RICHARD J. NEVES

# Proposed Guide for Conducting Acute Toxicity Tests with the Early-Life Stages of Freshwater Mussels

Submitted to:

Ecological Effects Branch
Office of Pesticide Programs

## U.S. Environmental Protection Agency

Washington, DC

Submitted by:

KBN Engineering and Applied Sciences, Inc.
Gainesville, Florida

November 1990

EPA Contract Number 68-02 4278

# PROPOSED GUIDE FOR CONDUCTING ACUTE TOXICITY TESTS WITH THE EARLY LIFE STAGES OF FRESHWATER MUSSELS

Submitted to

Ecological Effects Branch Office of Pesticide Programs
U.S. Environmental Protection Agency
Washington, DC

Submitted by

Isabel C. Johnson, M.S.
KBN Engineering and Applied Sciences, Inc.
Gainesville, Florida

In Association with

Steven G. Zam, Ph.D. and Anne E. Keller, Ph.D.[1]
University of Florida, Gainesville, Florida

EPA Contract Number 68-02-4278
87018 4-EEB-08

November 1990

[1]Dr. Keller is now at Tennessee Valley Authority, Chattanooga, Tennessee.

## ACKNOWLEDGMENTS

KBN Engineering and Applied Sciences, Inc. (KBN), extends its appreciation to the staff of the University of Florida, Gainesville, Florida, for access to laboratory space and equipment. KBN also appreciates the technical support provided by Mr. Donald C. Wade and Dr. John J. Jenkinson, Tennessee Valley Authority, and Dr. Robert G. Hudson, Presbyterian College, Clinton, South Carolina. Appreciation is also extended to Dr. Fred Thompson, Florida Museum of Natural History, University of Florida, for assistance in the field collection of freshwater mussels in Florida and for all taxonomic verifications.

A special thanks is extended to Dr. Steven G. Zam, Microbiology Department, University of Florida, for the valuable assistance provided throughout this project. KBN also wishes to acknowledge Dr. Anne E. Keller's efforts and dedication throughout methods development and testing of this freshwater mussel. Appreciation is also extended to Mr. C. Steven Manning of Environmental Science and Engineering, Inc., for his assistance during pesticide testing.

The following chemical manufacturers provided the pesticides used during this research program: Rhone-Poulenc AG Company, ICI Americas, Inc., and Ciba-Geigy Corporation. This project was funded by the Office of Pesticide Programs (OPP), U.S. Environmental Protection Agency (EPA), Washington, DC (Contract Number 68-02-4278). Special thanks are extended to Mr. Henry Craven, EPA Project Officer, for his guidance and support throughout this effort, and to Dr. Arthur L. Buikema, Jr., on detail to EPA from Virginia Polytechnic Institute and State University, for his technical and editorial contributions.

## ABSTRACT

Testing techniques to evaluate the acute effects of pesticides to sensitive life stages of freshwater mussels have been developed for the Ecological Effects Branch (EEB), Office of Pesticide Programs (OPP), U.S. Environmental Protection Agency (EPA). The emphasis of this research effort was to develop techniques for testing pesticides using freshwater mussels and not to promote or endorse the mussel species used. The techniques developed include testing of the following early life stages: mature glochidia, recently transformed juveniles, and older juveniles.

Currently, organisms such as the freshwater cladoceran Daphnia magna and estuarine bivalves are being used to estimate the potential impact of pesticides to freshwater mussels. The availability of testing techniques to evaluate sensitive life stages of freshwater mussels will facilitate pesticide environmental impact assessments to this group of organisms, which includes numerous endangered and threatened species.

Anodonta imbecilis was the freshwater mussel selected for the development of these testing techniques because this species has been successfully cultured in vitro from the glochidial to the juvenile stage, precluding the fish host parasitic stage (Isom and Hudson, 1982). This mussel is abundant and widely distributed geographically; however, this species is not representative of all freshwater mussels. This species typically inhabits ponds and shallow impoundments but not flowing water systems.

A. imbecilis was amenable to acute testing methods development for the following reasons:

1. It is reproductively active throughout most of the year (Hudson, 1990, unpublished data);
2. It is easily collected in the field and maintained in the laboratory;
3. It adapts well to artificial conditions;
4. The glochidia have been successfully cultured in vitro, and high juvenile survival has been documented (Isom and Hudson, 1982; Hudson and Isom, 1984); and
5. The transformed juveniles do not require feeding or special conditions for short-term laboratory maintenance and testing.

The sensitivity of these young mussels was evaluated using three pesticides. The A. imbecilis acute toxicity data obtained through these exposures imply that the life stages tested are less sensitive to Cyhalothrin®, Carbaryl®, and Atrazine® than the standard freshwater species D. magna.

The techniques used are proposed as a guide for testing early life stages of freshwater mussels; the techniques were developed primarily for testing of pesticides but are applicable to a wide spectrum of chemicals. Additional testing using several other freshwater mussel species is needed in order to determine the sensitivity range of early life stages of this group of organisms. In order to predict potential impacts to endangered and threatened species, phylogenetically related surrogate mussel species should be used, if possible.

# TABLE OF CONTENTS
(Page 1 of 2)

TABLE OF CONTENTS
(Page 2 of 2)

# LIST OF TABLES

## LIST OF FIGURES

# 1.0 INTRODUCTION

Testing protocols have been promulgated for estuarine/marine species and are used routinely in the assessment of ecological impacts of pesticides and other chemicals. The protocols for mollusks are the 96-hour shell deposition test and the 48-hour embryo-larval acute toxicity test using primarily the eastern oyster (Crassostrea virginica).

The U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) uses the estuarine/marine mollusk protocols to support registration of end-use products intended for direct application to the estuarine or marine environment. Furthermore, these protocols are also used in the ecological risk assessment of pesticides expected to enter the aquatic environment in significant concentrations.

Standardized acute toxicity testing protocols for freshwater mollusk early life stages are not available at this time. Freshwater mussels represent an important ecological and commercial resource and, because of their widespread distribution, are representative of many freshwater systems. Overharvesting, as a result of demand for shells, contributed significantly to the decline of freshwater mussel resources in the United States. Contamination of aquatic systems also may have affected the propagation and survival of freshwater mussels. Furthermore, because of the mussels' parasitic stage during embryological development, their survival is also dependent upon the presence of an appropriate fish host. Currently, numerous mussel species are thought to be extinct, and others are listed as endangered or threatened (Hart and Fuller, 1974; Department of Interior, 1989).

Estuarine/marine mollusk data, as well as freshwater cladoceran data, have been used to estimate the potential effect of chemicals on freshwater mollusks. Methods to assess the effect of chemicals on potentially sensitive life stages of freshwater mussels would facilitate the accurate assessment of impacts on this important group of aquatic organisms.

Early life stage acute toxicity test methods were developed using the freshwater mussel Anodonta imbecilis. This species was chosen for the development of toxicity testing techniques for the following reasons:

1. It is reproductively active throughout most of the year (Hudson, 1990, unpublished data);
2. It is easily collected in the field and maintained in the laboratory;
3. It adapts well to artificial conditions;
4. The glochidia have been successfully cultured in vitro, and high juveni vival has been documented (Isom and Hudson, 1982; Hudson and Isom, 1984
5. The transformed juveniles do not require feeding or special condit or short-term laboratory maintenance and testing.

The current methods were based on research initiated and ongoing at the Tennessee Valley Authority (TVA) (Isom and Hudson, 1982; Hudson and Isom, 1984). The testing techniques described herein were developed specifically for testing of pesticides but are applicable to a large number of other chemicals and complex effluents. Mature glochidia and two age groups of juveniles were tested using acetone and three pesticides, Carbaryl®, Cyhalothrin®, and Atrazine®. Juveniles used in testing were reared in vitro from mature glochidia. Definitive 48-hour acute tests were conducted using A. imbecilis juveniles and Daphnia magna to determine the 48-hour median lethal concentrations (LC50). D. magna first instars (<24 hours old) were tested in order to compare directly the sensitivity of this widely used freshwater standard species to the freshwater mussel species used. Additionally, 24-hour mature glochidia tests were conducted using these three pesticides.

# 2.0 BACKGROUND

## 2.1 FRESHWATER MUSSELS

The Unionid mussels were among the first bivalves to colonize fresh water. The large number of genera in this order (110 genera) indicates the extent of freshwater bivalve adaptive radiation, as well as the importance of isolation and lack of competition in adaptive radiation (Purchon, 1977). Little is known regarding the sequence of events in the physiological adaptation of these bivalves that enabled them first to invade fresh water and eventually to adapt themselves wholly to this new environment.

One of the most significant physiological and ecological changes that freshwater bivalves have undergone relates to their reproduction strategy, which includes:

1. Modification of the embryological development, from free-living larvae (saltwater species) to parasitic glochidial larvae. There is only one known freshwater species that still produces the free- swimming veliger larvae, i.e., Dreissena polymorpha.
2. The incubation of eggs and young developmental stages.
3. Requirement of a fish host in order to complete embryological development (i.e., parasitic life stage).

In nature, sperm are released into the aquatic environment by a male mussel. The sperm are transferred into the female with water taken in during filter feeding. The fertilized eggs develop into simple glochidia, or veliger, in the gills of the female. The glochidia consist primarily of two shells, mantle cells, and one adductor muscle.

The glochidia of Unionidae, when liberated from the female, are attracted to certain species of freshwater fish and subsequently attach themselves to the skin as ecto-parasites. The glochidia then encyst in the fish tissues, where they complete their embryological development. During this parasitic period, the larval structures disappear and adult organs develop. When the glochidium has finalized its transformation process into a juvenile mussel (refer to Section 3.0, Figures 3-1 through 3-3), it breaks out of the cyst and drops to the bottom sediments and begins its benthic existence. The juveniles, like the adults, are filter feeders (Purchon, 1977).

## 2.2 RATIONALE FOR METHOD DEVELOPMENT

Several freshwater mussels of the family Unionidae were considered initially for testing, including two species of Anodonta (A. imbecilis and A. peggyae) and Elliptio icterina. A. imbecilis was selected for methods development due to the characteristics previously described. The intent of this study was to evaluate and develop techniques for acute toxicity testing of freshwater mussels and not to select or endorse a specific standard species. Consequently, the focus is not on A. imbecilis, but on the techniques used for testing mussels with similar reproductive strategies.

As previously discussed, freshwater mussels undergo a parasitic stage as part of their embryological development. Typically, a glochidium, once released by the female, must quickly find an appropriate host to encyst, otherwise it dies. If the glochidium successfully transforms into a free-living juvenile, it has the potential to grow and reproduce. Therefore, an assessment of the potential effect of contaminants to the early life stages of freshwater mussels would provide valuable information for the protection of this group of organisms. Thus, the period encompassing transformation and early life stages potentially critical to the survival of the species were selected for this study.

Prior to the development of testing techniques, an understanding of the growth and developmental patterns of the larvae was required. This included the ability to culture and maintain the organism under conditions amenable to pesticide testing. Survival of immature glochidia, mature glochidia, in vitro developing glochidia (parasitic stage), and juveniles was evaluated first. Excellent laboratory survival was found for these life stages, (refer to Appendix A, Table A-1). Immature glochidia were the only exception; if removed from the female and maintained in fresh water or incubated, they died. Therefore, testing of mature glochidia and juveniles was proposed for consideration. The process of organogenesis during parasitic development is a critical biological process that was not considered for testing. Although scientifically appealing due to the many ongoing physiological changes, it was not feasible to expose glochidia to pesticides during in vitro cultures due to the complexity of the culture medium and potential interactions with the pesticide. Furthermore, in nature, the transformation process typically occurs within the fish tissues, thus the embryo is not exposed to

ambient water concentrations. Effects of contaminants on encysted glochidia is being evaluated at Virginia Polytechnic Institute and State University, Blacksburg, Virginia (Jacobson et al., 1989).

