

**COPPER TOXICITY TO LARVAL *MERCENARIA MERCENARIA*
(HARD CLAM)**

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(ABSTRACT)

Toxicity of copper to larval *Mercenaria mercenaria* was evaluated with static non-renewal and continuous renewal methods that permitted daily observation of mortality, activity, development, and metamorphosis without subsampling. Clam larvae, 100 - 150 μm , were held for up to two weeks in small, 30 mm, sealed petri plates during static assays with excellent control survival, low evaporative losses, and relatively low between replicate variability. An eight day LC50 of 12 $\mu\text{g}/\text{l}$ for six day old organisms was determined as well as EC50s (active swimming). EC50s at 24 hours were as much as much as seven times lower than LC50s after 24 hours of exposure. Flow - through assays were conducted in a similar modified petri dish. Two sections from opposing sides of a 30 mm petri dish were removed and covered with 35 μm polyester screening. This dish (organism dish) was placed in an outer catch dish that captured the effluent toxin as it passed through the screening and routed it to a catch bottle for water quality analysis. The toxicant feed line entered through the catch dish cover and slowly dripped toxin into the organism dish. Water quality in the flow - through assay remained high. Control survival in the flow - through assay was lower than in static assays, but metamorphosis

was not delayed as had been observed in static assays. Data variability was low enough that statistical distinctions were made between the effects of copper on metamorphosis. A non-standard “M” shaped survival response was observed in all assays. The responses generating the “M” shaped response in the static petri assay were statistically different from each other. Activity, as judged by swimming, in organisms was not observed to follow the “M” shaped pattern, instead it decreased exponentially with increasing copper concentrations. Static experiments with unfed clams, observations of activity, and data from experiments in copper accumulation by algae led to a theory relating the unusual dose response to food consumption and its effect on the larval clams total mass balance of copper.

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Table of Contents

| | |
|---|------|
| Acknowledgements: | iv |
| List of Tables: | vii |
| List of Figures:..... | viii |
| Executive Summary..... | 1 |
| Issue | 1 |
| Review..... | 1 |
| Methods | 2 |
| Results | 3 |
| Theory on Toxicity of Copper in the presence of Algae (food)..... | 3 |
| Literature Review | 5 |
| Clam Culturing | 5 |
| Eastern Shore of Virginia Background..... | 7 |
| Testing methods..... | 9 |
| Flowthrough Methods..... | 10 |
| Measurement of Metals | 14 |
| Toxicity in Bivalves | 15 |
| Toxicity Specific to <i>M. mercenaria</i> | 21 |
| Algae Culture..... | 23 |
| Sub-Organism Toxicity | 23 |
| Copper Chemistry and General Toxicity | 25 |
| Algae uptake and transfer of metals in the food chain..... | 27 |
| Data Analysis..... | 29 |
| References | 31 |
| Copper Toxicity to Larval <i>M. mercenaria</i> | 47 |
| Acknowledgements: | 48 |
| Abstract..... | 49 |
| Introduction: | 50 |
| Objectives..... | 54 |
| Methods | 55 |
| Apparatus preparation | 55 |
| Artificial seawater | 55 |
| Toxicant solutions | 55 |
| Water Quality | 56 |
| Counting and observation..... | 57 |
| Algae culture | 57 |
| Copper uptake by algae | 57 |
| Metal Analysis..... | 58 |
| Organism transport and acclimation..... | 59 |
| Experiment setup | 59 |
| Control Survival | 60 |
| Statistical Analysis | 60 |
| Results | 61 |
| Copper uptake by algae | 61 |
| Evaluation of sealed 30 mm petri plate design | 62 |
| Range Finding Experiment..... | 62 |
| 5 - 495 ug/l range experiment..... | 64 |
| Copper Nitrate and Kocide 101 | 66 |

| | |
|---|------------|
| Copper Nitrate Response | 67 |
| Kocide 101™ Response | 67 |
| Interchemical comparison..... | 68 |
| Discussion..... | 69 |
| Chamber viability | 69 |
| Algae uptake of copper..... | 69 |
| Mortality and impairment..... | 71 |
| Sublethal effects | 72 |
| Kocide - CuNO ₃ Comparison | 72 |
| Toxicity mechanism | 73 |
| Conclusions | 77 |
| References | 78 |
| Figure Legends: | 87 |
| Table Legends: | 99 |
| | |
| A Flow Through Test Chamber For Larval <i>Mercenaria mercenaria</i>..... | 102 |
| Acknowledgements: | 103 |
| Abstract:..... | 104 |
| Introduction: | 105 |
| Eastern Shore Background | 107 |
| Flowthrough methods | 109 |
| Methods | 111 |
| Apparatus preparation | 111 |
| Toxicant Solutions..... | 111 |
| Water Quality | 112 |
| Counting, observation, and growth measurement..... | 113 |
| Metal Analysis..... | 113 |
| Organism transport and acclimation..... | 114 |
| Chamber Design | 114 |
| Statistical Methods | 116 |
| Results | 116 |
| Chamber Performance | 116 |
| Development and Mortality of <i>M. mercenaria</i> | 117 |
| Discussion..... | 120 |
| Flow - through chamber: Physical performance..... | 120 |
| Mortality and Impairment..... | 120 |
| Conclusions | 121 |
| References: | 123 |
| List of Figures..... | 129 |
| Table Legends: | 135 |
| | |
| Appendix A: Photo Database Index..... | 139 |
| Appendix B: Video Recording Index..... | 156 |
| Vitae:..... | 160 |

