work by Huson and Isom (1984) indicated that juvenile mussels survived better in water containing a minimum amount of silt (800 mg/L). The role of silt is not well understood but it may provide stimulation for the mussel digestive system or for the cilia around incurrent siphons (Hudson and Isom 1984).

In early screening tests I used bentonite, a clay, diatomaceous earth or river silt as a particulate. It was hard to see the mussels in the presence of bentonite and they seemed to do better in diatomaceous earth than in either of the other two particulates. Therefore, in definitive tests of survival success for juvenile mussels in water with various types of particulates I used silt from the Suwannee River and diatomaceous earth.

Test organisms

Glochidia. Some consideration was given to the choice of mussel life stage appropriate for use in toxicity tests. Glochidia (larvae) are exposed to ambient water only for a brief time before they attach to fish. While it was believed that juveniles would be the

better stage to measure toxicity, several experiments were also performed with glochidia. Glochidia were put in water containing one of several pesticides or metals for 24 hours to simulate short exposures to toxicants. They were then rinsed and put into culture media. The endpoint was transformation success of cultured glochidia after exposure to toxicants.

Maintaining the sterility of culture media is essential because the presence of bacteria or fungal spores from the air will cause contaminations of the cultures and death of the glochidia. Generally, mussel cultures were manipulated in a sterile hood in a tissue culture lab. However, because other non-mussel cultures were at risk by the presence of toxicants in the laboratory, it was impossible to perform glochidia exposure tests under sterile conditions. As a result, cultures became contaminated with microoganisms and transformation success was affected. It was impossible to determine whether toxicants had any impact on transformation success. With proper facilities, glochidia exposure tests might prove to be useful.

Juvenile mussels. During their development to juveniles, glochidia are embedded in fish tissue (Barnes 1963) and are isolated from waterborne toxicants. Therefore, I concluded that mussels were most vulnerable to the effects of pollutants as young juveniles. At that stage, they are extremely small and have a higher

metabolic rate associated with growth and maturation (Fuller 1974, Foster and Bates 1978). To maintain greater metabolic activity, juveniles need to feed more continuously than adults. The result is that water flow around and through their bodies is greater, increasing the contact with and potential uptake of pollutants. Juvenile mussels seemed to be the better choice for use in toxicity tests than were glochidia even without the culture contamination problems cited above.

Juvenile mussels used in test protocol development were cultured in vitro with fish plasma and the medium developed by Isom and Hudson (1982). Several test conditions had to be defined for each age group (Table 6-2). These included: (1) whether soft reconstituted freshwater widely used in other toxicity test protocols (Peltier and Weber 1984, Horning and Weber 1985) was an adequate dilution and control water for mussel tests, (2) whether silt or another particulate was required to maintain juvenile health during the tests, (3) whether juveniles had to be fed to maintain life and health during tests and (4) what type of food to use.

It was hypothesized that sensitivity might be greater for 1-day old juveniles than for 10-day olds, but since no data were available that allowed a comparison, both ages were used initially. If survival in control water was as good for 1-day old juveniles as it was for 10-day olds, the use of the younger animals would be

Table 6-2. Summary of conditions examined in test development.

Mussel Age
One-day old
Ten-days old
Test Water
Soft reconstituted freshwater
Diluted well water
Food
None
Chlorella
YTC (yeast, trout chow, Cerophyll)
Particulates
None
Silt
Diatomaceous earth

preferable because juveniles would not have to be maintained in the laboratory for 10 days prior to use.

To examine the importance of each of these conditions, survival tests were performed with one-day old juveniles using the following combinations: (1) soft reconstituted freshwater, with diatomaceous earth or silt, or without any particulates, (2) with each of the two particulates, three food sources were tested--no food, Chlorella, and YTC (a mixture of yeast, trout chow and Cerophyll that constitutes typical food for zooplankton tests) (Horning and Weber 1985). The same set of conditions were then duplicated using diluted well water. Finally, all tests were performed with 10-day old juveniles in the same way.

Juvenile survival tests were performed in 15 X 60 mm covered glass pyrex Petri dishes. Ten juveniles were placed in each test chamber containing 15 ml of dilution water. Two replicates, consisting of separate test chambers containing the same water type, food source or particulates, were used per treatment. Survival was recorded at the end of the 8-day tests.

Survival success was tested by three way ANOVA (with factor analysis) and Duncan's multiple range test (SAS 1986) to determine if age, type of dilution water, presence or absence of particulates, or food source had a significant effect.

Results

When both age groups were combined (1-day and 10-day old juveniles), soft reconstituted freshwater was the better dilution water based on survival (Table 6-3). Survival in soft reconstituted freshwater was 81.9%, while it was only 74.7% in diluted well water. Neither presence nor absence of food or particulates had a significant effect on juvenile survival.

In separate analyses, one-day old juveniles of Anodonta imbecilis survived better in all dilution water tests than did 10-day old juveniles (Tables 6-4 and 6-5). The younger animals were able to survive for the 8-day test period with almost no deaths (92% survival) and remained active throughout the test. Only 50% of the 10-day old juveniles survived under any food, water or particulate regime.

One-day old juveniles survived better (96 %) in soft reconstituted freshwater (Table 6-4) than they did in diluted well water (86.9 %), and better than 10-day olds in either water type. Water source had no significant effect on survival of 10-day old juveniles.

Neither food source nor presence or type of particulate had a significant ($p \leq 0.05$) effect on the survival of A. imbecilis juveniles of either age group (Table 6-3). One-day old juveniles had a mean survival rate of 91.2-93.1% whether or not they were fed, or had

Table 6-3. Effect of age, dilution water type, presence of particulates and food type on the survival of juvenile Anodonta imbecilis based on ANOVA and Duncan's multiple range test. Means within each category with the same letter are not significantly different from each other ($p \leq 0.05$).

CATEGORY	N*	MEAN (S.D.) % SURVIVAL @ 8 DAYS
Age in days (all other factors combined)		
One	58	92.1 (11.3)[a]
Ten	28	50.7 (6.6)[b]
Water type (all other factors combined)		
Reconstituted	46	81.9 (22.5)[a]
Well Water	40	74.7 (20.8)[b]
Particulates (all other factors combined)		
None	16	81.8 (21.7)[a]
Silt	36	78.0 (23.0)[a]
Diatomaceous earth	34	77.6 (21.3)[a]
Food (all other factors combined)		
None	38	79.2 (22.1)[a]
Chlorella	24	79.2 (22.8)[a]
YTC[1]	24	77.1 (21.5)[a]

[1] YTC=yeast, trout chow, Cerophyll mixture.
* N=total number of test chambers included in the analysis.

Table 6-4. Effect of dilution water type, food and particulate presence or absence on survival of one-day old juvenile Anodonta imbecilis mussels. Results are based on ANOVA and Duncan's multiple range test. Means with the same letter in a division are not significantly different ($p \leq 0.05$).

CATEGORY	N*	MEAN % SURVIVAL (S.D.) @ 8 DAYS
Water (particulates and food combined)		
Reconstituted	32	96.2 (4.91)[a]
Well Water	26	86.9 (14.6)[b]
Particulates (water type and food combined)		
None	12	91.7 (14.67)[a]
Silt	24	92.9 (9.99)[a]
Diatomaceous earth	22	91.4 (11.25)[a]
Food (water type and particulates combined)		
None	26	91.9 (12.65)[a]
Chlorella	16	93.1 (12.5)[a]
YTC[1]	16	91.2 (8.06)[a]

[1] YTC=yeast, trout chow, Cerophyll mixture.
* N=total number of test chambers included in the analysis.

Table 6-5. Effect of dilution water type, food and particulate presence or absence on survival of ten-day old juvenile Anodonta imbecilis mussels. Results are based on ANOVA and Duncan's multiple range test. Means with the same letter in a division are not significantly different ($p \leq 0.05$).

CATEGORY	N*	MEAN % SURVIVAL (S.D.) @ 8 DAYS
Water (food and particulates combined)		
Reconstituted	14	52.1 (6.99)[a]
Well Water	14	49.3 (6.15)[b]
Particulates (water type and food combined)		
None	4	52.5 (5.00)[a]
Silt	12	52.5 (7.53)[a]
Diatomaceous earth	12	48.3 (5.77)[a]
Food (water type and particulates combined)		
None	12	51.7 (8.34)[a]
Chlorella	8	51.2 (6.41)[a]
YTC[1]	12	48.7 (3.53)[a]

[1] YTC=yeast, trout chow, Cerophyll mixture.
* N=total number of test chambers included in the analysis.

particulates in their water. Similarly, 10-day old juveniles survived as well without food (51.2%) as with either food source, and as well with no silt (52.5%) as with either type of particulate.

Discussion

The better choice of age group for acute toxicity testing appears to be 1-day old juveniles. The 10-day old animals did not survive as well under any conditions as did the younger animals. It is more convenient to use 1-day olds, because in so doing one avoids the need for maintenance of cultures for long periods of time prior to their use. Transformed juveniles are simply put in dilution water for a day then used in toxicity tests.

It was shown conclusively that soft reconstituted freshwater (Peltier and Weber 1985) is a good control water. Both one-day and ten-day old juvenile mussels survived better in soft reconstituted freshwater than they did in diluted well water. Therefore, all subsequent toxicity tests were performed in the reconstituted water except for those comparing the effects of soft and moderately hard water on metal toxicities.

Since feeding the juveniles did not enhance their survival in dilution water over not feeding them, food was not administered during toxicity tests. It is an

advantage not to feed test organisms for two reasons. First, food often reduces the impact of toxicants on test organisms (Peltier and Weber 1985). Second, there is always the possibility that variations in the amount of food used will occur. In that case, differences in the sensitivities of organisms may be caused by the feeding regime and not the toxicant.

Although long-term survival and health may require mussels to have silt of some type (Hudson and Isom 1984), neither juvenile age group tested here demonstrated enhanced survival in the presence of particulates. It is easier to find and count survivors in the absence of particulates in the test chambers. Therefore, toxicity tests with metals and pesticides were performed in water with silt, diatomaceous earth or no particulate.

The test method (Table 6-6) developed here is simple, fast and appropriate to begin assessing the impact of dissolved pollutants on freshwater mussels. With laboratory cultured juveniles, test exposures are performed using inexpensive, easily obtainable materials and results can be obtained in 96-h. The verification of laboratory-derived toxicity by field tests is recommended and could be performed simultaneously with laboratory tests following initial evaluations.

Since many of the methods and materials used in this protocol are similar to those of other toxicity tests, it will be easy for other researchers to implement. Inter-

Table 6-6. Summary of acute toxicity test conditions using juvenile Anodonta imbecilis mussels.