In summary, test methods were developed for isolated mature glochidia, recently transformed juveniles, and older juveniles. Testing details, endpoints, and the test species are discussed in Section 3.0.

## 2.3 PREVIOUS WORK

### 2.3.1 ARTIFICIAL PROPAGATION OF FRESHWATER MUSSELS

Artificial propagation techniques for freshwater mussels were essential to the development of the proposed methods. Juveniles of a known age and taxonomic identity cannot be collected from field populations, therefore, laboratory rearing is a cornerstone for testing of newly transformed juveniles. Since visual determination of glochidia viability following exposure to chemicals is difficult and potentially unreliable, initiation of transformation of these glochidia facilitates accurate visual viability determinations.

Early efforts in artificial propagation of freshwater Unionid mussels were unsuccessful (LeFevre and Curtis, 1912). However, Ellis and Ellis (1926), using glochidia removed from fish gills after 18 to 96 hours of encystment, were able to culture the glochidia in an artificial medium through transformation to the juvenile stage. The composition of the medium was not described.

In an effort to replenish natural populations of threatened and endangered mussel species, TVA has supported research designed to improve laboratory culture techniques for freshwater mussels (Isom and Hudson, 1982; Isom and Hudson, 1984; Hudson and Isom, 1984; Isom, 1986). Isom and Hudson (1982) developed in vitro culture techniques for parasitic freshwater mussel glochidia. In their paper, they discuss the methods used to culture glochidia in the laboratory, precluding the fish parasitic stage. A growth medium consisting of Unionid Ringers solution, antibiotics and antimycotic agents, dextrose, amino acids, vitamins, fish blood plasma, and phenol red indicator was developed. Fish blood plasma was found to be necessary to stimulate development in all mussel species studied by Isom and Hudson (1982). The medium

preparation and glochidial incubation require sterile conditions. A temperature-controlled carbon dioxide ($CO_2$) incubator is used for the 9- to 11-day incubation period. An essential element in the successful culture of glochidia is the prevention of bacterial and fungal infestation during the incubation period. In vitro transformations in excess of 60 percent were reported by Isom (1986).

Juvenile rearing methods were discussed by Hudson and Isom (1984) and Isom (1986). Sterile conditions are not required for the successful culture of juveniles. Natural surface waters containing a diversity of phytoplankton for food were used for culture maintenance. Hudson and Isom (1984) found that juvenile cultures require the addition of organic and inorganic particulate silt for long-term laboratory survival.

### 2.3.2 TESTING OF GLOCHIDIA AND RECENTLY TRANSFORMED JUVENILES

Results of acute toxicity studies using glochidia and laboratory-reared juveniles have not been previously published, although some testing has been conducted. The following ongoing studies were presented at the Tenth Annual Meeting of the Society of Environmental Toxicology and Chemistry; manuscripts were not available for review.

Wade et al. (1989) conducted short-term laboratory studies to determine the toxicity of manganese present in the Tennessee River sediment to juvenile freshwater mussels (A. imbecilis) and to evaluate the potential for manganese inhibition on reservoir mussel recruitment during drought conditions. Following in vitro transformation of mussel glochidia into juveniles, toxicity tests were performed on 8-day-old juveniles. Additional toxicity screening tests of two aquatic herbicides [2,4-dichlorophenoxy acetic acid (2,4-D) and Aquathol K], a paper mill effluent, and a mosquito larvicide (BTI) were also conducted. Chronic toxicity tests using A. imbecilis to evaluate the effects of long-term exposure (90 days) to manganese have been completed recently at TVA. These data are not available for distribution (Hudson, 1990, personal communication). The effects of several metals and organic compounds to juvenile A. imbecilis have been evaluated by Keller and Crisman (1989, unpublished data).

Tests using bradytictic (winter breeders) freshwater mussels from southwestern Virginia have been conducted by Jacobson et al. (1989) to compare static copper exposures of encysted glochidia (from encystment through transformation) with static short-term (24- to 48-hour) exposures involving isolated glochidia and juvenile mussels. No impairment of transformation of encysted glochidia, as measured by juvenile recovery, was noted at concentrations up to 400 micrograms of copper per liter ($\mu$g Cu/L). Tests of isolated glochidia yielded LC50 values in the range of 50 to 100 $\mu$g Cu/L, compared with 100 percent mortality at 50 $\mu$g Cu/L for isolated juveniles of all species tested. The most sensitive stage was the juvenile mussel, whereas the most resistant stage was the encysted glochidia. These data were obtained through testing of more than 10 different mussel species. This study was sponsored by the American Electric Power Service Corporation, and data are not available for public distribution (Farris, 1990, personal communication).

# 3.0 MATERIALS AND METHODS

The primary objective of this research effort was to develop laboratory maintenance and testing techniques for early life stages of freshwater mussels. The materials and methods presented in this section were based on Isom and Hudson's numerous publications discussed in Section 2.3. Methods were changed as needed to facilitate the development of pesticide testing techniques.

Testing methods are described primarily for A. imbecilis, although the methods are applicable to other freshwater mussel species. Through the study of this readily available species, glochidia culture techniques were refined, or simplified, and prototype acute toxicity test methods were developed. For clarity, this section will be divided into two subsections: culture techniques required to obtain juvenile mussels and the acute toxicity test methods.

## 3.1 CULTURE AND MAINTENANCE TECHNIQUES

The following is a description of in vitro culture techniques for mature glochidia. The culture techniques discussed provided sufficient juveniles of a known mussel species and of the same age class for testing. Furthermore, these in vitro culture techniques provided a means to assess the viability of mature glochidia before following pesticide exposure. The species used, adult collection and maintenance procedures, glochidia procurement, culture medium preparation, and glochidia incubation procedures are presented below.

### 3.1.1 TEST ORGANISMS

Source--Freshwater mussel species are widely distributed and can be easily collected along stream and river banks and in the littoral zone of lakes. They can be transported to the laboratory in coolers without water or substrate. A. imbecilis, the species chosen for this project, was obtained from the Haleyville City Lake, Haleyville, Alabama, by Dr. Paul Yokley, University of North Alabama, Florence, Alabama. Dr. Fred Thompson, Florida Museum of Natural History, University of Florida, Gainesville, Florida, conducted all taxonomic verifications and maintains the project's reference collection.

A. imbecilis can be easily collected, shipped, and maintained in the laboratory. The glochidia culture techniques described in later sections were straightforward and successful. Basic

maintenance requirements for glochidia and juveniles were amenable to the requirements for pesticide testing. However, it is important to note that A. imbecilis is most commonly found in shallow ponds and is not representative of all freshwater mussels. This species was used to evaluate and develop the laboratory techniques described herein, not to promulgate its use as a standard species.

Elliptio icterina, A. peggyae, Lampsilis ovata, and Ligumia recta have been cultured successfully in vitro (Isom and Hudson, 1982; Isom and Hudson, 1984; Hudson and Isom, 1984; Isom, 1986). These and other species are good candidates for testing. In order to evaluate the potential impact of contaminants to endangered and threatened mussels, phylogenetically related surrogate species should be selected for future testing.

Adult Maintenance--Adult mussels should be kept moist during transport to the laboratory. In the laboratory, the adult mussels are maintained in aerated aquaria with water and sediment from their natural habitat. Alternatively, the mussels can be held in other suitable water and sediments. Gravid mussels with immature and mature glochidia can be maintained in the laboratory for up to 1 month without feeding; this does not appear to affect the viability of the glochidia. However, algal additions to the holding tanks enhance adult survival. The ability to easily maintain gravid females in the laboratory until their glochidia are mature (or hold females with mature glochidia) is a great advantage for laboratory testing.

Glochidia Collection--During their early embryological development, glochidia are carried in the gills of the female mussel. The ripeness or maturity of the glochidia can be deduced by the color of the female's gills. Gills containing mature glochidia are enlarged and brown in color, whereas enlarged beige or white gills may contain immature glochidia. Visual examination of the live female mussel to determine glochidial ripeness is done by pressing the two ends of the shells together (in A. imbecilis), thus gaping the shell open. If ripe, a sample of the glochidia may then be taken by inserting a sterile Pasteur pipette into the outer gill and suctioning the glochidia. The developmental maturity of the glochidia is determined through microscopic examination. Mature glochidia are free of embryonic membranes and can be seen sporadically opening and closing their shell valves.

Mature glochidia are collected by excising the gills of the female and manually shaking the gills in sterile distilled water. The glochidia may stick together forming strings (due to byssal fiber release); these aggregates can be broken by carefully aspirating them in and out of a sterile Pasteur pipette. The glochidia are then allowed to settle to the bottom of the container, and the supernatant is removed. Typically, mature glochidia will settle, and the immature ones will remain in the supernatant. This washing process should be repeated at least three times to remove debris and immature glochidia. The remaining mature glochidia can then be added to the freshly prepared culture medium or immediately used for testing.

### 3.1.2 CULTURE MEDIUM

The following is a detailed description of culture medium preparation. The key factor in the success of glochidial in vitro culture is the sterility of all components of the media.

Equipment and Materials--All reagents used in the preparation of the culture medium should be of tissue culture quality. This is necessary in order to eliminate contamination of the culture medium by heavy metals or other inhibitors of cell growth. Reagents used are available from Sigma Laboratories, Flow Laboratories (subsidiary of Flow General, McLean, Virginia), Gibco Laboratories (Grand Island, New York), and others.

Prior to use, all solutions should be sterilized either by autoclaving or by membrane filtration [0.22 micrometers ($\mu$m)]. To verify the sterility of each component, a small sample of each solution should be placed in a separate sterile test tube and allowed to incubate at room temperature for 24 to 48 hours. If no microbial or fungal growth is visible after the incubation period, the solutions can be considered sterile. This should be done before preparation of the final stock solution and each time a new solution is made.

Table 3-1 lists the reagents (salts) and amounts used in the preparation of Unionid Ringers solution. These salts are dissolved in distilled water and sterilized by autoclaving or filtering through a 0.22-$\mu$m filter apparatus.

Table 3-1. Reagents and Amounts Used in Unionid Ringers Solution Preparation

| Reagent | Concentration (mg/L) |
|---|---|
| Calcium chloride | 1,200 |
| $MgCl_2 \cdot 6H_20$ | 1,000 |
| Sodium chloride | 1,530 |
| Potassium chloride | 99 |
| Sodium bicarbonate | 2,200 |

Note: mg/L = milligrams per liter.

Table 3-2 lists the essential and nonessential amino acids required. These amino acids may be mixed from dry ingredients or purchased from Flow Laboratories or Gibco Laboratories Eagles Essential Amino Acids (50X) and Eagles Nonessential Amino Acids (100X).

The amino acids taurine and L-ornithine, listed in Table 3-2, are not constituents of either of the Eagles Amino Acids solutions and must be added separately. For simplicity, these amino acids may be added to the Nonessential Eagles Amino Acid solution. It is important that they be purchased in tissue culture quality and are available from Sigma. The vitamins listed in Table 3-2 are also required. Preparation of these vitamins in a composite 100X solution facilitates addition to the final stock medium.

Antibiotics and antimycotic agents required for culture maintenance are described in the following paragraphs.