List of Tables:

Manuscript One: Copper Toxicity to Larval *Mercenaria mercenaria*

Table 1: 100
ANOVA, LC50, and EC50 summary of 7 day old *Mercenaria mercenaria* survival to copper exposure in sealed plastic petri plates with initial *Isochrysis galbana* concentrations of 100,000 cells/ml. Control, artificial seawater, copper concentration was 1 µg/l.

Table 2: 101
Comparison of ANOVA on Kocide 101™ and CuNO₃

Manuscript Two: A Flow Through Test Chamber for Larval *Mercenaria mercenaria*

Table 1: 136
Typical size of *Mercenaria mercenaria* during first two weeks of life

Table 2: 137
Summary of Water Quality Data

Table 3: 138
Summary Of Inwater and Outwater Time Weighted Copper Concentrations

List of Figures:

Manuscript One: Copper Toxicity to Larval *Mercenaria mercenaria*

Figure 1: 90
Survival and metamorphosis (setting) of larval *Mercenaria mercenaria* in artificial seawater containing no added copper in sealed plastic petri dish with 100,000 cells/ml *Isochrysis galbana* added. Clams were 5 days old at initial exposure (N = 10). Bars represent 1 standard error.

Figure 2: 91
Average survival of 6 day old larval *Mercenaria mercenaria* exposed to copper in artificial seawater containing 100,000 cells/ml of *Isochrysis galbana* in a sealed 30 mm petri plate (N=3). Bars represent one standard error. Survival of controls (not shown) at 24 hours was not significantly different from those clams exposed to 29 or 459 µg/l Cu.

Figure 3: 92
Mercenaria mercenaria response to copper. Clams were contained in sealed petri plates in 1.5 mls of test solution and had an initial *Isochrysis galbana* density of 100,000 cells/ml. Clams were 6 days old at initial exposure. Bars represent one standard error.

Figure 4: 93
Comparison of *Mercenaria mercenaria* response over time to copper in sealed petri plates with 100,000 cells/ml initial *Isochrysis galbana*. Bars represent 1 standard error.

Figure 5: 94
Comparison of survival and setting (top) and active swimming (bottom) between larval *Mercenaria mercenaria* in control groups fed an initial *Isochrysis galbana* density of 100,000 cells/ml and control larvae that were not fed *I. galbana*. Clams were 7 days old at initial exposure. No significant difference was observed in survival or swimming. Artificial seawater without added copper was the matrix. Bars represent one standard error.

Figure 6: 95
Comparison of larval *Mercenaria mercenaria* (initially treated when 7 days old) survival (top) and active swimming (bottom) at 14 and 29 µg/l soluble copper with and without feeding of an initial 100,000 cells/ml *Isochrysis galbana*. No significant difference in survival was observed between fed and unfed clams exposed to 14 µg/l soluble copper.

Figure 7:..... 96
Survival of *Mercenaria mercenaria* in artificial seawater with copper dosing with CuNO₃ and Kocide 101™. Clams were held in unsealed petri dishes in a total water volume of 1100 µls with an initial density of 100,000 cells/ml *Isochrysis galbana*. Control copper content = 2.4 µg/l. Bars represent 1 standard error.

Figure 8:..... 97
Theoretical relative clam exposure and clam activity. Activity impairment threshold 3.9 µg/l Cu. Zero activity threshold 29 µg/l Cu. Exponential decay rate (F = 3.9) and algae potentiation factor of 3.9.

Figure 9: 98
Observed Survival % vs. Effective Copper ug/l

Manuscript Two: A flow through test chamber for Larval *Mercenaria mercenaria*

Figure 1:..... 130
Larval clams: Left; straight hinge, 48 hours. Right; umboned, 6 days.

Figure 2:..... 131
Diagram of flow - through testing apparatus

Figure 3:..... 132
Survival of *Mercenaria mercenaria* in flow - through chamber with continuous flow of test solutions and *Isochrysis galbana* at 100,000 cells/ml. Bars represent one standard error.

Figure 4:..... 133
Mercenaria mercenaria metamorphosis from umbonate to pediveliger "setting " in flow - through petri plate apparatus. Organisms were continuously dosed copper and fed *Isochrysis galbana* at 100,000 cells/ml for the duration of experiment. Bars represent one standard error.