TEST TYPE	Static or static-renewal
TEMPERATURE	$22^{o} \pm 3^{o}$ C
PHOTOPERIOD	16-hour light, 8-hour dark
TEST CHAMBER SIZE	15 X 60 mm Petri dishes with lids
TEST SOLUTION VOLUME	15 ml
AGE OF TEST ORGANISMS	1-2 days old
NUMBER OF ANIMALS PER TEST CHAMBER	10
REPLICATES PER CONCENTRATION	2
ANIMALS PER CONCENTRATION	20
FEEDING REGIME	No feeding during the test
AERATION	None during the test; Dilution water was saturated with oxygen prior to test initiation.
DILUTION WATER	Soft reconstituted water
DILUTION FACTOR	60 percent
TEST DURATION	48-h to 96-h
EFFECT MEASURED	Survival (based on presence of heartbeat)

laboratory comparisons would provide additional information on precision and test utility. The method is also adaptable for use in the assessment of sediment-sorbed toxicants, requiring only the addition of contaminated sediments.

CHAPTER 7
THE TOXICITY OF SELECTED METALS TO THE FRESHWATER MUSSEL, *Anodonta imbecilis* AND THE ZOOPLANKTER, *Ceriodaphnia dubia*

Introduction

Pollution of the aquatic environment by metal wastes has been a problem of increasing proportions since the beginning of the industrial revolution (Leland and Kuwabara 1985, Hellawell 1986). Coal combustion and burning of fossil fuels that played a major role in industrialization resulted in the production of airborne metal pollutants (e.g. Cd, Hg, Cu). Even more important sources were smelting, mining and manufacturing activities. Atmospheric inputs of metals from smelters (Scheider et al. 1981), ore washing and flotation wastes, and metallurgical operations (Hellawell 1986) have resulted in the destruction of aquatic biota in lakes and streams in Great Britain, Canada, northeastern United States and Scandinavia. The establishment of more stringent emission standards and water quality criteria have decreased the output of these pollutants in many areas (Scheider et al. 1981). However, sediment-sorbed metals can continue to have an impact on the structure

and function of aquatic systems long after effluents have been eliminated.

The heavy metals of principal interest in aquatic ecosystems are zinc, copper, lead, mercury, cadmium, nickel and chromium (Hellawell 1986). Not surprisingly, the greatest attention has been focused on the response of species of importance to man as food or for recreation, i.e. fish and their food sources (Mount 1966, Doudoroff and Katz 1953, Alabaster and Lloyd 1982, Clubb et al. 1975). However, as our level of environmental awareness has become more sophisticated, concern about other components of the aquatic ecosystem has increased.

A number of studies have examined the toxicity and impact of metals on aquatic biota (Khangarot and Ray 1987, Baudouin and Scoppa 1974, Gupta et al. 1981) . As a result, we know that mercury and cadmium are usually the most toxic metals while zinc and nickel are less toxic (Khangarot and Ray 1987). One scheme of ordering the toxicity of metals commonly found in aquatic systems is: Hg > Cu > Cd = Zn > Ni > Fe > Mn (Jones 1964). This order was based on literature values and was explained on the basis of the solubility and reactivity of the metal ions. Another ordination related metal toxicity with the electron configurations of their outer electron orbitals (Kaiser 1980). The most toxic group was comprised of Sn^{2+}, As^{3+}, Se^{4+} and Pb^{2+} which have filled d and s orbitals, but unfilled p orbitals. The least toxic

group--Na^{+}, Be^{2+}, Ba^{2+}, Al^{3+} and Cr^{6+}, had configurations like inert gases. However, responses to heavy metals vary considerably according to the organisms involved (Hellawell 1986), therefore only trends in toxicity can been identified.

Another area of interest in toxicity testing is the impact of metal mixtures on biota. While water quality criteria are established on the basis of single compound toxicity test (EPA 1979), many metals enter aquatic systems as mixtures. For example, zinc and cadmium occur together both in uncontaminated waters (Lake 1979, NRC 1979) and in industrial effluents (Casarett and Doull 1975, Hemelraad et al. 1987). Nickel, cadmium and mercury may be discharged due to the manufacture of batteries (Occhiogrosso et al. 1979). Thus, there is a need for information on the impact of metal mixtures to aquatic biota.

Although many studies have been performed to determine the toxicity of heavy metals to invertebrates, relatively few have used animals from flowing waters (Whitton and Say 1975). It is particularly important to determine such effects because rivers and streams have been the recipients of much industrial waste over the years. The older literature attributed faunal declines in contaminated streams to the presence of various metals, but did not quantify the relationship (Carpenter 1924, Jones 1940 and 1958).

During the last 20-30 years, a marked decline in species diversity and density of freshwater mussels has been observed in many streams that receive mining and industrial effluents (Havlik and Marking 1987, Clarke 1970). Although molluscs are among the most sensitive to heavy metal pollution (Wurtz 1962), few experimental data quantifying their susceptibility to metals are available.

It has been noted in numerous field surveys that mussel species are declining (Rasmussen 1980), but the fact that they often carry high body burdens of metals (Anderson 1977, Foster and Bates 1978, Jones and Walker 1979) suggested that metals were killing the mussels. In fact, almost no verifying data exist (Imlay 1971) even for adults, much less earlier life stages. With the placement of over 70 species of freshwater mussels on the threatened or endangered species list (USFWS 1989), the establishment of acceptable exposure limits for these species has become a priority.

In accordance with the need for basic experimental data, a series of acute toxicity (96-h) tests were performed to determine whether juvenile mussels were sensitive to metal pollution. Possibly they bioaccumulate significant concentrations of metals, but are unable to withstand the same levels in direct exposure. On the other hand, the loss of mussels from metal polluted rivers and streams may be caused by

habitat destruction, sedimentation, pesticides or other factors rather than metal toxicity.

Six of the seven most toxic heavy metals were chosen for testing--mercury, zinc, nickel, cadmium, copper and chromium (Hellawell 1986). Using methods developed earlier in this dissertation, juvenile mussels were exposed to each of these metals separately and in several mixtures to determine their 96-h LC50s (lethal concentration to 50% of the organisms).

Of the many environmental characteristics that can modify the toxicity of metals to biota, water hardness is among the most important (Sprague 1985). Water hardness protects against the toxicity of heavy metals in two possible ways. First, metals become less soluble in hard water as they form complexes with carbonates. Second, water hardness, caused primarily by Ca^{2+} and Mg^{2+}, may decrease membrane permeability and therefore uptake of metals from water (Everall et al. 1989). Therefore, the effect of water hardness on metal toxicity to mussels was also examined.

Finally, separate experiments were performed to compare the sensitivity of mussels and the zooplankter Ceriodaphnia dubia to metal-contaminated sediments and an industrial effluent containing chromium. It was desirable to determine how similar their sensitivities were because zooplankton are commonly used in toxicity tests as surrogates for mussels (EPA, personal

communication). Data from these experiments with single metals in soft and moderately hard water metal mixtures, and the contaminated sediments can be used both to determine whether ambient water levels of the metals could have caused the loss of mussel species and to help set more appropriate water quality criteria. They may also validate the use of zooplankton toxicity data in setting safe exposure limits for mussels.

Materials and Methods

Test Organisms

One- to two-day old juveniles of the freshwater mussel Anodonta imbecilis were used as test organisms. Glochidia (larvae) of these mussels were cultured in vitro using one ml of horse serum and two ml of DME (Dulbecco's Modified Eagle's Medium) in a CO_2 incubator. Details of the culture method are contained in Chapter 5 of this dissertation. The transformation process was observed periodically during culture until activity of the mussel's foot was visible through the shells. At that time (usually 6-10 days), transformation was complete and cultures were transferred to soft reconstituted freshwater.

Test Methodology

Dissolved Metals. After 24 to 48 hours in water, juveniles were randomly distributed in test chambers which consisted of 15 X 60 mm pyrex Petri dishes. Ten

animals were placed in 15 ml of solution in each of two replicates per test concentration.

Metals used in toxicity tests were: $Cu \cdot 5\ H_2O$, $ZnSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 6H_2O$, $HgCl_2$ and $CdCl_2$. A stock solution of each metal was made in deionized water and diluted to test concentrations with either soft or moderately hard reconstituted freshwater (Peltier and Weber 1985). Five dilutions (in a 60% dilution series) plus a control (soft or moderately hard reconstituted freshwater) were used for each metal.

Mussels were not fed during the tests, nor were test solutions renewed. The test endpoint, death, was determined based on absence of a visible heartbeat. The total number of survivors was recorded by replicate and concentration each day, and used to calculate a 96-h LC50.

Metal Mixtures. Four mixtures of metals were evaluated for toxicity to Anodonta imbecilis juveniles. The combinations were chosen to include a pair of very toxic metals (Cd and Cu), a pair comprised of a metal of low toxicity (Zn) and one of moderate toxicity (Ni), a pair that consisted of two moderately toxic metals (Hg and Cr), and one made of a very toxic metal (Cd) and a minimally toxic metal (Zn). The level of toxicity assigned to each single metal was based on results of earlier tests with single metals.

The mean LC50 from single metal toxicity tests with A. imbecilis was chosen as the highest concentration of each metal used in the mixture tests. Other concentrations were prepared by a series of 60% dilutions. Exposures were performed in soft reconstituted freshwater under conditions matching those described earlier for single metal tests.

Sediment Tests. Since no experimental data were available to determine whether mussels were more sensitive to dissolved or sediment-bound metals, two experiments tested the effect of sediment-sorbed cadmium and copper on Anodonta imbecilis juveniles. In each test, five grams of washed and dried (80^{o} C) Miami River sediment (3% organic content) was put in a 50 ml glass vial with a teflon-lined lid. The metal solution was added (30 ml), the vial capped and then mixed overnight on a wrist-action shaker. Five dilutions of cadmium or copper in soft reconstituted freshwater and a control were prepared in this manner for each test. Shaking the solutions in the presence of sediments was done to load the sediments with the metal while removing them from the aqueous phase.

After being shaken overnight, the sediments and solutions were transferred to 50 ml beakers and allowed to settle for at least 24 h prior to the introduction of juvenile mussels into the chambers. Mussels were not fed nor was the water aerated during the 96-h tests.

Five neonatal (<24 h old) Ceriodaphnia dubia were added to each chamber as reference organisms. These animals are very sensitive to metals and because they are pelagic organisms of a different taxonomic group, their sensitivity relative to that of A. imbecilis should enhance the usefulness of test results. If C. dubia has a sensitivity to metals at least as great as that of the mussels, it would be appropriate to use the zooplankter as a surrogate for mussels in future tests of metal toxicity. Since there are already numerous test organisms in use, it would be preferable not to add another unless it is necessary. The number of survivors of both groups were tallied each day and behavior was noted.