Carbenicillin (Sigma)--Add 4 milliliters (mL) of sterile Unionid Ringers to a 1-gram (g) vial of carbenicillin to make Stock Solution A. As needed, add 0.2 mL of Stock A to 25 mL of sterile Unionid Ringers or distilled water. The diluted stock (Stock B) contains 2 milligrams per milliliter (mg/mL) carbenicillin. The allowable concentration of carbenicillin in the culture medium ranges from 0.1 mg/mL to 0.5 mg/mL; the concentration of 0.1 mg/mL was used in this study. This is obtained by adding 0.15 mL of Stock B to each 3 mL of complete (with plasma) culture medium.

Gentamicin Sulfate (Sigma)--Using a sterile 1-cubic-centimeter (cc) syringe and 22-gauge needle, remove 1 mL from the gentamicin sulfate stock vial (Stock A, purchased as a liquid with 40 mg/mL) and dilute to 40 mL with sterile Unionid Ringers. This diluted stock (Stock B) contains 1 mg/mL. Gentamicin sulfate has been used in the culture medium at a final concentration ranging from 0.1 mg/mL to 0.5 mg/mL (Isom and Hudson, 1982). The concentration of 0.05 mg/mL was successfully used during this study. This is achieved by adding 0.15 mL of Stock B to each 3 mL of complete (with plasma) medium.

Rifampin (Sigma)--Add 300 milligrams (mg) of powdered rifampin to 150 mL of sterile distilled water or Unionid Ringers. This stock (Stock A) contains 2 mg/mL. The range of rifampin

Table 3-2. Amino Acids and Vitamins Used in the Preparation of the Culture Medium

| Compound | Concentration (mg/L) |
|---|---|
| Essential Amino Acids | |
| L-arginine | 105 |
| L-cystine | 24 |
| L-histidine | 31 |
| L-isoleucine | 52 |
| L-leucine | 52 |
| L-lysine | 58 |
| L-methionine | 15 |
| L-phenylalanine | 32 |
| L-threonine | 48 |
| L-tryptophane | 10 |
| L-tyrosine | 36 |
| L-valine | 46 |
| Nonessential Amino Acids | |
| L-alanine | 8.9 |
| L-asparagine | 13.2 |
| L-aspartic acid | 13.3 |
| Glycine | 7.5 |
| L-glutamic acid | 14.7 |
| L-proline | 11.5 |
| L-serine | 10.5 |
| Other Amino Acids | |
| Taurine | 31.0 |
| L-ornithine | 10.0 |
| Vitamins | |
| Choline Chloride | 1.0 |
| Folic Acid | 1.0 |
| Inositol | 2.0 |
| Nicotinamide | 1.0 |
| Calcium Pantothenate | 1.0 |
| Pyridoxal | 1.0 |
| Riboflavin | 0.1 |
| Thiamine | 1.0 |

Note: mg/L = milligrams per liter.

concentrations used in culturing is 0.1 to 0.5 mg/mL. A final concentration of 0.1 mg/mL is recommended. This concentration is achieved by adding 0.15 mL of Stock A to each 3 mL of complete (with plasma) medium.

Amphotericin B (Sigma)--Add 10 mL of sterile distilled water or Unionid Ringers to a 50-mg vial of powdered Amphotericin B (Stock A, 5 mg/mL). Dilute Stock A 10 times by putting 1 mL into 9 mL Ringers. This diluted solution (Stock B) contains 0.5 mg/mL. Amphotericin B is more toxic to the glochidia than are the antibiotics. The acceptable final concentration in the complete culture medium (with plasma) is 0.005 to 0.025 mg/mL. The use of 0.005 mg/mL is recommended. This is equivalent to adding 0.03 mL of Stock B to each 3 mL of complete culture medium.

Other culture medium components are:

| Compound | Concentration |
|---|---|
| Dextrose | 20 percent weight-to-volume |
| Phenol red | 1 percent weight-to-volume |

Procurement and Preparation of Blood Plasma--Fish blood can be obtained from a number of sources, including aquaculture facilities, fish processing plants, fish farms, or other places where large numbers of fish are readily available on a continuous basis.

If the fish are large enough, blood may be obtained by severing the tail and catching the blood in a heparinized container. An alternative method is to collect the blood by cardiac puncture using a heparinized syringe and 18-gauge needle. Once the blood is collected in heparinized containers, it should be transported on ice to the laboratory and centrifuged immediately. Separation of the blood into cellular and noncellular components is accomplished by centrifugation at 1,000 revolutions per minute (rpm) (Sorvall centrifuge, SS34 head) for 10 minutes, followed by 10 minutes at 5,000 rpm. The plasma should be drawn off with a pipette and frozen at -30 degrees Celsius (°C) until needed. Fish blood is approximately 50 percent plasma.

When the culture medium is being prepared, the thawed plasma should be centrifuged at 5,000 to 7,000 rpm for 10 minutes. This process allows removal of the coagulated proteins, which may cause the medium to gel during incubation.

Culture Medium Preparation--A basic stock solution containing all reagents (except fish plasma and antibiotic/antimycotic agents) is prepared by combining the separate components in the proportions presented in Table 3-3. This is done in a laminar flow hood to maintain sterility.

The stock solution may be acidic (yellow) and should be checked. Titration with a dilute sodium hydroxide (NaOH) solution (1 to 2 N) to the required pH of 7.2 to 7.4 will turn the solution to a bright red. If the component solutions are made and combined under sterile conditions, it is not necessary to resterilize the final basic stock. The stock can be kept for several months refrigerated at 4°C.

Two additional components must be added to the basic stock to complete the culture medium, the antibiotic/antimycotic agents and fish plasma. This final medium should be prepared on an as-needed basis. The appropriate volumes of antibiotics/antimycotics needed were presented previously and depend on the volume of final stock being prepared.

The thawed fish plasma should be added to attain a 33 percent volume-to-volume concentration in the final culture medium. Since plasma is viscous, it is virtually impossible to filter sterilize when undiluted (even with larger-pore 0.45-$\mu$m filters). Therefore, the plasma and the basic stock should be mixed and the final culture medium should then be filter sterilized using a 0.45-$\mu$m filter. This is done to decrease microbial contamination caused by the nonsterile plasma. The use of a pre-filter is recommended. Once the final culture medium has been sterilized, it can be kept under refrigeration for 1 month or more, as long as sterility is maintained.

### 3.1.3 INCUBATION PROCEDURE

Three milliliters of culture medium (at 23 to 26°C) are added to each sterile disposable 60- by 15-millimeter (mm) Petri dish in a laminar flow hood. The use of the laminar flow hood is essential to maintain culture sterility. One milliliter of concentrated, rinsed glochidia (100 or

Table 3-3. Culture Medium Basic Stock Solution (Excluding Fish Plasma and Antibiotic/Antimycotic Agents)

| Compound | Volume (mL) |
|---|---|
| Dextrose | 5 |
| Unionid Ringers | 960 |
| Essential Eagles Amino Acids (50X) | 20 |
| Nonessential Eagles Amino Acids (100X)[a] | 10 |
| Vitamins (100X) | 10 |
| Phenol Red | 1 |

Note: mL = milliliters.

[a]Containing taurine and L-ornithine supplements.

more) is placed in each Petri dish containing culture medium. Each culture dish is then marked with the date, test species, and other pertinent information.

Culture dishes are placed in a $CO_2$ incubator (set at 5 percent $CO_2$) and maintained at 24 to 26°C. A. imbecilis glochidia require 9 to 11 days to transform to juveniles under these environmental conditions. Daily examination of the cultures is suggested in order to follow the developmental process and to detect bacterial/fungal contamination, if present. Early detection of infection allows for glochidial transfer to new medium, precluding loss of the culture.

Transformation Process Description--The transformation (also referred to as metamorphosis) of parasitic glochidia to free-living juveniles is a slow developmental process. Mature glochidia recently removed from the female's gills will open and close their valves sporadically. These sporadic contractions stop upon introduction of the glochidia to the culture medium. Once in the medium, the mussel valves close and remain closed for the duration of the incubation period. If a glochidium does not close its valves within the first 24 hours of incubation, it does not continue its embryological development and dies.

Mature glochidia appear transparent under the microscope using transmitted light. An opaque area in the center of the glochidium is prominent. This area is the adductor muscle. Figure 3-1 is a photograph of a live mature glochidium. The process of organogenesis can be monitored daily by removing the culture dishes containing glochidia and placing the unopened dishes under a dissecting microscope. Within 2 to 3 days of incubation, the outlines of various organs and internal movements are easily observed. As organogenesis continues, the glochidia become more and more opaque, although hemolymph can be observed circulating through the future gill area. On days 9 to 11 of the incubation period, movement of the foot within the valves is initiated and easily observed.

Completion of the transformation process to the juvenile stage can be evaluated by removing a few young from the culture medium (under sterile conditions) using a sterile pipette and placing them in water. If the animals have completed the transformation process, mantle cells are extruded from the juveniles. In approximately an hour, the valves open and the foot is

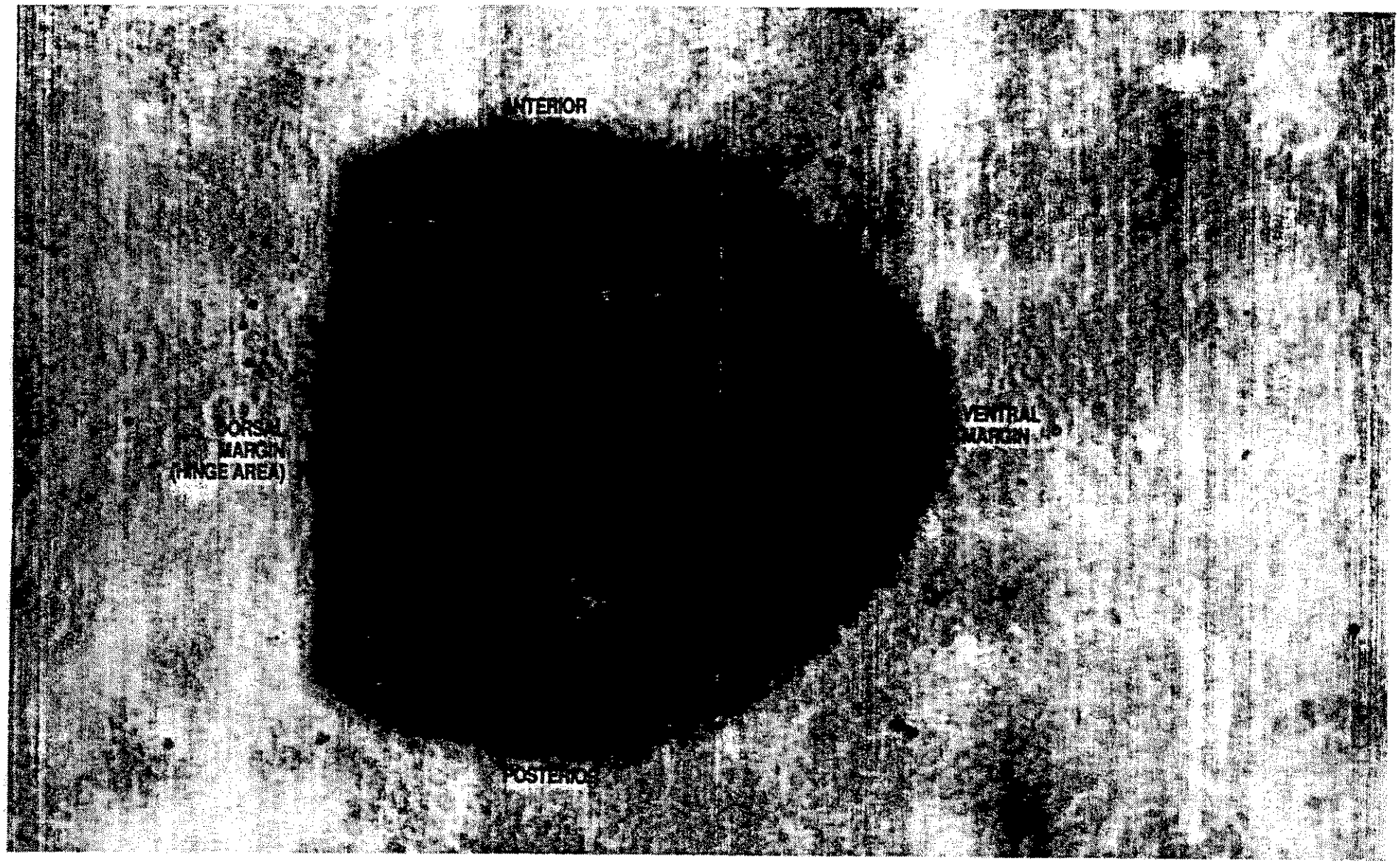


Figure 3-1 *ANODONTA IMBECILIS* — EXTERIOR VIEW OF LIVE MATURE GLOCHIDIUM

seen extending out of the mussel perimeter (Figure 3-2). If the glochidia do not exhibit these behavioral characteristics, the cultures tested should be placed back in the incubator and examined the next day in the same manner. It is important to discard any glochidia removed from the culture so the sterile, untransformed organisms are not contaminated. Transformed juveniles can be used in toxicity tests immediately or maintained in the laboratory for a few days prior to testing.