Figure 5:..... 134
Comparison of size in set *Mercenaria mercenaria* after 84 hours of continuous exposure to copper in flow - through chamber. Control and toxin contained *Isochrysis galbana* at density of 100,000 cells / ml. Measurements are of the set clams greatest width. Bars represent one standard deviation.

Executive Summary

Issue

In the 1990s, expansion in the use of plastic mulches, plasticulture, in the watersheds of the Eastern Shore of Virginia corresponded with increased mortalities in shellfish hatcheries in these same watersheds. Investigation found high levels of copper and other agricultural chemicals in runoff from these fields. While plasticulture is a best management practice (BMP), and can reduce nutrient runoff and the need for some agricultural chemicals, runoff of some chemicals that must be applied to the leaves surface can be enhanced due to the reduced permeability of the field and tilling practices designed to minimize standing water.

Review

Review of literature pertaining to copper and bivalve toxicity found an abundance of material on adult bivalves, less material on larval bivalves and very little material on larval *Mercenaria mercenaria* (*M. mercenaria*), the hard clam. Several factors may contribute to the scarcity of data on larval *M. mercenaria*. They have a very high natural mortality rate and ASTM standards recognize this and report a minimum acceptable control survival of 60% for only two days of embryo exposure. They are a very small organism, less than 100 μm in width in early stages. They exist in two different portions of the water column during the first two weeks of life as they gradually change from a free swimming organism to a sedentary bottom dwelling one. The toxicity citation of reference is from research performed in 1977 by Calabrese *et al.* (a). Copper at 16.4 $\mu\text{g/l}$

is cited as the LC50, concentration at which 50% of organisms died, after 8 to 10 days of exposure. Calabrese *et al.*, 1977(a) used a static renewal approach with preservation and subsampling at the conclusion of the assay followed by observations for normal development and growth. Hatchery operators stated that when the clams were not outright killed they would appear to develop normally but never "set" or metamorphose to the sedentary stage. This study was initiated to investigate both the toxicity of copper to larval clams and gain a more detailed insight to the low level effects of copper on larval clams.

Methods

Initial research with methods similar to those employed by Calabrese *et al.*, 1977(a) were too time consuming and inconclusive when applied to daily observation of activity, setting, and mortality. Next, a flow through device consisting of a small square screened cage designed for ghost shrimp was adapted to the smaller *M. mercenaria*. Factors of scale and optical properties of the smaller screening proved this design inappropriate. Static non-renewal assays in 30 mm petri plates provided a simple convenient test method that produced statistically significant discrimination between toxicant concentrations. These static methods were further refined by sealing the petri dishes with petroleum jelly to minimize evaporative losses thus permitting the extension of tests beyond 500 hours. The qualities of the static dish assay were incorporated into a flow - through assay by removing two opposing sections from the dish side wall and covering them with screening. Toxicant was continually added by drip addition and waste products left via the side screens.

Results

An eight day LC50 of 12 µg/l copper was determined for larval clams first exposed at six days of age. This is 4 µg/l lower than Calabrese *et al.*, 1977(a)'s eight to ten day LC50 of 16 µg/l copper for clams first exposed at two days of age. Activity, as measured by swimming, was shown to be impaired at copper concentrations up to seven times lower than copper concentrations causing death. Other non-lethal effects observed included growth impairment, metamorphosis failure, and deformations at concentrations less than 29 µg/l copper. Similar to literature reports, algae were shown to accumulate copper in excess of aquatic concentrations. The accumulation of copper by algae, which are food for the clams, combined with the reduction of activity observed in clams at increasing soluble copper concentrations produced a theory explaining the unusual "M" shaped dose response observed in all tests.

The flow - through chamber maintained water quality at ideal levels and provided a convenient means of observing the clams. Metamorphosis occurred without delays observed in static assays. Statistically significant differences could be made between low (less than 65 µg/l) copper doses. Survival of controls was lower than that observed in static assays which could be attributed to the more dynamic environment of the flow through chamber combined with the high natural mortality of larval clams.

Theory on Toxicity of Copper in the presence of Algae (food)

Briefly, the theory is as follows: the total copper content of a clam can be split into two general sources: Soluble copper or dissolved copper in the water column that the clam is always exposed to; and, particulate associated copper or copper that is bound

to bacteria, algae, and other materials that the clam may consume. Copper is necessary for life and the first peak in the “M” shaped response is explained by this factor. Copper becomes toxic when nutritional requirements are exceeded and this generally explains most all other single factor toxicity responses. However, as soluble copper content increases, activity (swimming) decreases, and likewise active feeding decreases. As feeding decreases, consumption of particulate associated copper decreases, therefore the effective copper dose to the clam is actually slightly lower as soluble copper content in the water column increases over the range of about 10 to 20 $\mu\text{g/l}$ soluble copper. The decreased effective copper dose translates to increased survival in the clams which explains the second peak in the dose response curve.