Metal Effluent. An effluent from an airplane maintenance company (Flying Colors) provided by the City of Gainesville, Florida, was also tested for toxicity to juvenile mussels. This particular effluent was thought to be contaminated with metals based on tests with Microtox and B-galactosidase (C. Maziji, G. Bitton, and B. Koopman, unpublished data). Sample analyses later verified the presence of 6,430 mg/L of chromium but found no other contaminants. A 96-h toxicity test was simultaneously performed with mussels and C. dubia. Ten mussels and five C. dubia neonates were placed in replicate 30 ml plastic test chambers containing 15 ml of effluent. Five dilutions (3%-0.4%) of the effluent

determined from a screening test and a control consisting of moderately hard reconstituted freshwater were used.

Test Chemicals

The metals used in these tests were prepared from reagent grade salts dissolved in soft or moderately hard reconstituted freshwater. They included $CuSO_4 \cdot 5H_2O$, $CdCl_2$, $ZnSO_4 \cdot 7H_2O$, $K_2Cr_2O_7$, $HgCl_2$ and $NiSO_4 \cdot 6H_2O$. Stock solutions were diluted in a 60% series and added to test chambers. Metal concentrations were measured as total metal using a Perkin-Elmer atomic absorption spectrophotometer Model 5000 with single element lamps, following EPA guidelines (U.S.E.P.A. 1983). Since determinations of mercury concentrations required the use of equipment not available at this time, mercury concentrations are given as nominal rather than measured. All single metal tests in each type of water were performed at least three times, while metal mixture toxicity was determined twice.

Data Analysis

Survival data were analyzed by several methods. A set of EPA (Peltier and Weber 1985) computer programs calculated the LC50. These programs, known as the TOX-DAT Multimethod, calculate the LC50 using moving average angle, probit and binomial methods. LC50s were then analyzed by ANOVA and Duncan's multiple range test to

determine if there were differences in toxicity among metals and metal mixtures.

Mixture toxicity was calculated based on the concentration of individual metals. These values were then compared to the LC50 for the same metal in single toxicant tests to determine if synergistic, antagonistic or additive toxicity were evident. Calculations of the additive index followed the system of Marking and Dawson (1975). The sum toxic action (S) is calculated as follows:

$$\frac{Am}{Ai} + \frac{Bm}{Bi} = S$$

For $S \leq 1.0$, Additive Index $= \frac{1}{S} - 1.0$;

For $S \geq 1.0$, Additive Index $= S(-1) + 1.$

Am = LC50 of metal A in the mixture.
Ai = LC50 of the same metal A alone.
Bm = LC50 of metal B in the mixture.
Bi = LC50 of metal B alone.

Index values (S) of zero indicate a simple additive effect of the two metals compared to their toxicity when tested individually. Index values greater than zero result from mixtures that have synergistic toxicity. Those mixtures whose index values are less than zero are said to contain antagonistic toxicants (Marking and Dawson 1975).

All statistical analyses except calculation of LC50s utilized the SAS statistical package (SAS 1986) available

at the Northeast Regional Data Center, University of Florida, Gainesville.

Results

Dissolved Metals

In general, 96-h LC50s for the mussels were lower than those measured at 48-h, and metal toxicity was reduced in moderately hard water compared to soft water (Tables 7-1 and 7-2). These findings are in agreement with those from studies using other aquatic organisms (Alabaster and Lloyd 1982, Petrocelli 1985).

In soft water, the order of metal toxicities to A. imbecilis at 48-h was: Cd > Cu > Hg > Ni > Cr > Zn. The 48-h LC50 for Cd^{2+} (0.057 mg/L) was approximately three times lower than the value for Cu^{2+} (0.171 mg/L0, four times lower than Hg^{2+} (0.216 mg/L) and 5-6 times lower than the LC50s for Ni^{2+}, Cr^{6+} and Zn^{2+} (Table 7-2). The greatest increases in toxicity between 48-h and 96-h were seen for copper and chromium. While the other metals exhibited a 1.3-3 fold decrease in LC50s over that time period, the toxicities of chromium and copper increased approximately six and 7.5 times, respectively (Table 7-2).

Because there are no published studies of metal toxicity to juvenile mussels, direct comparisons with my results cannot be made. However, the trend toward the

Table 7-1. Comparative toxicities to Anodonta imbecilis juveniles of single metals at 48-h and 96-h in soft and moderately hard water. Toxicities for waters within the same time and metal category with the same letters were not significantly different based on ANOVA and Duncan's multiple range test ($p \leq 0.05$).

	Type of Water	
Metal	48-h	96-h
Cd	S^{a1} MH^{b2}	S^{a} MH^{b}
Cr	S^{a} MH^{b}	S^{a} MH^{b}
Cu	S^{a} MH^{b}	S^{a} MH^{b}
Hg	S^{a} MH^{a}	S^{a} MH^{a}
Ni	S^{a} MH^{b}	S^{a} MH^{a}
Zn	S^{a} MH^{b}	S^{a} MH^{a}

[1] Soft water. [2] Moderately hard water.

Table 7-2. Mean (s.d.) 48-h and 96-h LC50 values for juvenile Anodonta imbecilis mussels exposed to six metals in soft reconstituted freshwater. The number of test replicates is indicated by N.

Metal	Time (h)	N	Mean LC50 (s.d.) (mg/L)
Cd^{+2}	48	3	0.057 (0.006)
	96	3	0.009 (0.003)
Cr^{+6}	48	3	0.295 (0.060)
	96	3	0.039 (0.034)
Cu^{+2}	48	3	0.171 (0.087)
	96	3	0.086 (0.031)
Hg^{+2}	48	3	0.216 (0.086)
	96	3	0.147 (0.035)
Ni^{+2}	48	5	0.240 (0.093)
	96	5	0.190 (0.097)
Zn^{+2}	48	3	0.355 (0.108)
	96	3	0.268 (0.095)

relative greater toxicities of mercury, cadmium and copper versus those of nickel, zinc and chromium is in accordance with the literature on other invertebrate organisms. In tests with Lymnaea acummata, Khangarot et al. (1982) determined the toxicity order to be Hg > Cu > Cd > Ni > Cr > Zn. Anderson (1950) exposed D. magna to several metal solutions. Their relative toxicities to D. magna arranged in decreasing order were Hg > Cu > Cd ≥ Zn > Ni. For Tubifex tubifex, the order was Hg > Cd > Cu > Cr > Zn > Ni (Brkovic-Popovic and Popovic 1977). In a comparison of heavy metal toxicity to the freshwater snail Biomphalaria glabrata, Ravera (1977) found copper to be more toxic than cadmium, and cadmium was more toxic than chromium. Gupta et al. (1981) exposed the mollusk Viviparus bengalensis to five metals and found their relative toxicities in the following order: Cu > Zn > Cr > Cd > Ni.

Variability in the response of different taxa to individual metals may be accounted for by physiological differences in the organisms. Many metals are needed in trace amounts as cofactors or as components of specific enzymes. The extent to which an animal is affected by the presence of high levels of metals may depend on the importance of specific enzymes and transport systems in its metabolic processes.

Cadmium, because of its role as an antagonist to the uptake of calcium by induction of metallothionein

(Hammond and Beliles 1980), causes skeletal deformities and nervous disorders (Alabaster and Lloyd 1982). It may also cause ion imbalances and interrupt energy production (Hiltibran 1971, Larsson et al. 1976).

Copper is a necessary component of many enzymes. However, copper at high concentrations can cause precipitation of mucus on fish gills and to branchial cell damage (Ellis 1937, Baker 1969). Tissue levels of copper rise upon exposure to sublethal and sublethal concentrations (Calamari and Marchetti 1973, Kariya et al. 1967) resulting in liver and kidney damage (Leland and Kuwabara 1985). Little else is apparently known of the harmful mode of action of copper on fish (Alabaster and Lloyd 1982).

Zinc is ubiquitous in the natural environment and an essential trace element for normal cell differentiation (Leland and Kuwabara 1985). It is part of a number of metalloenzymes and serves as a cofactor for many other enzymes. Zinc levels in cells can affect carbohydrate, fat and protein metabolism as well as other metabolic processes. Ion imbalances, skeletal deformities and nervous system disorders have been noted in fish exposed to high concentrations of zinc (Bengtsson 1974a, Bengtsson 1974b, Lewis and Lewis 1971).

The primary site of action of mercury on cells is the sulfhydryl groups on surface membrane proteins (Luckey and Venugopal 1977). As almost all enzymes

depend on the normal orientation of their sulfhydryl groups for proper conformation and function, the potential impact of mercury on cell processes is clearly evident (Leland and Kuwabara 1985).

Neither nickel nor chromium are very toxic to most animals (Hellawell 1986). No functional action of nickel or chromium has been described (Hammond and Beliles 1980).

The toxicities of all six metals except mercury were significantly lower ($p \leq 0.05$) in moderately hard water than they were in soft water (Table 7-1). Once again, cadmium was the most toxic to A. imbecilis with a 48-h LC50 of 0.137 mg/L and a 96-h LC50 of 0.107 mg/L Cd^{+2} (Table 7-3). Mercury was the second most toxic metal in moderately hard water. At 48-h, the LC50 was 0.223 mg/L Hg^{+2}, and its toxicity increased only slightly at 96-h to 0.171 mg/L. These values for mercury are not significantly different ($p \leq 0.05$) from comparable measures in soft water. The toxicity of copper to A. imbecilis in moderately hard water was almost half that observed in soft water. Except for the 96-h LC50 for chromium, Ni^{2+} and Cr^{6+} were also half as toxic in moderately hard water as in soft water. Finally, zinc toxicity increased from 0.588 mg/L to 0.438 mg/L at 48-h and 96-h respectively, in moderately hard water. This was an increase of 1.37 times.

Table 7-3. Mean (s.d.) 48-h and 96-h LC50 values for juvenile Anodonta imbecilis mussels exposed to six metals in moderately hard reconstituted freshwater. The number of test replicates is indicated by N.