## 3.2 TEST METHODS

Acute toxicity test methods were developed for mature glochidia and laboratory-reared juveniles. Standard EPA and American Society for Testing and Materials (ASTM) methods were followed in the development of the present guide. The test compounds used will be discussed first, followed by the juvenile mussel methods, and finally the mature glochidia methods. A general discussion of statistical analyses used is presented, followed by the description of standard parallel tests conducted using D. magna.

### 3.2.1 TEST MATERIALS

Pesticide-grade acetone and three pesticides were used for testing the sensitivity of the test organisms. The pesticides were provided by the chemical manufacturers listed in Table 3-4.

The testing methods were adjusted according to the properties of the test compounds. Carbaryl® and Cyhalothrin® are known to be unstable in aqueous solutions; therefore, they were tested under 24-hour static renewal conditions and the test concentrations were measured, whenever possible. Due to the stability of Atrazine®, testing for this compound was conducted under static conditions and the concentrations were not analytically verified. The Carbaryl® concentration verifications were conducted by Hunter Environmental Services, Inc. (now Environmental Science and Engineering, Inc.), Gainesville, Florida, and the Cyhalothrin® analyses were conducted by ICI Americas, Inc., Bracknell, England.

### 3.2.2 TEST CONDITIONS

Test conditions were selected based on ASTM guidelines for acute toxicity tests of pure compounds (ASTM, 1980). The test conditions evaluated as part of the methods development

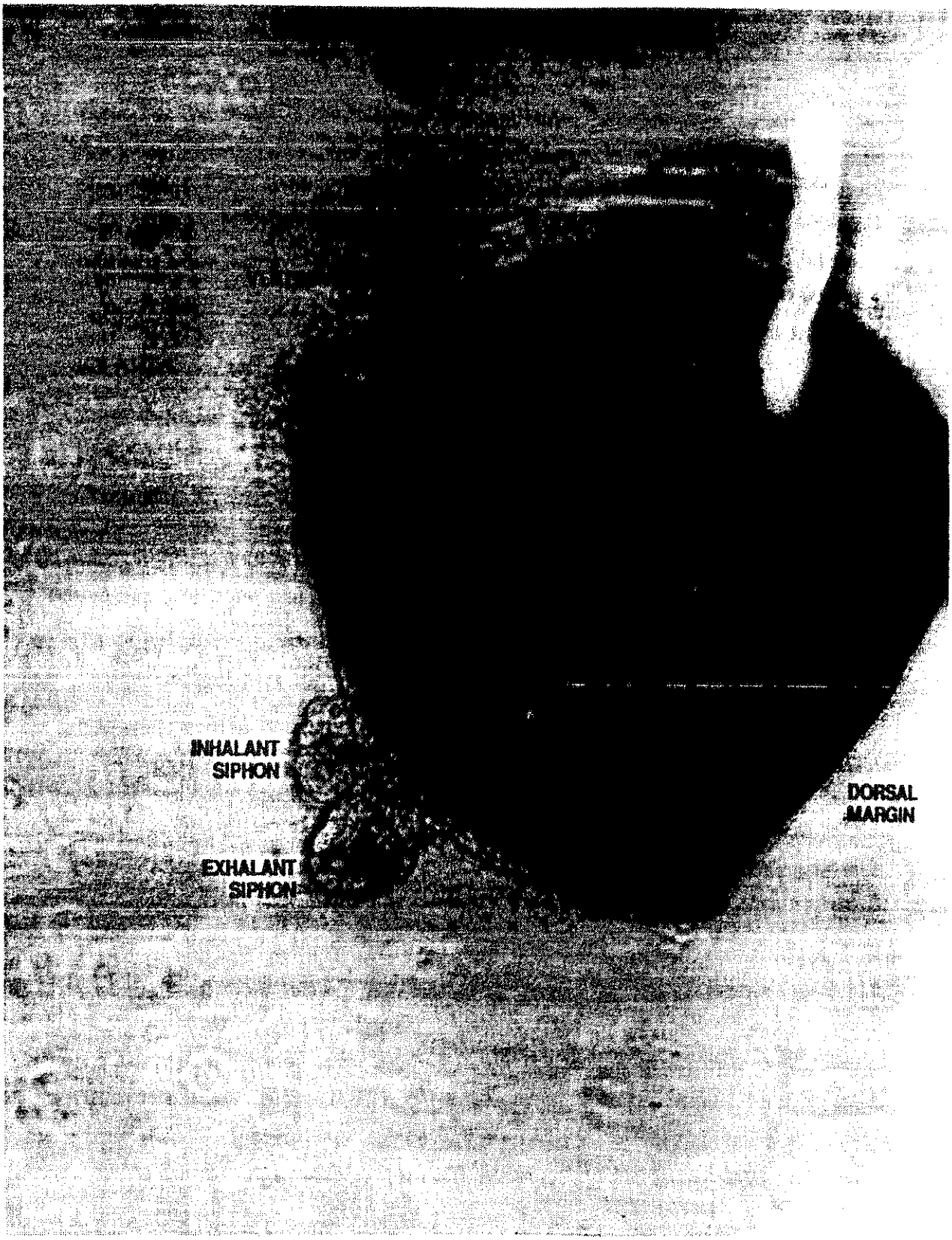


Figure 3-2 *ANODONTA IMBECILIS* — EXTERIOR VIEW OF LIVE POST-TRANSFORMATION JUVENILE

KBN

Table 3-4. Pesticides Used for Acute Toxicity Tests

| Manufacturer | Compound | Percent Active Ingredient |
|---|---|---|
| Rhone-Poulenc AG Co.<br>Triangle Park<br>North Carolina | Sevin Brand | 99 percent technical Carbaryl® Research Reference Number NA 2757 |
| ICI Americans, Inc.<br>Goldsboro<br>North Carolina | Cyhalothrin®<br>Technical | 99 percent pure PP321, ICIA0321 (Cyhalothrin®)<br>Reference Number ASJ/123 |
| Ciba-Geigy Corp.<br>Greensboro<br>North Carolina | Atrazine®<br>Technical | 97.3 percent pure Atrazine®<br>Batch Number FL-850942 |

were acceptability of soft reconstituted water for survival, feeding regime (no food during testing), and test endpoints for glochidial testing.

Survival of juveniles was evaluated under standard ASTM laboratory conditions (ASTM 1980) without food and using soft reconstituted water. Survivability of juveniles under these conditions was monitored for 8 days; post-transformation (<24 hours old) and older juveniles (7-day old) were tested. As shown in Table A-1 (Appendix A), excellent survival (≥90 percent) was observed after 8 days of maintenance for both juvenile age groups. Thus, it was concluded that a 48-hour exposure under such conditions would be acceptable.

The development of testing techniques using mature glochidia was also explored. Based on observations during the glochidia culture process, an accurate visual determination of glochidial viability was established. Following removal from the female's gills, viable mature glochidia typically remained open, closing and opening only sporadically. When these glochidia were placed in the culture medium, viable individuals would close the shell valves permanently and initiate the transformation process. Individuals not closing within the first 24 hours did not develop further (transform) and died. Consequently, a test was developed where the mature glochidia were exposed to the test material for 24 hours and then incubated in culture medium for an additional 24 hours. Following the incubation period, individuals with closed valves were considered alive at the end of the 24-hour test material exposure. Individuals with open valves were considered not viable. This is a quantifiable endpoint.

### 3.2.3 JUVENILE MUSSEL METHODS

Table 3-5 summarizes the conditions used for testing A. imbecilis juveniles. As shown, similar conditions were used for the reference species, D. magna.

In nature, transformed juveniles drop from the host fish to the bottom of the stream or pond and begin their benthic existence. Thus, they are potentially exposed to environmental contaminants throughout their free-living lifespan. Testing young juveniles 1 to 2 days old

Table 3-5. Summary of Recommended Test Conditions for the Freshwater Mussel A. imbecilis Juveniles and the Cladoceran D. magna Acute Toxicity Tests

| Parameter | Condition |
|---|---|
| Test Type | Static or static-renewal |
| Temperature | 20 ±1°C |
| Light Quality | Ambient laboratory light |
| Photoperiod | 16 hours of light, 8 hours of darkness |
| Test Chamber Size | 125 mL |
| Test Solution Volume | 100 mL |
| Renewal of Test Concentration<br>None for static test | Daily for renewal test |
| Age of Test Organisms | Mussels--juveniles 1 to 10 days old<br><br>Cladoceran--first instar<br>(<24 hours) |
| Number of Animals Per Chamber | 10 |
| Replicates Per Concentration | 2 |
| Animals Per Concentration | 20 |
| Feeding Regime | None for mussels; cladocerans fed at 24 hours |
| Aeration | None |
| Dilution Water | Reconstituted soft fresh water |
| Dilution Factor | 60 percent |
| Test Duration | 48 hours |
| Endpoints | Survival, behavior |

Note: mL = milliliters.

(Figure 3-2) and slightly older juveniles 7 to 10 days old (Figure 3-3) may provide a good indication of their sensitivity to environmental contaminants. Unlike the adults, young juveniles cannot close the shell valves for long periods of time; therefore, preventing direct exposure to chemicals in the water column is impossible. Taking into account these life history observations, the following test procedures were developed. They can be considered a guide for pesticide and other pure compound testing using this and other freshwater mussel species.

Test Organisms--Freshwater mussels used were laboratory-reared from mature glochidia (refer to Section 3.1). Following transformation, the juveniles were placed in soft, fresh reconstituted water with a hardness of 40 to 50 milligrams per liter (mg/L) as calcium carbonate ($CaCO_3$). Juveniles should be tested at ages ranging from 1 day old up to 10 days old; older juveniles may require feeding and their nutritional requirements have not been fully investigated. During holding, the juvenile mussels were fed an algal suspension of Chlorella at approximate concentrations ranging from 2 to 5 x $10^5$ cells per 100 mL of holding water. The juveniles were not fed during testing. Juveniles should be maintained at 20 $\pm$1°C, using fresh water identical to the dilution water used in the acute tests. Active juveniles were selected for testing and were randomly distributed to counting chambers. Due to their small size, all counts were conducted under a dissecting microscope.