Literature Review

Clam Culturing

Mercenaria mercenaria (*M. mercenaria*), the hard clam, or quahog has a range from the Atlantic Coast of North America from the Gulf of St. Lawrence to Texas in waters with salinities above 12 ppt. Spawning typically occurs between May and October when the water temperature is above 20 to 23 degrees C (Funderburk *et al.*, 1991). The first 48 hours following fusion of sperm and egg include an embryonic state with no apparent appendages when sperm and egg may still be visible as distinct entities. This is followed by the trochophore stage where the clam appears as an ovaloid mass with cilia. Finally the straight hinge or prodissoconch stage is reached where a properly developed clam will have a clearly defined shell with a straight portion about which the two halves of the shell pivot (ASTM, 1994). The straight hinge clam initially spends most of the time moving about in the water column but as it matures it spends greater portions of time on the bottom until the clam changes from the straight hinge shell to the ovate-trigonal shell. The metamorphosis or "setting" occurs typically between 8 and 14 days following fertilization but can be completed in as little as a week or can be extended to as long as 24 days if inadequate substrate is present or conditions are lacking (Funderburk *et al.*, 1991).

Marine bivalve culture has increased greatly in the past decade along with the growth of aquaculture world wide (Grant, 1996). Historically oysters were harvested from the Chesapeake Bay and other areas on the U.S. Atlantic coast from large shoals without regard for the capacity of the clams to regenerate themselves. Overharvesting combined with several destructive storms resulted in a sharp decline in clam harvests

(Wennerston, 1981). As watershed development increased, watersheds formerly holding natural clam beds became inhospitable to natural reproduction and clam populations did not return to former numbers. In response to this, hatcheries began the controlled induced spawnings of clams along tidal streams and creeks to replenish clam stocks.

Hatcheries typically maintain a select group of brood stock that have been chosen for rapid growth, resistance to disease, and shell markings (Castagna, 1981). The later permit easier field recognition of clams and identification of poachers at market (Kamm, 1998). Brood stock are brought in from holding sites in late winter or early spring and conditioned for spawning by holding clams between 20 and 25 degrees C, the exact temperature varies depending on the geographical origin of the clam (Castagna, 1981; ASTM 1994). Clams are fed regularly and when the gametes are fully ripened, the clams may be induced to spawn. Spawning can be induced by rapidly raising the temperature 5 to 10 degrees C and by the addition of heat killed sperm to the conditioning water (ASTM, 1994). Spawning can be delayed in conditioned clams for up to 8 months by holding them in water between 16 and 20 degrees C.

After spawning, the brood stock are removed from the spawning tank and the embryonic clams are diluted to 30 eggs per mL of water. Fertilized eggs develop best in salinities between 26 ppt and 30 ppt and temperatures from 18 to 28 C with faster development occurring at higher temperatures but with a greater risk of bacterial infection. The optimum temperature range is 23 to 25 degrees C. The embryonic clams are left undisturbed until reaching the straight hinge stage at which point they are counted and sorted by sieve according to size and resuspended in new water. Separation by size helps reduce competition and increases total yields. While a single clam may release as many as 60,000,000 eggs over an entire breeding season, survival to a size that may be placed in the field is typically less than 1% and additional losses are expected after

setting the clams in the field. Survival from the field to market size, generally between 18 and 24 months, is approximately 60% (Castagna, 1981).

Grant, 1996, provided a broad view of the role of food character in the consumption of food by bivalves and the influence of temperature, water circulation and a variety of other natural factors on bivalve energy levels and growth. The application of models including these parameters towards commercial aquaculture was addressed.

Eastern Shore of Virginia Background

In the 1990s the use of plastic mulch or "plasticulture" increased on the Eastern Shore of Virginia. During the same period, hatcheries operating in watersheds where plasticulture was employed experienced increases in clam larvae mortality. Operators of these hatcheries claimed that the two events were related and that chemicals in the agriculture runoff were responsible for the clam deaths.

Plasticulture is an agricultural method that can enhance productivity and reduce the need for insecticides and pesticides. Fields are tilled to prevent water buildup and crop rows are mounded. On top of the mounds irrigation and fertilization lines are installed and then the entire crop row is covered by plastic. This process of "fertigation" can reduce nutrient runoff from fertilizers and allows control over the soil water not offered by traditional cultivation methods (Aylsworth, 1997). Plasticulture is very effective in controlling disease, weeds, and pests (Garnaud, 1994). While plasticulture does have several benefits, it does accelerate the runoff of rain (Scott *et al.*, 1990). Copper containing fungicides, like Kocide 101 that contains 50% copper (Griffith, 1996), and other materials that are applied to the surface of plant leaves can be carried with this runoff into adjacent streams.