Metal	Time (h)	N	Mean LC50 (s.d.) (mg/L)
Cd^{+2}	48	3	0.137 (0.034)
	96	3	0.107 (0.128)
Cr^{+6}	48	3	1.187 (0.313)
	96	3	0.618 (0.168)
Cu^{+2}	48	3	0.388 (0.036)
	96	3	0.199 (0.006)
Hg^{+2}	48	3	0.223 (0.061)
	96	3	0.171 (0.038)
Ni^{+2}	48	3	0.471 (0.035)
	96	3	0.252 (0.050)
Zn^{+2}	48	2	0.588 (0.035)
	96	2	0.438 (0.132)

The impact of water hardness on metal toxicity has been noted in studies with other aquatic species. Chromium toxicity to the worm Tubifex tubifex was reduced from 1.5 mg/L at 48-h in soft water to 4.8 mg/L Cr^{6+} in hard water (Brkovic-Popovic and Popovic 1977). There was a 2-20 fold decrease in chromium toxicity to fathead minnows and bluegill as water hardness was increased 1800% (Pickering and Henderson 1966). Similar increases in LC50 values were noted for lead toxicity to rainbow trout (Davies et al. 1976), the effects of zinc on stickleback (Jones 1938) and rainbow trout (Lloyd 1960), copper toxicity to rainbow trout and the carp, Cyprinus carpio, (Tabata 1969), and cadmium's lethality to goldfish (Alabaster and LLoyd 1982) and rainbow trout (Brown 1968). Metal solubility and therefore bioavailability are decreased in hard water (Sprague 1985).

In summary, at 48-h, three metal toxicity groups were identified in soft water. Zinc was the least toxic ($p \leq 0.05$). Cu, Cr, Ni and Hg formed a moderately toxic set. Cadmium was the most toxic. The same trend was evident at 96-h, but the moderately toxic group--Hg, Ni, Cu, Cr--changed order. Cadmium remained the most toxic metal in soft water after 4-day exposures and zinc was the least toxic (Tables 7-4 and 7-5). In moderately hard water, chromium was the least toxic at both time periods while cadmium was the most toxic. With longer

Table 7-4. Anodonta imbecilis toxicity data for metals dissolved in soft reconstituted freshwater by ANOVA and Duncan's multiple range test. The test organism was Anodonta imbecilis. Metals connected by the same line are not significantly different ($p \leq 0.05$). Metals were increasingly toxic going from left to right.

SOFT WATER

48-h LC50s

Zn	Cr	Ni	Hg	Cu	Cd

96-h LC50s

Zn	Hg	Ni	Cu	Cr	Cd

Table 7-5. Results of analysis of Anodonta imbecilis toxicity data for metals dissolved in moderately hard reconstituted freshwater by ANOVA and Duncan's multiple range test. Metals connected by the same line are not significantly different ($p \leq 0.05$). Metals were increasingly toxic going from left to right.

MODERATELY HARD WATER

48-h LC50s

Cr	Zn	Ni	Cu	Hg	Cd

96-h LC50s

Cr	Zn	Ni	Cu	Hg	Cd

exposure, the toxicities of individual metals to Anodonta imbecilis in moderately hard water became more similar. At 96-h, the toxicities of Cr and Zn were significantly different from all other metals and each other. However, Ni, Hg, Cu and Cd were not significantly different from each other.

Metal Mixtures

The toxicity of a particular metal can increase (synergism), decrease (antagonism) or remain unchanged (additive) when combined with another metal (Marking 1985). Which of these responses will occur depends on whether the presence of one facilitates the uptake of the other metal or whether or not they compete for the same transport sites on the membrane (Sprague 1985).

In the present study, there was a trend toward a greater toxicity of metals in combinations containing Ni, Zn, Hg or Cu than for single metal exposures (Tables 7-6 and 7-7). However, the increases in toxicity were not significant ($p \leq 0.05$) based on ANOVA and Duncan's multiple range test. In contrast, cadmium had a higher 96-h LC50 in mixtures with Zn (0.029 mg/L) and Cu (0.012 mg/L) than it did alone (0.009 mg/L) and the LC50 for chromium alone, 0.039 mg/L, increased to 0.148 mg/L in combination with Hg.

Of the four mixtures tested, the combination of cadmium and copper produced the greatest toxicity to A.

Table 7-6. Comparisons of single metal LC50s at 48-hours to the same metal in combination with another for Anodonta imbecilis. Values represent the mean (s.d.) LC50 for two tests.

Mixture	48-h LC50 (mg/L) Individual	In Combination	Additive[a] Index
Ni	0.240(0.093)	0.128(0.043)	-0.14
and			
Zn	0.355(0.108)	0.217(0.055)	
Cd	0.057(0.006)	0.050(0.14)	-0.58
and			
Zn	0.355(0.108)	0.249(0.071)	
Hg	0.216(0.086)	0.094(0.014)	-0.43
and			
Cr	0.295(0.060)	0.170(0.054)	
Cd	0.057(0.006)	0.037(0.020)	-1.36
and			
Cu	0.171(0.087)	0.062(0.035)	

[a] Calculation of Additive Index followed Marking and Dawson (1975).

Table 7-7. Comparisons of single metal LC50s at 96-hours to the same metal in combination with another for Anodonta imbecilis. Values represent the mean (s.d.) LC50 for two tests.

Mixture	96-h LC50 (mg/L) Individual	In Combination	Additive[a] Index
Ni	0.190(0.097)	0.088(0.032)	-0.07
and			
Zn	0.268(0.095)	0.162(0.051)	
Cd	0.009(0.003)	0.029(0.029)*	-2.76
and			
Zn	0.268(0.095)	0.145(0.025)	
Hg	0.147(0.035)	0.088(0.012)	-1.32
and			
Cr	0.039(0.034)	0.148(0.064)	
Cd	0.009(0.003)	0.012(0.001)	-0.58
and			
Cu	0.086(0.031)	0.021(0.003)	

* Significantly different from LC50 of individual metal ($p \leq 0.05$). All other combinations and individual LC50s were not significantly different. [a]Calculation of Additive Index followed Marking and Dawson (1975).

imbecilis. The 48-h LC50 for copper individually was 0.171 mg/L while in combination with cadmium, copper had an LC50 of 0.062 mg/L (Table 7-6). Likewise, the individual 48-h LC50 for cadmium was 0.057 mg/L, but in the mixture with copper it decreased to 0.037 mg/L.

Marking and Dawson (1975) developed an index to determine whether chemicals in mixtures exerted an antagonistic, synergistic or additive effect on each other. Index values statistically indistinguishable from zero are representative of additive effects. Synergistic effects are identified by index values greater than zero and antagonism of chemicals is indicated by values less than zero. If the calculated index range contains zero, it is said to be indistinguishable from zero.

The additive index (Marking and Dawson 1975) for Cu and Cd after two days' exposure of A. imbecilis was -1.36 with a range of -1.27 to +1.66 (Table 7-6). Therefore, Cu and Cd exert an additive toxicity effect on each other. At 96-h, copper and cadmium had generally lower LC50s individually and in the mixture than at 48-h (Table 7-7). However, cadmium was slightly less toxic at 96-h in combination with copper. Because of this, copper and cadmium were determined to be slightly antagonistic to each other based on the Marking and Dawson (1975) index. The only other antagonistic effect was measured between Hg and Cr at 96-h. No synergistic effects were seen in any of the four mixtures.

Similar evaluations of metal mixture toxicity by other investigators have produced various results. Attar and Maly (1982) determined that Cd and Zn were antagonistic to each other in toxicity tests with D. magna. Thompson et al. (1980) found that the toxicity of Zn-Cu mixtures to bluegills was additive. Lloyd (1961) found the combined effect of Cu and Zn on rainbow trout was additive at low concentrations and synergistic at higher levels. The presence of high levels of zinc inhibited the uptake of cadmium in adult Anodonta cygnea (Hemelraad et al. 1987) and no lethality was noted.

Sediment Tests

No toxicity to either A. imbecilis or C. dubia was detected in sediment tests with copper and cadmium. Based on earlier results with aqueous exposures, the nominal aqueous concentration range of 1,000 ug/L (200 mg/Kg sediment) to 130 ug/L (26 mg/Kg sediment) would have killed both species without the presence of sediments. However, in the presence of sediments all mussels and zooplankton survived for the duration of the 96-h tests. Changes in mussel behavior were noted. Mussels did not burrow into the substrate in chambers with metals, in contrast to observations of their behavior in control chambers, and offspring were seen developing in C. dubia in lower metal concentrations (< 600 ug/L), while in containers with higher metal levels

no reproduction was visible. Zooplankton molts were visible on the bottom of test chambers containing <600 ug/L metal and food was seen in their guts.

The non-lethal effect of the metals on both organisms was probably due to sediment binding of metals. There was virtually no metal measured in the aqueous phase of any test chamber. However, based on measurements of acid-digested samples, sediment metal concentrations were lower than expected. It is possible that some metal was lost by sorption to glass during preparation of the sediments prior to initiation of the tests.

Effluent Toxicity

The "Flying Colors" effluent was very toxic to both A. imbecilis and C. dubia. Their 48-h LC50s were 0.95% effluent and 0.57% effluent, respectively (Table 7-8). At 96-h, the effluent toxicity to both animals increased. A concentration of less than 0.6% was lethal to half the A. imbecilis juveniles (LC50=0.58%), while 0.43% was lethal to half of the C. dubia neonates. Analysis of this effluent by the City of Gainesville showed that it contained 6.43 mg/L Cr^{+6}. The LC50 of 0.6% for mussels is equal to 0.039 mg/L, the same as the 96-h LC50 in soft reconstituted water but much lower than

Table 7-8 . The toxicity of "Flying Colors" effluent to *Ceriodaphnia dubia*, *Anodonta imbecilis* and Microtox. LC50 values are in per cent whole effluent.

Organism	Time (h)	LC50 % effluent	95% C.I.
Ceriodaphnia dubia	48	0.57	0.10-0.95
	96	0.43	0-0.67
Anodonta imbecilis	48	0.95	0.74-1.18
	96	0.58	0-0.040
Microtox*	0.25	0.07	N/A

* From C. Maziji, unpublished data.

the value for moderately hard water at 96-h (0.618 mg/L). However, metals are usually less toxic in hard water than in soft water as evidenced by data from this dissertation as well as by literature data.

Discussion

The freshwater mussel A. imbecilis is as sensitive to dissolved metal pollution as are zooplankton and may be more sensitive than some insects (Table 7-9). These data show that while mussels can accumulate high levels of metals from their environment and live for some time, they may also be adversely affected by much lower concentrations. Before using a surrogate animal to determine safe exposure levels for another, it is prudent to verify that they have comparable sensitivities. Having done so in the laboratory, it appears that zooplankton can be used to identify waters that may be toxic to mussels. However, the habitats of these two organisms is so different that they may not be exposed to the same level of toxicant in nature.