Test System--Acute toxicity tests were conducted under static or renewal conditions. The tests conducted during this research program were performed in a temperature-controlled chamber, and the temperature was monitored continuously using a Rustrack® recorder calibrated at test initiation. The photoperiod was 16 hours of light and 8 hours of darkness. Aeration was not required for any of the tests. Test chambers were 125-mL crystallizing dishes with 100 mL of test solution.

Test Design--Range-finding tests were conducted using four or five pesticide concentrations. Based on the results of the range-finding tests, typically six pesticide concentrations and controls (dilution water only) were used for the definitive tests. A solvent control (dilution water containing the highest level of acetone used) was included as required by the solubility of the pesticide. All concentrations were duplicated, and 10 juveniles were tested per test chamber. Juveniles were not fed during testing, as recommended by ASTM methods. Test temperature

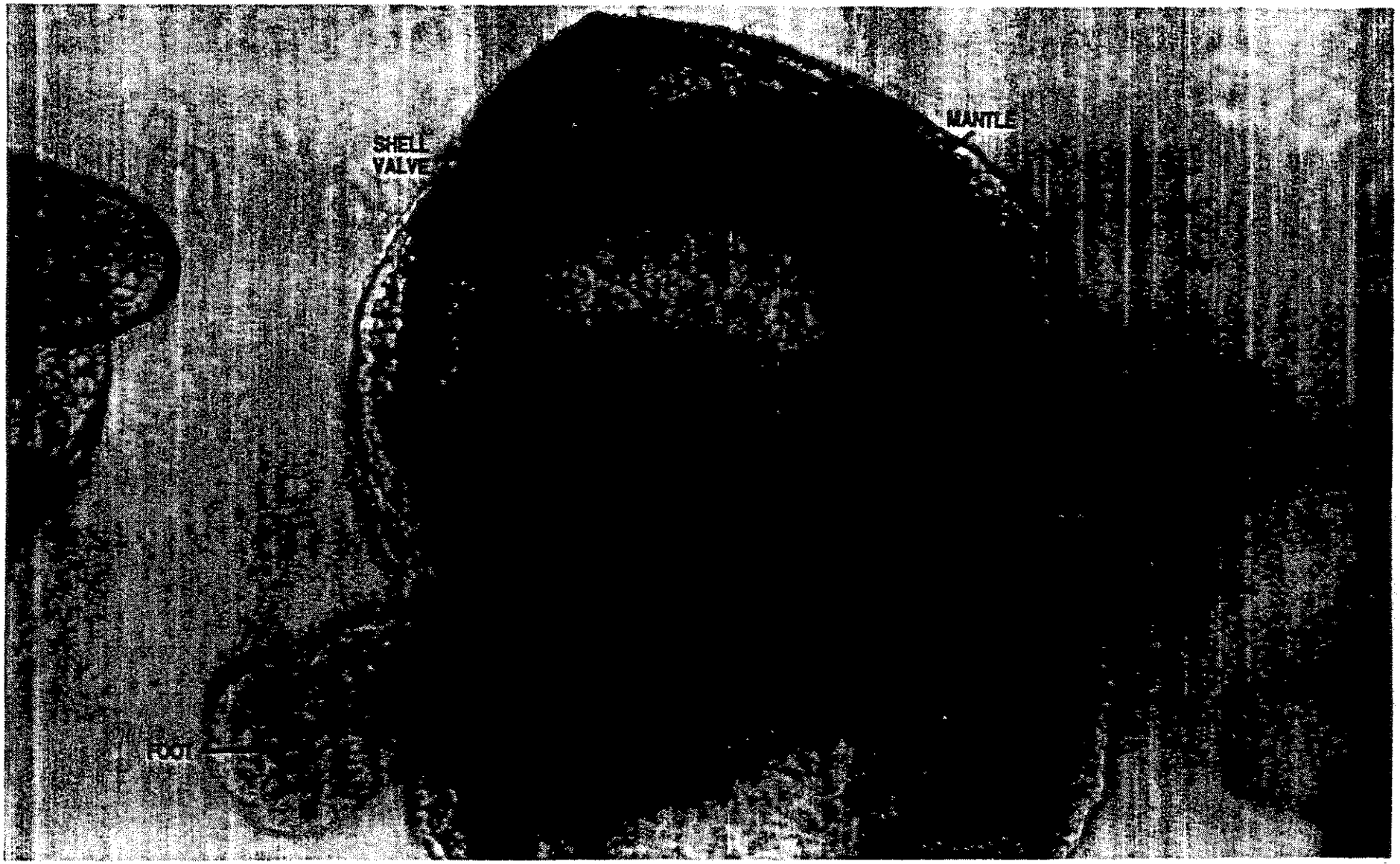


Figure 3-3 *ANODONTA IMBECILIS*— INTERIOR VIEW OF LIVE JUVENILE (7 TO 10 DAYS OLD)

KBN

was 20 ±1°C. Test chambers were covered using clear wrap to prevent evaporation. Definitive tests were conducted for 48 hours.

Because of their small size, juveniles were placed into the test chambers using pipettes, while being observed under a microscope. The number of juveniles present in each test chamber was checked before test initiation. Tests were monitored daily for survival, pH, and dissolved oxygen. Test temperature was monitored continuously. Dilution water hardness was measured at test initiation.

Tests were conducted using two juvenile age groups, 1 to 2 days old and 7 to 10 days old. This was done in order to determine whether juvenile sensitivity changed drastically with the age of the test organisms. This was considered important because older juveniles are more active; thus, viability can be more easily determined in the 7- to 10-day-old juveniles than in 1- to 2-day-old ones.

Endpoints--Survival is the easiest endpoint to monitor. Active juveniles in good physical condition are observed with their shell valves open and mantles extended (see Figure 3-3); under closer examination, internal movements are also seen through the transparent mussel shells. These behaviors are apparent for all juveniles, but they are more easily observed in older juveniles (7 to 10 days old). If prodded, a live mussel responds by partially contracting the mantle; rarely will the shell valves close. Signs of stress or near death include partially clogged mantles, and little response to prodding. Signs of death include lack of movement and closed shells; if shells are open, the mantle is typically clogged with debris.

### 3.2.4 GLOCHIDIAL METHODS

Table 3-6 summarizes the general conditions recommended for testing mature glochidia. The rationale for the glochidia test is slightly different from the juvenile protocols. As discussed in an earlier section, mature glochidia are released into the aquatic environment and, if successful, find a fish host. During embryological development (transformation process), the glochidia are not exposed to contaminants in the aqueous medium because they are encysted in the fish tissues. Thus, glochidia potentially can be exposed to aquatic contaminants while in the gills of the female, as well as during the short period following release from the female. A test was

Table 3-6. Summary of Recommended Test Conditions for Freshwater Mussel (A. imbecilis) Glochidia Acute Toxicity Tests

| Parameter | Condition[a] |
|---|---|
| Test Type | Static |
| Temperature | 20 ±1°C |
| Light Quality | Ambient laboratory light |
| Photoperiod | 16 hours of light, 8 hours of darkness |
| Test Chamber Size | 100 mL |
| Test Solution Volume | 50 mL |
| Renewal of Test Concentration | None |
| Lifestage of Test Organisms | Mature glochidia recently removed from female's gills |
| Number of Animals Per Chamber | 10 |
| Replicates Per Concentration | 2 |
| Animals Per Concentration | 20 |
| Feeding Regime | None |
| Aeration | None |
| Dilution Water | Reconstituted soft fresh water |
| Dilution Factor | 60 percent |
| Test Duration | 24 hours in exposure chamber, followed by 24 hours in culture medium (Test 1) or 9 to 11 days in culture medium (Test 2) |
| Endpoints | Test 1: Survival (ability to close shell valves in culture medium after 24 hours of incubation)<br>Test 2: Successful transformation (ability to complete embryological development) |

Note: mL = milliliters.

[a]During pesticide exposure, not during incubation.

designed to encompass the interval between release from the female's gills and successful encystment on the host. Laboratory observations show that the glochidia are active during the first few hours following removal from the female. By 24-hours after removal, they are less active and appear lethargic. In nature, following release from the female to the aquatic environment, the glochidia are expected to remain in the water column for a limited time before settling on the bottom sediments. Thus, the glochidia are more likely to find an appropriate host within a few hours after release from the female and while suspended in the water column. Thus, a toxicity test method was developed to simulate a short environmental exposure (24 hours) of mature glochidia to water column contaminants.

In order to evaluate the effects of pesticides on mature glochidia, a reliable endpoint is required. When mature glochidia are removed from the female and placed in fresh water, they are very active and sporadically open and close their shell valves. Thus, it is easy to determine their viability. However, after several hours in water, glochidia become less active, making survival determinations difficult. Therefore, visual survival determinations following their exposure to pesticides can be unreliable.

To obtain reliable measures of acute toxicity to mature glochidia, the following endpoint determination is proposed. Following the 24-hour pesticide exposure, all glochidia should be transferred to the culture medium and incubated for 24 hours. If viable, the shell valves close within 24 hours of incubation; if affected, they remain open and die. In this manner, viable young can be easily enumerated.

This short-term test (Test 1 in Table 3-6) was conducted as part of this project. A longer term method (Test 2 in Table 3-6) also can be conducted. During Test 2, the exposed glochidia are incubated (beyond the initial 24 hours) until transformation, and successful transformation in the treatments, as compared to controls, is the effect (endpoint) measured.

Test Organisms--Test organisms were obtained from gravid females as discussed in Section 3.1. The glochidia were maintained in soft, fresh reconstituted water (40 to 50 mg/L as $CaCO_3$ hardness) until testing. Mature glochidia were placed into small counting watchglasses. The

byssal fibers in some cases were still attached to the glochidia, and clumping occurred, making their transfer difficult. Therefore, all counts were conducted under a dissecting microscope.

Test System--Acute toxicity tests using mature glochidia were conducted under static conditions. The tests conducted during this research program were performed in a temperature-controlled chamber. Temperature was monitored continuously using a Rustrack recorder calibrated at test initiation. The photoperiod was 16 hours of light and 8 hours of darkness. Aeration was not required for any of the tests. The initial 24-hour pesticide exposure was conducted in 100-mL crystallizing dishes with 50 mL of test solution, follwed by a 24-hour incubation period was conducted in Petri dishes containing culture medium. During incubation, the Petri dishes were maintained in a $CO_2$ incubator at the appropriate culture temperature.

Test Design--Acute toxicity exposures were conducted under static conditions for 24 hours (simulating a theoretical short exposure to the aqueous environment). Following this exposure, the glochidia were transferred to the culture medium and incubated for an additional 24 hours. After 24 hours of incubation in the medium, the number of live glochidia (those with closed valves) was counted in each test concentration. If a glochidium did not close its valves upon incubation in the culture medium, it was considered dead.

This incubation period (following pesticide exposure) can be extended, and exposed glochidia can be allowed to continue development, undisturbed, until transformation. At the time that glochidia in the control group are observed transforming, the number of transformed juveniles should be counted in all test concentrations. The number transformed should then be statistically compared among treatments and controls. This represents Test 2 exposure and endpoints in Table 3-6. Test 2 exposures were not conducted as part of this project.

### 3.2.5 STATISTICAL ANALYSES

EPA's TOXANAL computerized program was used to estimate all LC50 values and their 95-percent confidence limits. The program includes the Binomial Method, the Moving Averages Method, and the Probit Method. This program or other similar analyses are recommended for the calculation of LC50 values. TOXANAL can be conducted using Abbott's correction to account for control mortality.