A variety of contaminants can be found in plasticulture runoff. Azinphosmethyl, fenvalerate, endosulfan, and chlorothalonil were measured at concentrations from (<1 to > 100 µg/l) in samples from Virginia and South Carolina (Scott *et al.*, 1990; Dietrich *et al.*, 1996). Dietrich *et al.*, 1996 reported high levels of copper in plasticulture watersheds as well. Concentrations up to 1450 µg/l copper were measured in streams bordering on plasticulture fields following rain events. For reference, the Virginia Department of Environmental Quality standard for endosulfan in saltwater is 0.0087 µg/l, 0.01 µg/l for azinphosmethyl in salt water, and 2.9 µg/l soluble copper in saltwater (Virginia DEQ, 1992).

Clam hatcheries are a significant part of the aquaculture economy. In the annual report of production of crabs, trout, catfish, clams, and oysters in 1995, gross sales of Virginia aquaculture products accounted for \$19.6 million of which \$14.4 million was in marine sales. Clams supplied \$8 million of the total gross sales (Virginia Agricultural Statistics Service, 1996)

Pesticides and practices used on nine crops in 1991, including tomatoes, grown in Virginia were reported by Weaver and Fatima, 1995. The report covered all portions of crop production including pre-planting herbicides, insecticides, seed treatment, fungicides, nematocides, alternative pest controls, and safety recommendations. Worthing, 1987 provided detailed information on the pesticides at issue on the Eastern Shore. Nomenclature, structure, properties, uses, toxicology data, and formulations were presented.

A scientific/research subcommittee was formed by the Eastern Shore Vegetable and Shellfish Growers Advisory Committee to perform a literature review on plasticulture and water quality concerns and recommend research issues to be accomplished relative to the Eastern Shore issue. The report addressed those issues and

within the report explained the process of plasticulture, the potential effects of plasticulture, and management practice necessary for the practices. Research of shellfish mortality in hatcheries was reported to be limited and caution was suggested in proceeding with attempts to research the issue due to the expertise requirements, time, and cost of assembling an effective assay for marine bivalve larvae (Wilson *et al.*, 1997). Similar issues with copper were addressed in Florida by Trefry *et al.*, 1983 where significant inputs of copper were made by copper containing anti-fouling paints.

Testing methods

The three basic physical approaches to testing include static, static renewal, and continuous renewal. Static methods are the least technically involved and least costly of the methods, but are limited in length by oxygen depletion and toxicant transformations. Static renewal methods can control oxygen depletion and reduce variation in toxicant character by regular replenishment of test media, but require more toxicant volume and can involve more manipulation of organisms. Continuous renewal techniques can maintain toxicant concentrations at constant levels and dissolved oxygen can be maintained. Continuous renewal typically is the most technically and financially demanding of the three methods.

Six short term assays for *Atherinops affinis* (*A. affinis*), common top smelt, *Holmesimysis costata* (*H. costata*), the Bannerfish shiner, *Strongylocentrotus purpuratus* (*S. purpuratus*), the purple sea urchin, *Dendraster excentricus* (*D. excentricus*), the sand dollar, *Haliotis rufescens* (*H. rufescens*), the red abalone, *Crassostrea gigas* (*C. gigas*), the Pacific oyster, and *Macrocystis pyrifera* (*M. pyrifera*), the giant kelp, were reported in Chapman *et al.*, 1995. Static renewal and static non-renewal testing techniques were

presented as well as general testing guidelines and an explanation of a variety of statistical treatments. Weber *et al.*, 1988, and Weber, 1995, presented similar reports and techniques with different organisms.

ASTM standard E724 - 94 presented methods for acute (48 hour) testing of embryos of *C. gigas*, the pacific oyster, *Crassostrea virginica* (*C. virginica*), the eastern oyster, *M. mercenaria*, and *Mytilus edulis* (*M. edulis*), the blue mussel. Guidelines were provided for apparatus, dilution water, test material, test organisms, procedures, analytical methods, acceptable results, and result calculations. General terminology as well as illustrations and photos of the different relevant stages during the first 48 hours of the bivalve life were reported.

His *et al.*, 1997, developed a simplified version of the ASTM standard test procedure for acute testing of embryos of bivalve larvae. Their test utilized Coulter Counter accuvettes to retain embryos and allowed observation and counting without subsampling and delivered 48 hour control survival from embryos to straight hinge of greater than 90% for *C. gigas* and *Mytilus galloprovincialis* (*M. galloprovincialis*), the Mediterranean mussel. Other features included induced natural spawning and application of toxicants earlier than the ASTM method.

Flowthrough Methods

When adapting flow - through methods designed for one organism to a different organism, it is essential to consider the size of the organisms for which the method was designed. For comparison, 21 day old *Daphnia magna* (*D. magna*), are approximately

eight mm in longest dimension and *Ceriodaphnia dubia* (*C. dubia*), are approximately 1.4 mm in longest dimension (Pennak, 1953; Ferrando *et al.*, 1995). *Mercenaria mercenaria* at 48 hours range from 0.090 to 0.140 mm (Funderburke, 1991).