Certainly, laboratory findings using controlled conditions may produce conservative estimates of toxicity. No accounting of substrate, water flow, interactions with other organisms or the effect of various food types was made. These factors may have substantial impact on the determination of LC50s (Sprague

Table 7-9. Comparative toxicities of selected metals in soft water to several invertebrates and fish.

Organism	Metal	Water Hardness (mg/L)	Time (h)	LC50 (mg/L)	Ref.*
A. imbecilis	Cd	39	48	0.057	-
Daphnia	Cd	45	48	0.065	3
Chironomus	Cd	25	48	8.05	5
Bluegill	Cd	20	96	1.96	2
A. imbecilis	Cr	39	48	0.295	-
Daphnia	Cr	--	48	1.800	6
Chironomus	Cr	25	48	11.80	4
Bluegill	Cr	36	24	0.280	4
A. imbecilis	Cu	39	48	0.171	-
Daphnia	Cu	45	48	0.065	3
Chironomus	Cu	25	48	0.327	5
Bluegill	Cu	44	96	0.884	1
A. imbecilis	Hg	45	48	0.216	-
Daphnia	Hg	45	48	0.005	3
Chironomus	Hg	25	48	0.029	5
Fathead minnow	Hg	45	96	0.168	1
A. imbecilis	Ni	39	48	0.240	-
Daphnia	Ni	45	48	0.510	3
Chironomus	Ni	25	48	0.327	5
Bluegill	Ni	25	48	69.50	1
A. imbecilis	Zn	39	48	0.355	-
Daphnia	Zn	45	48	0.100	3
Chironomus	Zn	25	48	8.20	5
Bluegill	Zn	20	96	5.38	2

* References: (1) Mayer and Ellersieck 1986. (2) Mount 1966. (3) Biesinger and Christensen 1972. (4) Smith and Heath 1979. (5) Khangarot and Ray 1989. (6) Khangarot and Ray 1987. All others from this study.

1985). However, the acute toxicity test is accepted as giving the most precise results and is still the most widely used method for determining relative sensitivity to pollutants.

Water hardness has a major effect on metal toxicity to mussels. This has been demonstrated for many other organisms and is related to metal chelation and to physiological responses of the organisms (Sprague 1985). The affect of water hardness on the toxicity of metals to mussels is important to know because many of the streams with depauperate mussel fauna have received acid mine drainage or industrial effluents over the years. They also have low water hardness (Jones 1940 and 1958, Wurtz 1962, Dieffenbach and Ryck 1976). While measured concentrations of metals in these streams are lacking for the most part, there has been enough circumstantial evidence to support the assertion that metals were responsible for the faunal decline.

Comparison of LC50 values for metals and ambient water quality guidelines established by EPA (USEPA 1976) indicates that current standards may be adequate to protect mussel species. In most cases, the maximum limit allowed to be released during a 30-day period (Table 7-10) is lower than the mussel 96-h LC50 in moderately hard water. The only exception was seen in the case of nickel. However, since this value was based on total

Table 7-10. Comparisons between EPA[a] water quality criteria for metals and LC50 values for A. imbecilis (in moderately hard freshwater).

Metal	EPA Guidelines (ug/L) 24-h Max.	Max. Limit	48-h LC50 for A. imbecilis (ug/L)
Cd	0.038	4.6	137
Cr	0.290	21	1,190
Cu	5.6	32	388
Hg	0.2	4.1	223
Ni	130	2500	471
Zn	47	450	588

[a] U.S.E.P.A. 1976. Based on water hardness of 150 mg/L.

metal concentration in water with a hardness of 150 mg/L $CaCO_3$, and my data were generated at a somewhat lower hardness (39 mg/L $CaCO_3$), the values are not likely to be different in terms of biological impact. Standards are not set for waters with low alkalinity. Since higher water hardness decreases metal toxicity (Sprague 1985) perhaps receiving stream hardness should be considered in determining safe levels of metals in effluents.

Determination of the impact on mussels of in situ chronic exposures (several months) to low metal concentrations is critically needed. Metals are sometimes believed to contribute to the decline of mussel species in rivers meeting water quality standards. This assumption is supported by information from both the Tennessee Valley Authority on metal concentrations in rivers judged to be good mussel habitat based on species diversity (Jenkinson and Heuer 1986) and from long-term studies on the Clinch River, Virginia, which is one of the best remaining mussel habitats (American Electric Power Service Corp. 1989). However, water quality standards do not take into account the potential for bioaccumulation or sublethal responses. Long-term exposure could affect fecundity or behavior, e.g. filtering capacity or ability to burrow, that could lead to species decline.

The impact of low concentrations of metal mixtures is not considered in the development of water quality

criteria (U.S.E.P.A. 1976). From this research, however, metals can be more toxic to mussels at lower concentrations in combination than they may be singly. This conclusion is in agreement with the literature on other aquatic organisms. Since sources of metal pollution rarely produce pure metal wastes, it seems reasonable to assess the impact of mixtures in setting effluent concentration limits.

Comparisons of mussel sensitivities to metals with those of other invertebrate species, particularly zooplankton, suggest that setting water quality standards based on tests with the latter will adequately protect mussel fauna. Similarly, the responses of A. imbecilis and C. dubia, a standard effluent toxicity test organism, indicate that the latter animal may serve as an acceptable surrogate for mussels in metal effluent toxicity tests. The two organisms appear to be equally sensitive to chromium waste. However, analyses of the toxicity of other metal effluents should be performed to determine how broadly the responses of these two organisms overlap.

CHAPTER 8
THE TOXICITY OF SEVERAL PESTICIDES, ORGANIC COMPOUNDS AND A WASTEWATER EFFLUENT TO THE FRESHWATER MUSSEL, *Anodonta imbecilis*, THE ZOOPLANKTER, *Ceriodaphnia dubia* AND THE FATHEAD MINNOW, *Pimephales promelas*

Introduction

Pesticides (insecticides, herbicides, molluscicides, piscicides and nematicides) have been used to eliminate or control pests since at least Grecian times (Edwards 1973, Ruzicka 1973). Pliny the Elder advocated the use of arsenic to control insects in A.D. 70. Other inorganic compounds were used for centuries, and although many were persistent in soils resulting in crop damage (Edwards 1973), they were not as refractory or globally distributed as the modern organic pesticides.

Development and use of organic chemicals to control pests increased as a result of the growth of the petrochemical industry in the 1940s (Nimmo 1985). New pesticides were effective in reducing crop losses caused by insects, competitive weeds and soil microbes, and the incidences of malaria, typhus, dysentery and other diseases (Edwards 1973). However, because of their biocidal capacity, they also posed a serious threat to non-target organisms (Hellawell 1986). Therefore, it is surprising that little thought was given to the impact of

the widespread use of such pesticides might have until the 1960s (Carson 1962, Rudd 1964, Butler and Springer 1963).

In the past 20 years, vast amounts of data have accumulated showing the effects of pesticides on non-target organisms, particularly fish and zooplankton (Mayer and Ellersieck 1986, Johnson and Finley 1980). Due to differences in chemical structure, there are wide ranges in the sensitivities of non-target organisms to pesticides. However, some generalizations can be made. Organochlorine insecticides (e.g., DDT, lindane, toxaphene, chlordane) are the most persistent and most toxic to fish of all organic pesticides. They are very insoluble in water (1 ug/L-7 mg/L), tending to partition into lipids and organic substrates (Nimmo 1985). Thus, they accumulate in biota and the ecosystem. While the mode of action of organochlorine insecticides has not been clearly established (Ware 1978), they cause spontaneous nerve impulses that eventually lead to convulsions and death.

Organophosphate insecticides (e.g., malathion, diazinon) which were developed after the organochlorines, tend to be more water soluble (24-2500 mg/L), less persistent and less toxic to fish (Nimmo 1985). The organophosphate insecticides prevent the breakdown of acetylcholine by acetylcholinesterase. In so doing, they

interfere with the normal passage of nerve impulses and cause paralysis (Ware 1978).

The newer carbamate insecticides (e.g., carbaryl, carbofuran) are water soluble (40-700 mg/L) highly unstable and toxic to fish in only very high concentrations (Nimmo 1985). Like the organophosphates, carbamate insecticides are cholinesterase inhibitors (Ware 1978).

Pyrethroid insecticides such as Karate (ICI Americas) are derivatives of pyrethrum, a natural botanical insecticide (Hellawell 1986). They inhibit normal sodium and potassium conductance in the neuron that eventually blocks the passage of nerve impulses. Paralysis of muscles follows (Murphy 1980). They are highly toxic to fish and invertebrates, but are rapidly degraded by photolysis and therefore do not persist (Ware 1978, Johnson and Finley 1980).

Pentachlorophenol (PCP) has been used as a wood preservative (fungicide), and as a molluscicide in some parts of the world where snails serve as vectors of human parasites (Cheng 1974, Ware 1978, Hellawell 1986). It is the second most heavily used pesticide in the United States (U.S.E.P.A. 1986a). Being highly chlorinated, PCP is very toxic to both plants and animals. Its mechanism of action is via a combination of plasmolysis, protein precipitation and uncoupling of oxidative phosphorylation (Ware 1978).

Modern herbicides are also less persistent than chlorinated insecticides and less toxic to non-target aquatic biota than were the defoliants widely used in 1960s (2,4-D and 2,4,5-T) (Edwards 1973). The triazines, e.g. atrazine, are used extensively in agriculture and silviculture to control broadleaf and grassy weeds (Ware 1978, Weed Science Society of America 1979). Triazines are strong inhibitors of photosynthesis (Ware 1978, Weed Science Society of America 1979).

Endothall herbicides, e.g. Hydrothol-191 and Aquathol-K, are among the most effective for use in the control of the aquatic weeds Hydrilla verticillata, Valisneria americana and Myriophyllum spicatum in canals, lakes and streams (Dumas 1976). Endothalls have half-lives of 3-10 days depending on the formulation and are biodegraded by microorganisms (Rienert and Rodgers 1987). The inorganic endothall herbicides, such as Aquathol and Aquathol-K, are less toxic to non-target biota but are not as effective as the organic endothall, Hydrothol-191 (Reinert and Rodgers 1987).

Organic compounds other than pesticides are released into the aquatic environment. Included among these are sodium dodecyl sulfate (SDS), a bacteriocidal surfactant, and ethylediamine tetraacetate (EDTA) a chelating agent used in shampoos and detergents. Surfactants disrupt cell membranes (Ware 1978), while EDTA removes cations from solution. A number of organic

solvents are used in industry, and as diluents of hydrophobic pesticides for use in aquatic toxicity tests. Two of the most common are acetone and methanol.