### 3.2.6 STANDARD REFERENCE SPECIES

Forty-eight-hour acute tests were also conducted using the standard cladoceran species D. magna. These tests were conducted according to 1980 standard ASTM guidelines and EPA's Standard Evaluation Procedures (EPA, 1985). A major deviation from ASTM was that the D. magna were fed at the end of 24 hours. These methods are summarized in Table 3-5.

### 3.2.7 SOLVENT TEST

Based on the anticipated low water solubility of some pesticides, the acute toxicity of acetone to juvenile mussels was quantified (Table A-2, Appendix A). These tests followed the conditions outlined in Table 3-5.

## 3.3 PESTICIDE TEST PREPARATIONS

### 3.3.1 CARBARYL®

Carbaryl® was tested under static-renewal conditions. A saturated Carbaryl® solution was prepared by mixing overnight 100 mg of Carbaryl® with 1 liter (L) of dilution water (Carbaryl® solubility in water is 40 mg/L at 30°C). The supernatant was filtered before the test concentrations were prepared. Tests were conducted for 48 hours and were renewed with freshly prepared pesticide concentrations after 24 hours of exposure. The only exception was the glochidial test. In this case, after 24 hours of pesticide exposure, the glochidia were transferred to the culture medium and incubated for 24 hours. Carbaryl® concentrations were measured for the D. magna test, but not for the freshwater mussel tests.

### 3.3.2 CYHALOTHRIN®

Cyhalothrin® was tested under static-renewal conditions. Acetone was the solvent used. Each test was conducted for 48 hours and was renewed with freshly prepared test solutions after 24 hours of exposure. The only exception was the glochidial test. In this case, at 24 hours, the glochidia were transferred to the culture medium and incubated for 24 hours. Samples were taken for chemical verification from all tests. This was done because of the insolubility and instability of Cyhalothrin® in water. The solubility of Cyhalothrin® in water is 5.0 $\mu$g/L (at 20°C). Limited preliminary concentration verification data are available from ICI Americas, Inc., but are not available for public distribution. Therefore, nominal concentrations are reported. It is important to note that the concentrations tested are several orders of magnitude

higher than the reported solubility of the compound. The measured concentrations (once available in final form) may not reflect the concentrations of Cyhalothrin® in solution. This is because the sampling method for chemical analyses efficiently traps both dissolved and particulate Cyhalothrin® in suspension. Thus, because of the high Cyhalothrin® concentrations tested (above solubility), the measured concentrations (when available) will reflect both dissolved and suspended particulate matter.

### 3.3.3 ATRAZINE®

Atrazine® was tested under static conditions. A saturated Atrazine® solution was prepared by mixing overnight 100 mg of Atrazine® with 1 L of dilution water (Atrazine® solubility in water is 33 mg/L). The supernatant was filtered before the test concentrations were prepared. Each test was conducted for 48 hours. The only exception was the glochidial test. In this case, at 24 hours, the glochidia were transferred to the culture medium and incubated for 24 hours. Test concentrations were not measured because of the apparent stability of Atrazine® under the conditions tested.

# 4.0 TOXICITY TEST RESULTS

Toxicity tests using juvenile mussels and D. magna were conducted for 48 hours; mature glochidial exposures were conducted for 24 hours. Test conditions are presented in Section 3.0. The endpoint for all tests was death, thus all values presented are LC50s. In the following subsections, the toxicity data are presented by chemical. The pesticides tested were selected by OPP personnel; the pesticides were provided by the chemical manufacturers. A minimum of six pesticide concentrations was tested per chemical. Results of acetone acute toxicity tests (96-hour exposures) using juvenile mussels are also presented.

## 4.1 CARBARYL®

### 4.1.1 REFERENCE SPECIES DATA

Table B-1 of Appendix B presents the results of the D. magna acute test. Control survival was 95 percent. The 48-hour LC50 based on nominal concentrations is 5.4 mg/L Carbaryl® (3.7 and 8.8 mg/L, 95-percent confidence limits, slope 1.67). The 48-hour LC50 based on the initial 24-hour mean measured concentrations is 1.9 mg/L Carbaryl® (1.3 and 2.9 mg/L, 95-percent confidence limits, slope 1.76). These values were calculated without the lowest test concentration value, which was considered an anomaly. The Probit Method using Abbott's correction was used for LC50 calculations.

Test concentrations were chemically measured at test initiation, at 24 hours (old solutions), at 24 hours (fresh solutions), and at 48 hours (old solutions). Because of logistical problems at the analytical laboratory, the 48-hour final samples were lost. Therefore, average measured concentrations were calculated for the first 24 hours by averaging the test concentrations measured at test initiation and the 24-hour-old test solutions. This provides a good estimate of the approximate Carbaryl® concentrations to which the daphnids were exposed during the 48-hour exposure. The LC50 estimate, based on measured concentrations, is approximately 35 percent of the LC50 calculated based on nominal additions. This is expected, based on the solubility of Carbaryl® in water (40 mg/L at 30°C) and the serial dilutions prepared from a nominal 100 mg/L saturated solution. Table B-2 presents the Carbaryl® measured concentrations.

### 4.1.2 FRESHWATER MUSSEL DATA

Table B-3 summarizes the juvenile mussel data. Control survival was 95 percent or higher during all tests. Two juvenile age groups were tested; post-transformation (1 to 2 days old) and older juveniles (7 to 10 days old). The 48-hour LC50 values for post-transformation juveniles and older juveniles were 23.7 mg/L Carbaryl® (20.8 and 26.9 mg/L, 95-percent confidence limits, slope 8.91) and 25.6 mg/L Carbaryl® (22.1 and 29.5 mg/L, 95-percent confidence limits, slope 6.93) based on nominal concentrations, respectively. As shown, both age groups displayed similar sensitivities to Carbaryl®.

Table B-4 presents the data from the mature glochidial test. Based on the number alive after the 24-hour exposure to Carbaryl® and 24 hours of incubation, the 24-hour LC50 estimate is 30.1 mg/L Carbaryl® (23.1 and 47.7 mg/L, 95-percent confidence limits, slope 2.9) based on nominal concentrations. All glochidia exposed to Carbaryl® appeared alive at the end of the 24-hour exposure period. The number shown at the initiation of the incubation period is the actual number of mussels transferred into the culture medium. As shown in Table B-4, because of a transfer technique error, not all animals were transferred. This was not discovered until test completion. The LC50 value for this test was calculated using the actual number of mussels incubated.

### 4.1.3 SUMMARY

Table 4-1 summarizes all Carbaryl® acute toxicity data. All LC50 values reported were calculated using the Probit Method, including Abbott's correction as needed. The freshwater mussel life stages tested appear to be less sensitive than the daphnids to Carbaryl®. The most sensitive mussel life stage appears to be the post-transformation juveniles with a 48-hour LC50 of 23.7 mg/L (nominal), compared to the daphnid 48-hour LC50 of 5.4 mg/L (nominal). The typically cited 48-hour effective concentration (EC50) for D. magna is 5.6 $\mu$g/L; this value is based on immobilization (not death) using nominal concentrations. These raw data were obtained from the Fish and Wildlife Service's National Fisheries Contaminant Research Center. These data show that this test was conducted at 60°F (15.6°C) and that alcohol was used as the solvent. Furthermore, although the data sheet states "number dead", Buckler (1989) believes that when this test was conducted (1969) "it was common practice with invertebrates to not differentiate between dead and immobile organisms." KBN's data showed that all daphnids were immobilized (but not dead) in all Carbaryl® concentrations tested ($\geq$443 $\mu$g/L).

Table 4-1. Calculated LC50 Values for A. imbecilis and First Instar D. magna Exposed to Carbaryl®

| Test Species | Age | 48-Hour LC50 (mg/L) | 95-Percent Confidence Limits (mg/L) |
|---|---|---|---|
| D. magna[a] | | | |
| First Instar | <24 hours old | 5.4 | 3.7 to 8.8 |
| | | 1.9[a] | 1.3 to 2.9[a] |
| A. imbecilis | | | |
| Juveniles | 1 to 2 days old | 23.7 | 20.8 to 26.9 |
| | 7 to 10 days old | 25.7 | 22.1 to 29.5 |
| Mature Glochidia | <24 hours old | 30.1[b] | 23.1 to 47.7 |

Note: The lowest test concentration in the D. magna test series was not included in the analysis; it was considered an anomaly.

All estimated LC50 values were calculated using the Probit Method including Abbott's correction as needed.

mg/L = milligrams per liter.

[a]Average measured concentrations (first 24 hours), all others are nominal concentrations.
[b]24-hour LC50.

## 4.2 CYHALOTHRIN® (PP321)

### 4.2.1 REFERENCE SPECIES DATA

Table B-5 presents the results of the D. magna acute test. The 48-hour LC50 based on nominal concentrations is 27 µg/L Cyhalothrin® (23.1 and 34.4 µg/L, 95-percent confidence limits). These values were estimated using the Moving Average Method including Abbott's correction. The LC50 based on measured concentrations is expected to be lower (the solubility of Cyhalothrin® in water is 5µg/L).

### 4.2.2 FRESHWATER MUSSEL DATA

Table B-6 summarizes the juvenile mussel data. Control survival was 100 percent. The 48-hour LC50 for post-transformation juveniles and older juveniles was >1 mg/L Cyhalothrin® (the highest nominal concentration tested). Both age groups are relatively insensitive to Cyhalothrin®.

Table B-7 presents the data from the mature glochidia test. Based on the number alive after the 24-hour exposure to Cyhalothrin® and the 24-hour incubation period, the 24-hour LC50 estimate is also >1 mg/L Cyhalothrin® (nominal concentration).

### 4.2.3 SUMMARY

All freshwater mussel life stages tested are less sensitive than daphnids to Cyhalothrin®. Table 4-2 summarizes the Cyhalothrin® LC50 data. The unpublished 48-hour EC50 for D. magna, based on measured concentrations, is 0.36 µg/L Cyhalothrin (Farrelly et al., 1984).

## 4.3 ATRAZINE®

### 4.3.1 REFERENCE SPECIES DATA

Table B-8 presents the results of the D. magna acute test. The 48-hour LC50 based on nominal concentrations is 9.4 mg/L Atrazine® (3.9 and 20.5 mg/L, 95-percent confidence limits, slope 2.4). These values were calculated using the Probit Method.

Table 4-2. Calculated LC50 Values for A. imbecilis and First Instar D. magna Exposed to Cyhalothrin® Based on Nominal Concentrations

| Test Species | Age | 48-Hour LC50 | 95-Percent Confidence Limits (µg/L) |
|---|---|---|---|
| D. magna | | | |
| First Instar | <24 hours old | 27 µg/L | 23.1 to 34.4 |
| A. imbecilis | | | |
| Juveniles | 1 to 2 days old | >1 mg/L | NA |
| | 7 to 10 days old | >1 mg/L | NA |
| Mature | <24 hours old | >1 mg/L[a] | NA |
| Glochidia | | | |

Note: NA = not applicable.
mg/L = milligrams per liter.
µg/L = micrograms per liter.

[a]24-hour LC50.

### 4.3.2 FRESHWATER MUSSEL DATA

Table B-9 summarizes the juvenile mussel data. Control survival was 95 percent or higher. The 48-hour LC50 for post-transformation juveniles and older juveniles was >60 mg/L Atrazine® (the highest concentration tested), based on nominal concentrations. Both age groups are relatively insensitive to Atrazine®. Table B-10 presents the data from the mature glochidial test. Based on the number alive after the 24-hour exposure to Atrazine® and the 24-hour incubation period, the 24-hour LC50 estimate is also >60 mg/L Atrazine®.