Several authors (Knoezovich and Harrison, 1987; Murty and Murthy *et al.*, 1988; Francis *et al.*, 1986; Diamantino *et al.*, 1997) employed a similar approach to flow-through toxicity testing. Bottles or beakers were capped with a stopper. Entry and exit tubes were placed in the stopper and covered with screening to prevent organism loss. Toxicant was pumped from a reservoir into the test bottle and collected from the exit tube for post exposure analysis. Sediment addition, gravity flow, headspace and the absence of headspace were among the subtle variations in methods.

Kersting and Wijngarnden, 1992, developed a more sophisticated system based on the same basic bottle flow-through concept. Three separate reaction vessels were connected and water circulated through each chamber by a peristaltic pump to produce a microcosm for the testing of chlorpyrifos. Each chamber represented a level in the food chain with algae as primary producer, *D. magna* as herbivores (consumers), and bacteria on sand as degraders. *Daphnia magna* were retained in their chamber via screening over entry and exit ports.

A very different approach was taken by Gallagher, 1988 to observe larval *M. mercenaria* with high speed videography. Two and 10 day old larval *M. mercenaria* were tethered by means of a microsuction pipette or by gluing the clams to a 10 μm glass rod with isocyanocrylate adhesive. The tethered clams were placed in a clear trough

where water was flowing at a velocity similar to the clams natural swimming speed. Data on feeding rates, particle selectivity, and food rejection rates were reported. A description of the process of food capture and ingestion was detailed.

Lauth *et al.* 1996 combined the bottle techniques with flow-through trough methods to produce a unique test method for *C. dubia*. Holes were bored in opposing sides of 35 ml polystyrene cups and covered with 120 μm Nitex mesh. These cups retained *C. dubia* and permitted observation by stereoscope. The cups were placed in troughs that contained flowing solutions of toxicant. The toxicant entered one side of the cup through the mesh, exited from the opposing side, and continued on to the next cup in the trough.

A larger scale flow-through approach was demonstrated by Hatakeyama, 1988. Small, 15,000 cm^3 aquaria were used for flow - through toxicity tests to *Polypedilum nubifer* (*P. nubifer*), midge. Exit routes were covered with screen to prevent loss of organisms. Troughs also provide an alternate flow - through test method. Kreutzweiser *et al.*, 1994 presented a method for the assessment of drift response of 11 aquatic insects to an insecticide. 70 x 30 x 10 cm troughs were constructed of plexiglass. Each trough was divided lengthwise into three separate lanes. One half of the trough, the upcurrent portion, contained natural substrates. Insects that detached from this natural substrate, in response to toxin, were observed in a downstream portion that contained no substrate. Reattachment was possible in the final 10 cm portion of the trough in a recessed area that shielded the insect from the current and contained a stone for reattachment.

Within each of the general physical types of test methods are many possible testing mechanisms. One significant alternative approach is anoxic and aerial survival. Anoxic and aerial survival techniques for the investigation of toxicity to mature bivalves were reviewed by de Zwaan and Eertman, 1996. Anoxic and aerial survival techniques are advantageous as they can accelerate the effects of perturbing influences and remove the bioconcentration effect of a toxicant. Bivalve survival in an anoxic environment demands energy conserving practices. Any factor that disturbs the organism's ability to conserve energy will result in death sooner than an undisturbed organisms. Practical considerations and recommended techniques were reported.

Measurement of Metals

Methods for the determination of total and soluble metals were included in USEPA SW-846 Standard Method 3005, 3010, and 3020 (USEPA, 1997) as well as in Standard Methods for the Examination of Water and Wastewater 3010 and 3030 (Eaton *et al.*, 1995). Hidmi and Edwards, 1998, reported sorption of copper on filters commonly used in soluble copper measurements at pH greater than 8. Edwards, 1997, reported similar effects. Crompton, 1989, includes methods of metals determination specific to saltwater matrices. Reagents for copper extraction include Ammonium pyrrolidine dithiocarbamate and diethylammonium diethyldithiocarbamate and Freon TF. Procedures include an initial extraction of metals from the salt matrix followed by back extraction and then analysis by atomic absorption spectroscopy. Coefficients of variation for the method were reported to be between 5 and 10% for concentrations between 12.5 and 350 ng / dm³.

Dithiocarbamate extraction with Atomic Absorption Spectrophotometry was employed for the sampling and analysis of Cu, Cd, Ni, and Zn at ng / L concentrations in seawater. A comparison of this method with samples concentrated on Chelex 100 resin showed that cadmium and zinc were efficiently absorbed by the Chelex but that copper and nickel were inefficiently removed from saltwater (Bruland *et al.*, 1979).

EDTA and sodium thiosulfate were employed to differentiate sources of acute toxicity to *C. dubia*. The addition of EDTA or thiosulfate was shown to reduce the toxicity of different groups of metals thus it is proposed that the two agents could be used

as a part of TIE (Toxicity Identification Evaluation) to categorize the metals expressing toxicity in an unknown sample (Hockett and Mount, 1996).