Even though more stringent effluent water quality criteria were instituted in amendments to the Clean Water Act of 1977 (National Pollution Discharge Elimination System, NPDES) it is necessary to monitor the impacts of effluents on aquatic biota on a continual basis. The Environmental Protection Agency (EPA) requires testing for such impacts on aquatic organisms as part of the registration and re-registration processes for pesticides (Zucker 1985a and 1985b), as part of the pre-manufacturing notification for toxic chemicals under the Toxic Substances Control Act, and for re-permitting of many municipal wastewater facilities (NPDES).

Receiving waters for wastewater and industrial effluents are usually streams and rivers. Likewise, water associated with agriculture is often flowing in canals and ditches. However, standard toxicity test batteries do not include representatives of the lotic fauna. The most common test organisms are fish and zooplankton which primarily inhabit lakes (Johnson and Finley 1980, Mayer and Ellersieck 1986, Peltier and Weber 1985, Buikema et al. 1982). Thus, little is known about the sensitivity of most benthic invertebrates.

A wastewater entering the aquatic environment affects hundreds or thousands of species. Therefore,

using just a few test species, i.e., fish and zooplankton, may lead to underestimation of the impact on the ecosystem. Patrick et al. (1968) and others have shown that macroinvertebrates and algae are often more sensitive to toxicants than are fish. Additionally, algae and macroinvertebrates are organisms on which fish depend for food (Buikema et al. 1982). Fish may be indirectly affected by a decline in the density or biomass of such organisms, regardless of their direct response to the toxicant.

There is concern over the status of one group of stream organisms in particular, the unionid mussels. With the recent designation of over 70 species of unionid mussels as endangered or threatened (USFWS 1989), it has become necessary to assess the impact of pesticides, herbicides and other organic pollutants on their survival. Significantly, more than 10 species of freshwater mussels found in Florida are candidates for inclusion on the threatened or endangered species list. Federal law (Endangered Species Act) mandates that pesticide and herbicide use must be limited to levels that are not detrimental to designated species in watersheds containing endangered or threatened organisms. It is impossible to set valid standards or limits without appropriate data. Finally, the use of indigenous species for toxicity testing is ideal when possible because the organisms are acclimated to ambient conditions and,

therefore, provide a good indication of biotic responses in their locale (Buikema et al. 1982).

Therefore, the EPA was interested in testing the toxicity to freshwater mussels of several pesticides (atrazine, carbaryl and Karate) undergoing evaluation for registration or re-registration. Determining the toxicity of other selected organic pollutants to mussels was essential in assessing the adequacy of current water quality standards and the development of more protective water quality criteria if necessary. It was also important to assess the comparability of zooplankton and mussel sensitivities because the EPA Office of Pesticide Programs is advocating the use of Daphnia magna as a surrogate for freshwater mussels in toxicity tests.

The goals of my research in this area were to: (1) determine the toxicity of several pesticides, organic compounds and an organic effluent to juvenile Anodonta mussels, and (2) compare their sensitivities with common test organisms such as D. magna, Ceriodaphnia dubia and Pimephales promelas, the fathead minnow.

Materials and Methods

Test Organisms

A. imbecilis glochidia were cultured in vitro using methods described earlier (Chapter 3). After their transformation, juveniles were put in soft reconstituted freshwater (Peltier and Weber 1985) and used for tests

usually within two days. Daphnia magna were obtained from a local source using EPA approved culture methods. Ceriodaphnia dubia were cultured in this laboratory. Pimephales promelas (fathead minnow) larvae used in the effluent toxicity test were obtained from EPA-Newtown, OH, via overnight mail and used immediately.

Test Conditions

Aqueous Exposures. Toxicity tests with pesticides and pure compounds were performed for 48-h using methods developed earlier in this dissertation. All tests were performed in an environmental chamber with 16 hours of light and eight hours of darkness, at a temperature of $22^{o} \pm 1^{o}$ C. Five test concentrations were used, plus a control which was soft reconstituted freshwater. Two replicates, each containing 10 juvenile mussels, were used per concentration in either 200 ml crystallizing dishes (Karate, carbaryl and atrazine) or 15 X 60 mm glass Petri dishes with lids. All tests were static except for those using carbaryl and Karate which were known to decompose rapidly. Solutions of these two pesticides were renewed at 24-h. Details of the test protocol were given in Chapter IV.

Hydrothol-191, an endothall derivative (Pennwalt Corp., Philadelphia, PA.), was dissolved directly in soft reconstituted freshwater to make a stock of 530 mg/L. Because lindane is only slightly soluble in water (10

mg/L), a stock solution was made in methanol. Small aliquots of the stock were added to the test chambers directly. Na · PCP has low solubility in water, as well. It was dissolved in 0.01 N NaOH and pH was adjusted to 7.0. Stocks of the remaining compounds--SDS, methanol, acetone and EDTA--were prepared by dissolving the reagents directly in soft reconstituted water. Dilutions used in definitive tests were made as appropriate based on range-finding tests. Test solutions were made by 60% dilution of the stocks with soft reconstituted freshwater. All toxicant concentrations given are nominal except for those of toxaphene and chlordane.

Karate, atrazine and carbaryl. Tests with Daphnia magna neonates (< 24 h) were performed separately but concurrently with mussel tests for Karate, carbaryl and atrazine. The EPA was particularly interested in comparisons between Daphnia magna and Anodonta imbecilis sensitivities to two of the pesticides, i.e. carbaryl and atrazine, because use of these pesticides is already being limited in watersheds with endangered mussels to levels deemed safe by zooplankton tests. However, there were no data to support the assumption that D. magna and unionid mussels have comparable sensitivities to carbaryl or atrazine. Additionally, the toxicity of Karate (ICI Americas), a pyrethroid insecticide, was assessed because it is currently being tested for registration.

Since carbaryl and atrazine have such low solubilities in water (40 mg/L and 33 mg/L at 30° C, respectively), a saturated solution of each was prepared as follows: 100 mg of the pesticide was added to 1 L of soft reconstituted water and stirred overnight; the supernatant was filtered and small aliquots of this stock were added directly to the test chambers.

For each 48-h toxicity test, ten D. magna neonates were randomly placed in each chamber which consisted of a 200 ml crystallizing dish containing 100 ml of toxicant solution. Two replicates were prepared for each of five dilutions and a control containing soft reconstituted freshwater (Peltier and Weber 1985). The D. magna were fed at 24-h during the tests.

Toxaphene and Chlordane Tests. Chlorinated pesticides are known to adsorb to soils or sediments where they can remain for many years (Ware 1978, Menzer and Nelson 1980). While both of these pesticides have been recently de-registered in the United States for most uses (52 C.F.R., 51 C.F.R), they persist in the sediments of many bodies of water. Little was known about the toxicity of sediment-sorbed pesticides to mussels, or whether such infaunal molluscs are differentially susceptible to sediment-bound or aqueous concentrations. Therefore, I made a comparison of the toxicity of toxaphene and chlordane in both water and laboratory-spiked sediments.

Two sets of chambers were prepared for each of these insecticides. One series was prepared to determine toxicity in the usual way, dissolved in water. In addition, the diminution of toxicity as a result of sediment adsorption was assessed using a series of test chambers that contained both water and sediments. Thirty ml of soft reconstituted freshwater were put into each 50 ml glass vial with or without sediment (5 g dried, 3% organic content). An appropriate volume of stock pesticide in acetone was injected directly into each vial using a micro-syringe, then the vial was capped with a teflon-lined top. The vials were mixed over night on a wrist-action shaker, after which their contents were emptied into 50 ml beakers. Both sets of test chambers (with and without sediments) were left for 24 h prior to addition of the mussels, to allow sediments to settle and contents to equilibrate. Ten juvenile Anodonta imbecilis and five Ceriodaphnia dubia neonates were added to each of two chambers at each test concentration both with and without sediment. Toxaphene concentrations ranged from 1,829 ug/L to 0 ug/L. Chlordane was used at concentrations of 905 ug/L to 0 ug/L.

Water samples from chambers used in chlordane and toxaphene tests were analyzed by gas chromatography to determine aqueous concentrations. Samples were extracted with three 10 ml aliquots of methylene chloride and later transferred to iso-octane. The extracts were blown down

to 1 ml with nitrogen and stored in crimpseal vials until analyzed on a Varian 3700 gas chromatograph. A 30 m DB-5 column with a 0.53 mm diameter and a 1 um coating was used with an initial temperature of 150° C ramped to 250° C at 5° C per minute. The injector temperature was 22° C and the detector was set at 300° C. The recovery rate for both pesticides was 28%.

Effluent Toxicity Test. An industrial effluent sample was obtained from the Buckman Street Wastewater Treatment Facility, Jacksonville, FL for use in assessing the relative sensitivity of juvenile A. imbecilis mussels versus those of standard effluent test organisms. The 7-d effluent toxicity tests were performed using moderately hard reconstituted freshwater (Horning and Weber 1986) as diluent and control water.

Forty A. imbecilis juveniles were exposed to each effluent concentration, 20 in each of two replicate chambers. Ten Ceriodaphnia dubia neonates and 20 Pimephales promelas larvae were exposed to each test concentration, the former in individual 30 ml plastic containers to permit monitoring of reproduction, the latter in two groups of 10 at each dilution in 1 L pyrex beakers.

Toxicity was assessed based on protocols established in the Ceriodaphnia dubia survival and reproduction test, and the fathead minnow survival and growth test (Horning and Weber 1986). Survival of test organisms was recorded

daily until termination at seven days. Zooplankton reproduction and larval fathead minnow growth were used as indicators of sublethal affects. Water was changed daily in all test chambers (Horning and Weber 1985).

Data Analysis

Survival data were analyzed by several methods. A set of EPA (Peltier and Weber 1985) computer programs calculated the LC50s. These programs, known as the TOX-DAT Multimethod, calculate the LC50 using moving average angle, probit and binomial methods. Results of these analyses (LC50s) were then used to determine differences in toxicity among the chemical with ANOVA and Duncan's multiple range test. All statistical analyses except LC50s were performed using the SAS statistical package (SAS 1986) at the Northeast Regional Data Center, University of Florida, Gainesville.

Results

Aqueous Exposures

Of the 12 organic compounds tested for toxicity to A. imbecilis, PCP was the most toxic, while methanol was the least toxic (Table 8-1). Forty-eight hour LC50s for A. imbecilis exposed to acetone and methanol were 37.02 and 36.3 mg/L , respectively (Tables 8-1 and 8-2). A solvent must be non-toxic at concentrations $\leq$10 ml/L to be used in toxicity tests. If a pesticide is not soluble

Table 8-1. Summary of acute toxicity test results for juvenile Anodonta imbecilis with twelve pesticides and organic compounds. N is the number of experiments performed with each toxicant. LC50 values with the same letters are not significantly different from each other ($p \leq 0.05$).