### 4.3.3 SUMMARY

All freshwater mussel life stages tested are less sensitive than daphnids to Atrazine®. Table 4-3 summarizes the Atrazine® LC50 data. The published D. magna 48-hour EC50, based on nominal concentrations, is 6.9 mg/L Atrazine (Macek et al., 1976).

## 4.4 ACETONE

Acetone was tested under static conditions using freshwater mussel juveniles during a 96-hour exposure. Five test concentrations were used. Table A-2 summarizes the survival data. Control survival at test conclusion was 95 percent. The 48-hour LC50s for post-transformation juveniles and older juveniles were 50.0 mg/L acetone (42.5 and 60.4 mg/L, 95-percent confidence limits) and 69.4 mg/L acetone (61.1 and 81.3 mg/L, 95-percent confidence limits), respectively. The 96-hour LC50 remained unchanged for the post-transformation juveniles, but decreased to 66.1 mg/L (57.7 and 77.8 mg/L, 95-percent confidence limits) for the older juveniles. These estimates were calculated using the Moving Averages Method.

Table 4-3. Calculated LC50 Values for A. imbecilis and First Instar D. magna Exposed to Atrazine® Based on Nominal Concentrations

| Test Species | Age | 48-Hour LC50 (mg/L) | 95-Per[illegible]t Confidence [illegible]mits (mg/L) |
|---|---|---|---|
| D. magna[a] First Instar | <24 hours old | 9.4 | 3.9 to 20.5 |
| A. imbecilis Juveniles | 1 to 2 days old | >60 | NA |
| | 7 to 10 days old | >60 | NA |
| Mature Glochidia | <24 hours old | >60[a] | NA |

Note: NA = not applicable.
mg/L = milligrams per liter.

[a]24-hour LC50.

## 5.0 DISCUSSION

Techniques were developed for pesticide acute toxicity tests using freshwater mussel early life stages. Two major objectives were achieved:

1. Using artificial mussel propagation techniques (in vitro cultures), sufficient juveniles of a known age and taxonomic origin were obtained; and
2. Test methods and endpoints appropriate for the species were derived.

Juvenile freshwater mussels of a known age and taxonomic group cannot easily be collected from the field. Although they can be obtained from an encysted (infected) fish, the mussel taxonomic identification is not known unless the fish is parasitized under laboratory conditions. Therefore, in vitro culture of mature glochidia facilitates the procurement of large numbers of individuals of a known age and species. Juvenile culture methods were not developed through this effort, but the juveniles were simply maintained in the laboratory for testing. Scientists at TVA currently are developing juvenile culture procedures, but their work has not been published. Hudson and Isom (1984) stated that the juveniles require the addition of silt and plankton for long-term survival. The studies conducted as part of this project show that, for acute testing of early life stages, the addition of silt and food is not required.

Standard ASTM (1980) test conditions were used for testing these early life stages of A. imbecilis and were shown to be appropriate. An endpoint for testing of mature glochidia was defined to determine whether the test organism was alive following pesticide exposure. Visual determination of glochidial viability is possible following removal from the female. The 24-hour in vitro incubation of glochidia, following test material exposure, provides a simple and quick visual determination of viability; if the glochidia are closed following 24 hours of incubation, they are considered alive; if open, they are considered not viable.

The mature glochidia test presented can be extended beyond the 24-hour incubation period described herein. Following the 24-hour exposure to the test material, the glochidia are incubated in the culture medium until transformation is observed in the controls. In this case, successful transformation of glochidia is the test endpoint. At test termination, the number of

transformed juveniles in the treatments (exposed glochidia) statistically can be compared to the number transformed in the controls. This longer test would determine not only glochidial survival, but also the ability to complete the transformation process following test mater exposure.

The juvenile and isolated glochidial methods proposed were evaluated by conducting acute tests using three pesticides: Carbaryl®, Cyhalothrin®, and Atrazine®. Table 5-1 summarizes the 48-hour LC50 values obtained from the pesticide tests using juvenile A. imbecilis and first instar D. magna, and Table 5-2 summarizes the glochidial test results. As shown in these tables, all early life stages of A. imbecilis were less sensitive than the cladoceran D. magna to the pesticides used. Atrazine® and Cyhalothrin® produced no acute LC50 toxicity values to A. imbecilis under the conditions tested. For comparative purposes, oyster test data are also included on these tables (Tables 5-1 and 5-2). Currently, the oyster (Crassostrea spp.) is the primary mollusk test species required to support registration of end-use products intended for direct application to the estuarine and marine environment. Comparison of these oyster data to this study's data is inconclusive because all the available oyster values are reported as greater-than numbers.

It is important to note that, although this work was done exclusively on A. imbecilis, it is not the intention to promulgate the use of this particular species. This organism was used based on the background information available and ease in handling, but A. imbecilis is not a representative freshwater mussel.

The techniques described are straightforward and should be easily duplicated. It is recommended that the pesticides used in this project be tested again using other freshwater mussel species in order to initiate the description of the sensitivity range of this important group of organisms. It is critical that freshwater mussel species similar to endangered or threatened species be included in testing as surrogates. Only after this is done can conclusions be drawn regarding the comparative sensitivity of freshwater mussel early life stages to other groups of organisms.

Table 5-1. Calculated Acute Toxicity Values for A. imbecilis Juveniles, First Instar D. magna and Two Oyster Species Exposed to Carbaryl®, Cyhalothrin®, and Atrazine®

| Test Species | Age | LC50/EC50 | | |
|---|---|---|---|---|
| | | Carbaryl® | Cyhalothrin® | Atrazine® |
| A. imbecilis Juveniles | 1 to 2 days | 23.7 mg/L | >1 mg/L | >60 mg/L |
| | 7 to 10 days | 25.6 mg/L | >1 mg/L | >60 mg/L |
| D. magna First Instar | <24 hours | 5.4 mg/L<br>1.9 mg/L[a] | 27 µg/L | 9.4 mg/L |
| Oyster[b] | | >2.0 mg/L[c] | >0.59 mg/L[a,d] | >1.0 mg/L[c] |

Note: mg/L = milligrams per liter.
µg/L = micrograms per liter.

[a]Measured concentration, all others are based on nominal concentrations.

[b]Crassostrea virginica was used for Carbaryl® and Atrazine® tests, and Crassostrea gigas was used for the Cyhalothrin® test.

[c]These values are 96-hour EC50s based on oyster shell deposition tests conducted using juvenile oysters (Mayer 1987).

[d]This value is a 48-hour LC50 based on an embryo-larval test using fertilized eggs (Hill 1985).

Table 5-2. Calculated Acute Toxicity Values for A. imbecilis Mature Glochidia and Two Oyster Species Exposed to Carbaryl®, Cyhalothrin®, and Atrazine®

| Test Species | Age | LC50/EC50 | | |
|---|---|---|---|---|
| | | Carbaryl® | Cyhalothrin® | Atrazine® |
| A. imbecilis | Mature Glochidia | 30.1[a] | >1[a] | >60[a] |
| Eastern Oyster | Juvenile | >2.0[b] | – | >1.0[b] |
| Pacific Oyster | Embryo | – | >0.59[c] | – |

Note: All calculations were based on nominal concentrations, except oyster LC50 using Cyhalothrin®.

mg/L = milligrams per liter.

[a]24-hour LC50 values.

[b]96-hour EC50 values, shell deposition test using C. virginica (Mayer 1987).

[c]48-hour LC50 value, enbryo-larval test (Hill 1985).

# APPENDIX A

# BACKGROUND INFORMATION

Table A-1. Survival of Anodonta imbecilis Juveniles Maintained Under Standard Laboratory Conditions

| Age at Initiation | Replicate | Survival | | |
|---|---|---|---|---|
| | | Day 0 | Day 4 | Day 8 |
| Post-Transformation (<24 hours old) | A | 10 | 10 | 10 |
| | B | 10 | 9 | 9 |
| | C | 10 | 10 | 10 |
| | D | 10 | 10 | 10 |
| Juveniles (7 days old) | A | 10 | 10 | 9 |
| | B | 10 | 10 | 9 |
| | C | 10 | 10 | 10 |
| | D | 10 | 9[a] | 8 |

Note: Standard conditions--Soft reconstituted water, no feeding, 16 hours of light, 8 hours of darkness, regime temperature 20 ±1°C, static system.

[a]One crushed unintentionally.

Table A-2. Survival of Anodonta imbecilis Juveniles Exposed for 96 Hours to Acetone Under Static Conditions

| Nominal Test Concentration (mg/L) | Number Alive | | | | | |
|---|---|---|---|---|---|---|
| | Juveniles (<24 hours old) | | | Juveniles (7 days old) | | |
| | 0 Hour | 48 Hours | 96 Hours | 0 Hour | 48 Hours | 96 Hours |
| Control | 20 | 19 | 19 | 20 | 19 | 19 |
| 10 | 20 | 20 | 20 | 20 | 20 | 20 |
| 18 | 20 | 19 | 19 | 20 | 19 | 19 |
| 32 | 20 | 20 | 20 | 20 | 19 | 18 |
| 56 | 20 | 5 | 5 | 20 | 19 | 18 |
| 100 | 20 | 0 | 0 | 20 | 0 | 0 |

# APPENDIX B

## RAW ACUTE TOXICITY DATA

Table B-1. Survival of Daphnia magna Exposed for 48 Hours to Carbaryl® Under Static-Renewal Conditions

| Test Concentrations (mg/L) | | Number Alive | | |
|---|---|---|---|---|
| Nominal | Average Measured[a] | 0 Hour | 24 Hours | 48 Hours |
| Control | NM | 20 | 20 | 19 |
| 1.0 | 0.443 | 20 | 17[b] | 9[b] |
| 1.7 | 0.637 | 20 | 18[b] | 14[b] |
| 2.9 | 0.985 | 20 | 19[b] | 12[b] |
| 4.8 | 1.540 | 20 | 16[b] | 13[b] |
| 8.0 | 2.561 | 20 | 15 | 10 |
| 13.0 | 5.285 | 20 | 19 | 2 |

Note: The 48-hour survival value for the lowest test concentration is considered an outlier and was not included in the LC50 calculations.

NM = not measured.

[a]Average measured concentration for the initial 24 hours of exposure.
[b]Test organisms alive but immobilized.