Toxicity in Bivalves

Metals

Cunningham, 1979 reviewed the use of bivalve molluscs in heavy metal research. Research at all development stages, embryos, larvae, juvenile, and adult were addressed. The work of Prytherch, 1934, was cited and reported early onset of setting in oyster larvae at concentrations from 10 to 600 µg/l copper. Similar to later reports by other researchers, higher concentrations of copper were reported to produce abnormal development of internal organs and death. Dorn 1976, reported feeding rates in adult *M. edulis* decreased with increasing mercury concentrations. It was suggested that the reduced feeding rate was due to inhibition of ciliary activity caused by nervous system disruption by mercury. It is also possible that the mussels closed their valves in response to the mercury thus reducing their exposure.

Doherty, 1986, reported on the behavior of *Corbicula fluminea* (*C. fluminea*), the asiatic clam, to a variety of metals. Valve closure and rate of closure was reported to be dependent on metal concentration. Cadmium uptake was increased with the addition of chlorine and reduction or increase of temperature. The distribution of cadmium within *C. fluminea* was dependant the route of exposure with dissolved Cd producing the highest concentrations in gill, mantel, and adductor while algae associated cadmium resulted in higher concentrations in visceral mass.

Ruiz *et al.*, 1995; reported 30 day static renewal exposures of tributyltin to *Scrobicularia plana*, (*S. plana*), tellinid bivalve, pediveligers (240 μm) in a range of 37 to 102 ng Sn /l (as tin) induced substantial mortalities and negligible shell growth in individuals. Concentrations as low as 14 to 32 ng Sn/l reduced shell growth by a factor of 4 relative to controls and produced gross shell deformations. The unique test method included a controlled substratum, pulverized uniform size sand, to reduce the stress bivalves have been reported to experience when attempting to settle without a suitable media to settle in. This method lead to the finding of a sublethal effect of TBT on shell development in pediveligers not reported in other bivalvia research. The author linked the deformed shells to the effect repeated burrowing had on the more pliable shells in the TBT exposed organisms. This effect in the wild would lead to their demise before detection by field investigations.

Beiras and His, 1994, utilized an epinephrine-metamorphosis inducement technique to reduce the variability associated with assessing metamorphosis impairment. An increase in tolerance to Hg in *C. gigas* larvae with age was observed as well as a sublethal effect of inhibition of swimming that occurred at concentrations approximately 30 times lower than lethal concentrations. At higher concentrations: 128, 256, and 512 $\mu\text{g/l}$ Hg they observed dead larvae with extruded, granulated tissues appearing to have "exploded". Metamorphosis, growth, and activity were all shown to decrease with increasing Hg concentration. They also noted observations of valve closure with the velum protruded at higher Hg concentrations and increased metamorphic rate at sublethal Hg concentrations (1 $\mu\text{g/l}$ nominal). Though not statistically significant this enhancement of settlement at low metal concentration resembled the data previously

reported on Cu^{+2} (1.6 to 4.7 μM ; Nell and Holliday 1986) as being beneficial for settlement and metamorphosis of some bivalve and gastropod larvae.

NTA (nitrilotriacetic acid) and copper were used to vary the cupric ion activities in seawater medium and filtered (0.45 μm) seawater over a range of 10^{-11} to $10^{-8.5}$ M by Zamuda and Sunda, 1982. The accumulation of dissolved copper in unfed *C. virginica* (Eastern oyster) was related to cupric ion activity and not the concentration of chelated copper. Rapid accumulation of copper occurred when cupric activities were in excess of 10^{-10} Molar while no measurable accumulation occurred at 10^{-11} Molar. In contrast, George and Coombs; 1977, reported that Cd complexation to EDTA, humic and alginic acids, and pectin doubled the Cd accumulation by *M. edulis*, while Harrison, 1979, reported that EDTA strongly decreased the accumulation of copper and zinc by oysters.

Crassostrea virginica larvae were cultured in cadmium and copper solutions for 24 hours following fertilization at concentrations below those perturbing embryogenesis (Ringwood and Brouwer, 1995). Metallothionein isoforms were detected via size exclusion HPLC on exposure to cadmium at 20 $\mu\text{g/l}$ and copper concentrations of 10 $\mu\text{g/l}$. Exposure to the metals simultaneously revealed inhibition of cadmium binding metallothionein and an increase of cadmium binding to copper metallothioneins. When copper concentrations of 20 $\mu\text{g/l}$ were tested, shell formation was inhibited and the concentration of zinc associated with metallothionein was significantly reduced. A theory explaining calcification inhibition was proposed. Metallothioneins were believed to be involved in the movement of zinc to zinc requiring enzymes involved in

calcification. The impaired transport of zinc thus hindered shell formation (Ringwood and Brouwer, 1995).