Chemical	N	48-h LC50 (mg/L)
Methanol	5	37.02 (4.7)[a]
Carbaryl	1	36.3[ab]
Acetone	2	33.83 (11.31)[ab]
Atrazine	1	33[ab]
SDS	3	19.04 (4.19)[c]
Lindane	3	> 10*[c]
Hydrothol	3	4.85 (2.29)[d]
EDTA	3	1.35 (0.35)[d]
Karate	1	> 1*[d]
PCP	5	0.61 (0.26)[d]
Chlordane	2	N/C[+]
Toxaphene	2	N/C

* Solubility in water.

Table 8-2. Comparisons between the acute toxicities of several organic compounds for A. imbecilis, D. magna and L. macrochirus.

	LC50 (mg/L)		
Chemical	A. imbecilis[a]	D. magna[a]	L. macrochirus[b]
Acetone	33.83	.0039[d]	--
Methanol	37.02	11[eg]	29.40[fg]
PCP	0.610	0.33[c]	0.240[d]
SDS	19.04	10.3[c]	--

[a] 48-h LC50. [b] 96-h LC50.
[c] Lewis and Weber 1985. [d] Macek and McAllister 1970.
[e] Ceriodaphnia dubia. [f] Salmo gairdneri.
[g] Poirier et al. 1986.

in 10 ml/L of solvent, it is considered to be insoluble in water and therefore not a threat to aquatic life. With that in mind, it is safe to use either of these solvents in pesticide tests with mussels because neither is lethal at the maximum allowable concentration. Forty-eight hour LC50 values for D. magna are 0.0039 mg/L acetone (Macek and McAllister 1970) and 11 mg/L methanol (Poirier et al. 1986). The sensitivity of S. gairdneri (rainbow trout) to methanol was 29.40 mg/L, intermediate between the values for the two invertebrates (Mayer and Ellersieck 1986).

SDS is often used as a reference toxicant in tests with zooplankton to verify that a particular set of test organisms is healthy in comparison with accepted norms. The 48-h LC50s for A. imbecilis exposed to SDS was 19.04 mg/L (Table 8-2). There are no established benchmark values for SDS toxicity to mussels. However, mussels were less sensitive to the surfactant than D. magna based on published values (Lewis and Weber 1985) (Table 8-2).

A. imbecilis was relatively insensitive to all of the herbicides and insecticides tested (Table 8-3). Lindane, an organochlorine insecticide, was not toxic to mussels at concentrations as high as its solubility limit in water, 10mg/L. Under such circumstances, a pesticide is said to be non-toxic to aquatic biota. Lindane is toxic to most other aquatic biota at low concentrations (Table 8-3). Based on literature values, lindane is

Table 8-3. Comparative 48-h LC50s of five pesticides for three aquatic species.

	LC50 (ug/L)		
Chemical	A. imbecilis[c]	D. magna[c]	L. macrochirus[d]
Carbaryl	36,300	56[f]	15,800[f]
Lindane	> 10,000*	485[e]	77[f]
Karate	> 1,000*	27	N/A
Atrazine	33,000	9,800	42,000[f]
Hydrothol	4,280	360[g]	940[h]

[f] Mayer and Ellersieck 1986. [g] Pennwalt Corp.
[h] Johnson and Finley 1980. [e] Henderson et al. 1959.
[d] 96-h LC50. [c] 48-h LC50. * Solubility in water.
[c] 48-h LC50.

toxic to both D. magna and L. macrochirus at <500 ug/L. However, Bluzat and Seuge (1979) calculated the 48-h LC50 for Lymnaea stagnalis to be 7,300 ug/L. Molluscs do not appear to be susceptible to lindane at concentrations that are normally found in water.

The toxicity of the aquatic herbicide Hydrothol-191, to mussels was also very low. At 48-h, the mussel LC50 was 4,280 ug/L. In comparison, I determined the 48-h LC50 for Ceriodaphnia dubia to be 190 ug/L and the 96-h value for fathead minnow larvae to be 468 ug/L in earlier tests (Chapter 4). Literature values for other aquatic organisms are also much lower than those of the mussels (Pennwalt Corp. 1980, Johnson and Finley 1980).

PCP was acutely toxic to juvenile A. imbecilis mussels (Table 8-2). It was the only pesticide to which the mussels responded at a level similar to other species. The 48-h LC50 was 610 ug/L for mussels, 330 ug/L for D. magna (Lewis and Weber 1985) and 240 ug/L for bluegill (Macek and McAllister 1970). PCP is known to be toxic to virtually all biota, including molluscs (Ware 1978) and has been used as a molluscicide for years. The 48-h LC50s for two snail species, Lymnaea stagnalis and Gillia altilis, were found to be 240 ug/L and 810 ug/L PCP (U.S.E.P.A. 1986a). Therefore, it is not surprising that PCP was toxic to A. imbecilis.

Karate, Carbaryl and Atrazine

Karate, while not toxic to mussels at its solubility limit in water (1 mg/L, ICI Americas), was lethal to half of the D. magna at a concentration of 27 ug/L (48-h LC50) (Table 8-3). No data are available yet on the sensitivity of other aquatic biota to Karate since it is still undergoing pre-registration testing.

The LC50 for the carbamate insecticide, carbaryl, was 36,300 ug/L at 48-h (Table 8-3). This value is much higher than literature values of 56 ug/L for D. magna and 15,800 ug/L for L. macrochirus (Mayer and Ellersieck 1986). A. imbecilis was also less sensitive to carbaryl than the snail Lymnaea stagnalis (Bluzat and Seuge 1979). Carbamates are typically very effective insecticides, but are non-toxic to mammals and non-insect arthropods. They are also transitory in the environment (Hellawell 1986, Mount and Oehme 1981). D. magna tested concurrently with A. imbecilis had a 48-h LC50 of 1.9 mg/L carbaryl.

The herbicide atrazine was virtually non-toxic to juvenile A. imbecilis mussels, having an LC50 of 33,000 ug/L (Table 8-3). Comparable values are 9.8 ug/L for D. magna neonates tested in this laboratory and 42,000 ug/L for bluegill sunfish (Mayer and Ellersieck 1986).

Toxaphene and Chlordane Tests

Determinations of the toxicities of toxaphene and chlordane to A. imbecilis indicated that mussels were

tolerant of these insecticides at concentrations several orders of magnitude higher than were C. dubia, D. magna or L. macrochirus (Table 8-4). Acute toxicity values for most aquatic organisms range from 2-40 ug/L for toxaphene and from 3-115 ug/L for chlordane (Johnson and Finley 1980). However, neither toxaphene (up to 1.83 mg/L) nor chlordane (ug to 0.90 mg/L) was toxic to A. imbecilis at 48-h in chambers without sediment. After four days' exposure, half of the mussels were killed by 0.74 $\pm$ 0.07 mg/L toxaphene and 0.88 $\pm$ 0.05 mg/L chlordane. There were no mussel deaths due to toxaphene or chlordane exposure in test chambers containing sediment (Table 8-5). In those chambers, aqueous concentrations were markedly lower than in their counterparts without sediment.

Ceriodaphnia dubia neonates were considerably more effected by both toxaphene and chlordane than were juvenile mussels (Table 8-4). After 48-h, there were no survivors in test vessels without sediments. In contrast, no C. dubia died during the first two days in test chambers that contained sediments. By 96-h, all zooplankton had died in the toxaphene + sediment tests, while the LC50 in the chlordane + sediment chamber was 0.450 mg/L. Mussels were 1-2 orders of magnitude less sensitive than are fish or zooplankton.

Table 8-4. Acute toxicities of toxaphene and chlordane in soft water to A. imbecilis, D. magna and L. macrochirus.

Organism	LC50 (mg/L)	
	Toxaphene	Chlordane
Anodonta imbecilis[b]	0.74 ± 0.07	0.88 ± 0.05
Ceriodaphnia dubia[c]	N/C[+]	N/C[+]
Daphnia magna[c]	0.010	0.029
Lepomis macrochirus[b]	0.018	0.092

[c] LC50 at 48-h. [b] LC50 at 96-h.
[a] Mayer and Ellersieck 1986. *D. pulex.
± N/C= not calculable because all died before 48-h.

Table 8-5. Measured concentrations of toxaphene and chlordane in replicate test vessels with and without sediments.

	Aqueous Concentration (mg/L)	
	No sediment	Sediment
Toxaphene	0	0
	340	0
	824	466
	910	331
	1009	625
	1829	559
Chlordane	0	0
	191	0
	350 ± 97.2	49.58
	754 ± 41.2	114.5 ± 109.8
	864 ± 45.2	0
	905.5 ± 75.7	205 ± 111.7

Effluent Toxicity Test

The Buckman Street Wastewater Treatment Facility effluent, known to contain several organic compounds including diazinon (Koopman et al. 1989, Dutton 1988), was less toxic to mussels than to either C. dubia or P. promelas (Table 8-6). The tested sample, which was not analyzed for specific chemical contents, contained a volatile organic compound based on its smell. Juvenile mussels were 4-5 times less sensitive than were the zooplankton, and even less sensitive than that compared to fathead minnows which died at the lowest effluent concentration (6%) in 24-h. Ninety-six hour LC50s for A. imbecilis and C. dubia were 35.35% and 7.08%, respectively, while at 7-d the LC50s had decreased to 16.24% for the mussels and 4.97% for C. dubia. Reproduction levels in the controls were too low to determine subchronic effects on C. dubia.

Discussion

In contrast to the results of toxicity tests with metal pollutants, A. imbecilis was found to be generally less sensitive to organic pollutants than are standard toxicity organisms such as D. magna, Ceriodaphnia dubia, the fathead minnow and bluegill sunfish. The reasons for the apparent tolerance of mussels to pesticides,

Table 8-6 . Comparative toxicity of an effluent from the Buckman Street Wastewater Treatment Facility, Jacksonville, Florida to A. imbecilis, C. dubia and Pimephales promelas.

	% Wastewater	
Organism	96-h LC50	7-d LC50
A. imbecilis	35.35	16.24
C. dubia	7.08	4.97
P. promelas	N/C*	N/C

*N/C=not calculable; all fathead minnows died in 24-h even in only 6% effluent.

herbicides and effluents, all having different chemical structures, characteristics and mechanisms is unknown.