Table B-2. Measured Carbaryl® Concentrations

| Nominal Concentration (mg/L) | Measured Concentrations (mg/L) | | | Average of 0 and 24-Hour (old) (mg/L) |
|---|---|---|---|---|
| | 0 Hour | 24-Hour (old) | 24-Hour (new) | |
| 1.0 | 0.77 | 0.119 | 0.609 | 0.443 |
| 1.7 | 1.18 | 0.094 | 1.020 | 0.637 |
| 2.9 | 1.90 | 0.070 | 1.400 | 0.985 |
| 4.8 | 3.00 | 0.080 | 2.220 | 1.540 |
| 8.0 | 4.88 | 0.242 | 4.190 | 2.561 |
| 13.0 | 9.00 | 1.570 | 7.790 | 5.285 |

Table B-3. Survival of Anodonta imbecilis Juveniles Exposed for 48 Hours to Carbaryl® Under Static-Renewal Conditions

| Nominal Test Concentration (mg/L) | Number Alive | | | | | |
|---|---|---|---|---|---|---|
| | Juveniles (1 to 2 days old) | | | Juveniles (7 to 10 days old) | | |
| | 0 Hour | 24 Hours | 48 Hours | 0 Hour | 24 Hours | 48 Hours |
| Control | 20 | 20 | 20 | 20 | 20 | 19 |
| 8.0 | 20 | 20 | 20 | 20 | 20 | 20 |
| 13.0 | 20 | 19 | 19 | 20 | 20 | 20 |
| 22.0 | 20 | 19 | 15 | 20 | 19 | 12 |
| 36.0 | 20 | 3 | 0 | 20 | 16 | 4 |
| 60.0 | 20 | 0 | 0 | 20 | 7 | 0 |
| 100.0 | 20 | 0 | 0 | 20 | 0 | 0 |

Table B-4. Survival of Anodonta imbecilis Mature Glochidia Exposed for 24 Hours to Carbaryl® Under Static Conditions, Followed by a 24-Hour Incubation Period

| Nominal Test Concentration (mg/L) | Number Alive[a] | | | |
|---|---|---|---|---|
| | Exposure Period | | Incubation Period | |
| | 0 Hour | 24 Hours | 0 Hour[b] | 24 Hours |
| Control | 20 | 20 | 15 | 13 |
| 8.0 | 20 | 20 | 18 | 17 |
| 13.0 | 20 | 20 | 20 | 18 |
| 22.0 | 20 | 20 | 19 | 11 |
| 36.0 | 20 | 20 | 17 | 7 |
| 60.0 | 20 | 20 | 15 | 1 |
| 100.0 | 20 | 20 | 16 | 0 |

[a]Survival is defined as: ability to open and close shell valves after exposure period and ability to remain closed during incubation.
[b]The number shown at 0 hour is the number of mussels transferred to the medium, not the number surviving the 24-hour Carbaryl® exposure.

Table B-5. Survival of Daphnia magna Exposed for 48 Hours to Cyhalothrin® Under Static-Renewal Conditions

| Nominal Test Concentration (µg/L) | Number Alive | | |
|---|---|---|---|
| | 0 Hour | 24 Hours | 48 Hours |
| Control | 20 | 19 | 19 |
| Solvent Control | 20 | 20 | 19 |
| 2.5 | 20 | 19 | 14 (2[a]) |
| 4.1 | 20 | 20 | 18 (10[a]) |
| 6.9 | 20 | 19 | 14 (14[a]) |
| 11.5 | 20 | 18 | 15 (15[a]) |
| 19.2 | 20 | 17 | 16 (10[a]) |
| 32.0 | 20 | 19 | 6 (6[a]) |

[a]Number alive but immobilized.

Table B-6. Survival of Anodonta imbecilis Juveniles Exposed for 48 Hours to Cyhalothrin® Under Static-Renewal Conditions

| Nominal Test Concentration (mg/L) | Number Alive | | | | | |
|---|---|---|---|---|---|---|
| | Juveniles (1 to 2 days old) | | | Juveniles (7 tp 10 days old) | | |
| | 0 Hour | 24 Hours | 48 Hours | 0 Hour | 24 Hours | 48 Hours |
| Control | 20 | 20 | 20 | 20 | 20 | 20 |
| Solvent Control | 20 | 20 | 20 | 20 | 20 | 20 |
| 0.08 | 20 | 20 | 19 | 20 | 20 | 20 |
| 0.13 | 20 | 20 | 18 | 20 | 20 | 20 |
| 0.22 | 20 | 20 | 20 | 20 | 20 | 20 |
| 0.36 | 20 | 19 | 17 | 20 | 20 | 20 |
| 0.60 | 20 | 19 | 19 | 20 | 20 | 20 |
| 1.00 | 20 | 19 | 19 | 20 | 20 | 20 |

Table B-7. Survival of Anodonta imbecilis Mature Glochidia Exposed for 24 Hours to Cyhalothrin® Under Static Conditions, Followed by a 24-Hour Incubation Period

| Nominal Test Concentration (mg/L) | Number Alive[a] | | | |
|---|---|---|---|---|
| | Exposure Period | | Incubation Period | |
| | 0 Hour | 24 Hours | 0 Hour[b] | 24 Hours |
| Control | 20 | 19 | 17 | 15 |
| Solvent Control | 20 | 20 | 20 | 20 |
| 0.08 | 20 | 20 | 18 | 18 |
| 0.13 | 20 | 20 | 20 | 20 |
| 0.22 | 20 | 17 | 14 | 14 |
| 0.36 | 20 | 20 | 19 | 19 |
| 0.60 | 20 | 20 | 20 | 15 |
| 1.00 | 20 | 20 | 19 | 15 |

[a]Survival is defined as: ability to open and close shell valves after exposure period and ability to remain closed during incubation.
[b]The number shown at 0 hour is the number of mussels transferred to the medium, not the number surviving the 24-hour Cyhalothrin® exposure.

Table B-8. Survival of Daphnia magna Exposed for 48 Hours to Atrazine® Under Static Conditions

| Nominal Test Concentration (mg/L) | Number Alive | | |
|---|---|---|---|
| | 0 Hour | 24 Hours | 48 Hours |
| Control | 20 | 20 | 20 |
| 2.9 | 20 | 20 (6[a]) | 16 (6[a]) |
| 4.8 | 20 | 17 (5[a]) | 14 (13[a]) |
| 8.0 | 20 | 18 (4[a]) | 17 (17[a]) |
| 13.0 | 20 | 15 (15[a]) | 7 (5[a]) |
| 22.0 | 20 | 11 (9[a]) | 4 (3[a]) |
| 36.0 | 20 | 6 (6[a]) | 0 |

[a]Number alive but immobilized.

Table B-9. Survival of Anodonta imbecilis Juveniles Exposed for 48 Hours to Atrazine® Under Static Conditions

| Nominal Test Concentration (mg/L) | Number Alive | | | | | |
|---|---|---|---|---|---|---|
| | Juveniles (1 to 2 days old) | | | Juveniles (7 to 10 days old) | | |
| | 0 Hour | 24 Hours | 48 Hours | 0 Hour | 24 Hours | 48 Hours |
| Control | 20 | 19 | 19 | 20 | 20 | 20 |
| 2.9 | 20 | 20 | 20 | 20 | 20 | 20 |
| 4.8 | 20 | 20 | 19 | 20 | 20 | 20 |
| 8.0 | 20 | 20 | 20 | 20 | 20 | 20 |
| 13.0 | 20 | 20 | 20 | 20 | 20 | 20 |
| 22.0 | 20 | 20 | 19 | 20 | 19 | 19 |
| 36.0 | 20 | 19 | 19 | 20 | 20 | 20 |
| 60.0 | 20 | 19 | 18 | 20 | 20 | 20 |

Table B-10. Survival of Anodonta imbecilis Mature Glochidia Exposed for 24 Hours to Atrazine® Under Static Conditions, Followed by a 24-Hour Incubation Period

| Nominal Test Concentration (mg/L) | Number Alive[a] | | | |
|---|---|---|---|---|
| | Exposure Period | | Incubation Period | |
| | 0 Hour | 24 Hours | 0 Hour[b] | 24 Hours |
| Control | 20 | 20 | 19 | 19 |
| 2.9 | 20 | 20 | 19 | 19 |
| 4.8 | 20 | 20 | 20 | 20 |
| 8.0 | 20 | 20 | 19 | 18 |
| 13.0 | 20 | 20 | 18 | 18 |
| 22.0 | 20 | 20 | 19 | 19 |
| 36.0 | 20 | 20 | 18 | 16 |
| 60.0 | 20 | 20 | 15 | 15 |

[a]Survival is defined as: ability to open and close shell valves after exposure period and ability to remain closed during incubation.
[b]The number shown at 0 hour is the number of mussels transferred to the medium, not the number surviving the 24-hour Atrazine® exposure.

## 6.0 REFERENCES
(Page 1 of 2)

American Society for Testing and Materials (ASTM). 1980. Standard Practice for Conducting Acute Toxicity Tests With Fishes, Macroinvertebrates, and Amphibians. E729-80. ASTM Committee on Standards, Philadelphia, PA.

Buckler, D.R. 1989. Personal Communication. United States Department of Interior, Fish and Wildlife Service, National Fisheries Contaminant Research Center, Columbia, Missouri.

Ellis, M.M. and M.D. Ellis. 1926. Growth and Transformation of Parasitic Glochidia in Physiological Nutrient Stock. Science 54:579-580.

Farris, J.L. 1990. Personal Communication. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Farrelly, E., M.J. Hammer, and I.R. Hill. 1984. Toxicity to First Instar Daphnia magna. ICI Americas Unpublished Report.

Hart, C.W. Jr. and S.L. Fuller. 1974. Pollution Ecology of Freshwater Invertebrates. Academic Press.

Hill, R.W. 1985. Determination of the Acute Toxicity to Larvae of the Pacific Oyster (Crassostrea giga). ICI Americas Unpublished Report.

Hudson, R.G. and B.G. Isom. 1984. Rearing Juveniles of the Freshwater Mussels (Unionidae) in a Laboratory Setting. Nautilus 98:129-135.

Hudson, R.G. 1990. Personal Communication. Presbyterian College, Clinton, South Carolina.

Isom, B.G. 1986. Systems Culture of Freshwater Shellfish (Bivalves). EIFAC/FAO Symposium on Selection, Hybridization and Genetic Engineering in Aquaculture of Fish and Shellfish for Consumption and Stocking. Bordeaux, France. May 27-30, 1986.

Isom, B.G. and R.G. Hudson. 1982. In vitro Culture of Parasitic Freshwater Mussel Glochidia. Nautilus 96:147-151.

Isom, B.G. and R.G. Hudson. 1984. Culture of Freshwater Mussel Glochidia in an Artificial Habitat Utilizing Complex Liquid Media. U.S. Patent 4,449,480.

Jacobson, P.J., J.L. Farris, D.S. Cherry, and R.J. Neves. 1989. Comparisons of Sensitivity Among Early Life Stages of Freshwater Mussels Exposed to Copper. Compendium for the Tenth Annual Meeting, Society of Environmental Toxicology and Chemistry, October 28 to November 2, 1989, Toronto, Ontario, Canada.

## 6.0 REFERENCES
(Page 2 of 2)

Keller, A.E., and T.L. Crisman. 1989. Acute Toxicity of Several Metals and Organic Compounds to the Freshwater Mussel Anodonta imbeciliis. Compendium for the T Annual Meeting, Society of Environmental Toxicology and Chemistry, October 28 November 2, 1989, Toronto, Ontario, Canada.

LeFevre, G. and W.C. Curtis. 1912. Studies on the Reproduction and Artificial Prop ion of Freshwater Mussels. Bull. U.S. Bur. Fish. 30:105-201.

Macek, K.J., K.S. Buxton, S. Sauter, et al. 1976. Chronic Toxicity of Atrazine to Selected Aquatic Invertebrates and Fishes. EPA-600/3-76-047.

Mayer, F.L. 1987. Acute Toxicity Handbook of Chemicals to Estuarine Organisms. EPA-600/8-87/017.

Purchon, R.D. 1977. The Biology of the Mollusca. Second Edition. Pergamon Press.

U.S. Department of Interior. 1989. Endangered and Threatened Wildlife and Plants. 50 CFR 17.11 and 17.12.

U.S. Environmental Protection Agency. 1985. Hazard Evaluation Division, Standard Evaluation Procedure. Acute Toxicity Test for Freshwater Invertebrates. Office of Pesticide Programs. Washington, DC. EPA-540/9-85-005.

Wade, D.C., R.G., Hudson, and A.D. McKinney. 1989. The Use of Juvenile Freshwater Mussels as a Laboratory Test Species for Evaluating Environmental Toxicity. Compendium for the Tenth Annual Meeting, Society of Environmental Toxicology and Chemistry, October 28 to November 2, 1989, Toronto, Ontario, Canada.