Pesticides and herbicides are generally used to eliminate specific organisms. In this role, their efficacy on target organisms is maximized and in recent times, their impacts on non-target organisms is minimized. Evidently, the physiology of the mussel A. imbecilis is sufficiently different from that of targeted plants and animals that they are not susceptible to chemicals that interfere with various processes in targeted biota.

The only compound to which A. imbecilis showed sensitivity was PCP, a known molluscicide. It appears that the mode of action of this particular chemical is general enough that it can kill a broad spectrum of living organisms, including molluscs (Ware 1978).

It is particularly noteworthy that A. imbecilis was tolerant of extraordinarily high concentrations of the organochlorine pesticides lindane, toxaphene and chlordane. Organochlorines, in general, interfere with ion balance in the neuron via inhibition of Mg^{2+}-Ca^{2+} and Na^{+}-K^{+}ATPases. Lindane also perturbs cell division and causes proliferation of lysosomes (Ramade 1987). As a result, such compounds are highly toxic to birds, mammals, fish and zooplankton (Ramade 1987, Johnson and Finley 1980). It is interesting that both the mussels I

tested and the gastropod mollusc Lymnaea stagnalis were unaffected by lindane (Bluzat and Seuge 1979).

Acute exposures of mussels to high concentrations of sediment-sorbed toxaphene and chlordane were not lethal. This is of interest because while the use of both of these pesticides has recently been discontinued (C.F.R. v. 51 and v. 52), they have been applied extensively to various agricultural crops over the last 30 years. In fact, toxaphene was the most heavily used pesticide in the 1960s and 1970s, replacing DDT for many uses after 1971 (U.S.E.P.A. 1986b). As a result, they remain in the sediments to which they have an affinity. However, this does not appear to be a threat to mussels.

Since D. magna is currently used by EPA as a surrogate for mussels in deriving safety limits for pesticide use and that species was shown to be more sensitive to pollutants than was A. imbecilis, it appears that mussels are being adequately protected. However, it is impossible to determine from these data what the effects of chronic exposures to pesticides or other organic compounds might be. Further testing is necessary to determine whether long-term exposure of mussels to pesticides or other organic compounds is responsible for the loss of mussels from rivers and streams where they were once plentiful.

In recent years, there has been a move toward the use of microcosms and mesocosms to evaluate the impact of

pollutants on systems more complex than single-species exposure chambers (Cairns 1985, Giesy 1985, Taub 1973). Because multi-species systems better mimic natural ecosystems, they are useful in measuring some of the inter-related responses of species. In a limited sense, such an approach was used during several phases of this dissertation. The response of Ceriodaphnia dubia and Anodonta imbecilis to organic compounds and metals was evaluated simultaneously. Since fish eat zooplankton, and freshwater mussels use fish as hosts for their larvae, there is a unique species interdependence. A more complex exposure system permitting longterm studies with all three species would have provided more concrete answers to questions about the real impact of pollutants on the survival of mussels, fish and zooplankton. However, several scenarios can be imagined.

In cases where host fish species died because of their sensitivty to pesticides or metals, mussel larvae could not transform into free-living juveniles that eventually grow into adults. Over time, mussels would decline and disappear even though they were not directly eliminated by pollutants.

If zooplankton were more sensitive to toxic substances than were fish, there would be a reduced food resource for fish fry potentially reducing growth and survival of young fish. The density of individual fish species might be lowered leading to changes in

competition and predation interactions among fish. Since this might change the availability of host fish for mussel larvae, the impact of pollutants on zooplankton could also affect mussel survival.

Finally, some species of freshwater fish rely heavily on mollusks as food, e.g. redear sunfish. To the extent that fish consume mollusks, they may be negatively impacted by the loss of mussels from the food chain. In such cases, fish are indirectly affected by the pollutants that kill mussels.

Aquatic toxicology is beginning to pass from its infancy into a discipline that can be increasingly adept at assuming an ecosystem-level perspective. We have many techniques to measure the affects of toxicants on single species of organisms in the laboratory. The current move toward development of laboratory micro- and mesocosm test protocols will make controlled tests more representative of natural ecosystems.

CHAPTER 9
CONCLUSIONS

The overall purpose of this study was to evaluate the sensitivity of the freshwater mussel Anodonta imbecilis relative to that of the typical toxicity test organisms, e.g. Pimephales promelas, Ceriodaphnia dubia and Daphnia magna. While macroinvertebrate animals comprise a major component of the fauna of flowing waters few but the insects have been used as test organisms. Currently many unionid mussels are listed as endangered or threatened species. However, no method to assess the impact of various toxicants on the survival of unionid mussesl has been available.

In the process of determining whether fish or zooplankton are good indicators of mussel sensitivity to pollutants, several other goals were accomplished. These included: determining the toxicity of the aquatic herbicide Hydrothol-191 to the fathead minnow and Ceriodaphnia dubia relative to temperature at application, the simplification of in vitro culture techniques for Anodonta imbecilis, and the development of a test method to assess the acute toxicity of toxicants to Anodonta imbecilis.

The conclusions from this study were as follows:

1. Hydrothol-191 is more toxic to Pimephales promelas, the fathead minnow, than several other aquatic herbicides that may be as effective as Hydrothol-191 in controlling most undesirable macrophytes. At levels one-twentieth (0.081 mg/L) the concentration allowed for field application (1-5 mg/L), Hydrothol significantly decreased the growth of larval fathead minnows. With a half-life of 10 days, normal use of this herbicide could have a measurable impact on fish growth and development. The 48-h LC50 for Anodonta imbecilis was 4.85 mg/L.

The effect of water temperature on the toxicity of Hydrothol-191 to fathead minnows was determined to be significant. That is, at 15° C larval growth was impaired by Hydrothol concentrations below those that impaired growth at 25° C.

2. Hydrothol-191 was found to be highly toxic to Ceriodaphnia dubia based both on survival and reproductive impairment. Acute toxicity was measured at 0.490 mg/L, while survival was significantly lower after seven days in concentrations as low as 0.190 mg/L. Reproductive capacity of C. dubia was affected at Hydrothol concentrations of 15 ug/L. Water temperature was not as important a factor in determining the toxicity

of Hydrothol-191 to C. dubia as it was for the fathead minnow.

3. The in vitro culture of Anodonta imbecilis was considerably simplified by the substitution of horse serum for fish plasma, and commercially available culture media for the idiosyncratic medium used previously. Fish plasma is difficult to obtain except in cases where an aquaculture facility is located nearby, while horse serum can be purchased from several commercial sources. Substitution of horse serum also reduced the incidence of bacterial and fungal contamination of the glochidia cultures. Commercial culture media were also found to be adequate as nutrient sources for Anodonta imbecilis.

In vitro propagation of freshwater mussels is an advantage for those interested in replenishing excised populations of endangered species because it obviates the necessity of finding a suitable host fish species, many of which have not been identified. If a population of endangered mussels were located, they could be cultured in the laboratory to the juvenile stage and then returned to suitable natural habitats. Being able to culture mussels in vitro is also an advantage for those who are interested in using them as a toxicity test organism. Since metamorphosis of the glochidia can be followed under a microscope, tests can be scheduled for a time when adequate numbers of juveniles will be available.

4. A simple, inexpensive and fast toxicity test protocol was developed for A. imbecilis. The procedure includes many of the same materials and methods used in methodologies already in widespread use. Thus, others interested in using freshwater mussels as test organisms can do so with ease.

5. Juvenile mussels were found to be sensitive to metal pollution. In comparison to data for zooplankton from toxicity tests performed both in this study and in others, A. imbecilis was at least as sensitive as Ceriodaphnia dubia and Daphnia magna to Cr^{6+}, Cd^{2+}, Ni^{2+} and Cu^{+2} . Mussels were generally more sensitive to metal toxicants than were fish and Chironomus, another benthic invertebrate. A. imbecilis was about as sensitive to a wastewater effluent containing Cr^{6+} as was C. dubia, but less sensitive than were fathead minnow larvae.

6. Anodonta imbecilis was not as sensitive to eleven of twelve organic compounds and pesticides as were fish, Daphnia magna, and other common test species. Mussels were tolerant of very high concentrations of toxaphene and chlordane, as well as lindane and atrazine. Their 48-h LC50 for Hydrothol-191 was eight times higher than was the same value for Ceriodaphnia dubia.

Juvenile mussels were found to be as sensitive to PCP as were both fish and zooplankton. PCP is a potent fungicide and molluscicide that is toxic to most organisms.

In general, relative to widely used pelagic organisms, the freshwater mussel Anodonta imbecilis was found to be sensitive to metals but not so to organic compounds. While the species used is not endangered or threatened, it was chosen to represent the unionid mussels in toxicity tests because it has a broad distribution, can be propagated in the laboratory and has a relatively long reproductive period. There was a significant need to determine the sensitivity of mussels to metal and pesticide pollution. Further testing should be performed to expand the database on mussels. Based on this dissertation research, it appears that using D. magna as a surrogate for mussels in toxicity tests is acceptable for organic compounds. Since mussels appear to be more susceptible to metal pollution than are D. magna or C. dubia, zooplankton are not good substitutes for mussels in such tests. However, since fish, zooplankton and mollusks are inter-related, the loss of fish or zooplankton could also have a negative impact on the survival and recruitment of freshwater mussels. The decline in mussel density could also effect molluscivorous fish species. More research using an

ecosystem level approach would enhance our understanding of the real effects of pollutants on natural systems.

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BIOGRAPHICAL SKETCH

Anne E. Keller was born January 6, 1952, in Quantico, Virginia. During her first 17 years, she and her military family travelled throughout the United States and Europe. Once she entered Lake Forest College, Illinois, family trips were replaced by self-created adventures. After graduation from Lake Forest College, Anne attended the University of South Florida where she received a master's degree in zoology in 1976 and was certified to teach.

From 1977 to 1982, Anne taught high school biology and chemistry in several Florida schools. Then, she returned to graduate school to pursue a Ph.D. in environmental engineering sciences at the University of Florida. In 1984, she received her M.S. degree having performed research on the response of Florida freshwater fish to changes in lake acidity. She is currently completing her Ph.D. in the speciality area of environmental toxicology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas L. Crisman, Chairman
Professor of Environmental
Engineering Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gabriel Bitton
Professor of Environmental
Engineering Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Frank G. Nordlie
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen G. Zam
Associate Professor of
Microbiology and Cell
Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Clay L. Montague
Associate Professor of
Environmental Engineering
Sciences

This dissertation was submitted to the Graduate Faculty of the College of Engineering and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dean, College of Engineering

Dean, Graduate School