A linear relationship between concentrations of nickel in seawater and nickel uptake by mature *M. edulis* and *C. virginica* was reported for exposures of up to 10 µg/kg Ni after 12 weeks of exposure. After 12 weeks of culture, *M. edulis* had an average concentration of 10.4 and 16.4 µg/g dry weight after exposure to 5 and 10 µg/l Ni respectively. *C. virginica* had a concentration of 9.6 and 13 µg/g dry weight after exposure to 5 and 10 µg/l Ni respectively (Zaroogian and Johnson, 1984).

O'Connor, 1995 reviewed the NOAA Mussel Watch Data collected from 1986 to 1993 and concluded that both cadmium and arsenic concentrations in organism tissue decreased from 1986 to 1993. Usage in the United States of both of these metals decreased between 1986 and 1993. Similarly mussel tissue concentrations of chlorinated hydrocarbons and tributyltin were decreasing and both of these materials had been banned for use in the United States. Copper and selenium were reported as possibly decreasing but no data was available to support diminished use of these metals

Thomann *et al.*, 1995, developed a model to relate the metal concentration in the adult marine bivalves *C. virginica* and *M. edulis* with metal concentration in sediments. Data from NOAA Mussel Watch were incorporated to produce a model that included: depuration, metal assimilation efficiency from food, feeding rate, and growth rate. The calibrated model indicated that food is a significant route of metal uptake for all metals and that food accounted for nearly all of Zn, Cd, and Cu accumulation. In the case of copper 94% of accumulation was through the food route.

Barium was shown to adversely affect shell calcification as well as embryo development in *Mytilus californius* (*M. californius*), the california mussel, at concentrations as low as 200 µg/l Ba. Larvae in the gastrulae stage were determined to be the most sensitive to barium followed by blastula and trochophore (Spangenberg and Cherr, 1996).

Crassostrea virginica and *Ischadium recurvum* (*I. recurvum*), the hooked mussel, were deployed in the Patuxent River Estuary, Maryland up and downstream from a power plant known to be a source of copper for the purpose of assessing metal sources and interspecies differences in metal accumulation. The power plant was determined to be only a minor contributor to the accumulation of copper in bivalves and sources such as atmospheric deposition, anti-fouling paints, and water treatment plants were theorized to share responsibility for the copper present in the watershed. *Crassostrea virginica* was reported to vary in copper accumulation throughout the study while *I. recurvum* copper concentrations remained very stable suggesting different copper management processes in each organism (Riedel *et al.*, 1995).

While not about bivalve toxicity, the research of Luckenbach *et al.*, 1996, was relevant as a portion of it was conducted in affected watersheds of the Eastern Shore. The effects of agricultural runoff and its components were studied by Luckenbach *et al.*, 1996. Bioassays with caged *Palaemonetes pugio* (*P. pugio*), a grass shrimp that is a keystone species in the estuarine food web, positioned in tidal creeks that received agricultural runoff from plasticulture fields on the Virginia Eastern Shore showed that

runoff could cause up to 100% mortality in the organism and that metal toxicity was a potential source of toxicity. Subsequent laboratory studies also demonstrated the toxicity of azinphosmethyl, fenvalerate, and endosulfan to *P. pugio*.

Organic toxicity

While most of the focus of this literature review was on metals toxicity other reports of organic toxicity relevant to the chemicals used on the Eastern Shore were collected as well. The sublethal effect of endosulfan, malathion, and methyl parathion on the adult *Villorita cyprenoides (cochinensis)*, was assessed by measurement of lipid depletion. Clams were exposed to concentrations from 200 to 1000 µg/l and lipid levels measured at 24, 48, 72, and 96 hours. Lipid levels decreased significantly due to these sub-lethal exposures and the order of toxicity was endosulfan > malathion > methyl parathion (Sujatha *et al.*, 1995). Rajendra and Venugopalan, 1991, reported accumulated concentrations in the gill, mantle, adductor muscle, foot, and remainder of organism following 10 day continuous renewal exposures to 3 sublethal concentrations (0.13, 0.63, and 1.25 µg/l) of endosulfan in *Crassostrea madrasensis* and *Katylis opima*. Concentration factors varied significantly between organs. The greatest concentration factor was reported for the adductor muscle.

Ernst *et al.*, 1991, reported a 96 hour LC50 for a preparation of Bravo 500, a chlorothalonil containing fungicide, to *M. edulis* of 5.9 mg/l. Tissue concentrations of chlorothalonil increased to up to 10 times the environmental concentration initially but returned to environmental concentrations by the end of the test period (96 hours).

Toxicity Specific to M. mercenaria

Behrens and Duedall, 1981(a), reported that the varying metal content of mature *M. mercenaria* was associated with biological processes of the clam. They reported that changing tissue levels of nickel, lead, and copper were associated with the spawning cycle while cadmium, chromium, and zinc levels were not. They also reported that any assessment of metals variation must consider the seasonal changes that occur in clam body weight. Behrens and Duedall, 1981(b), observed the depuration of metals from clams transplanted from areas of high metal concentrations to low metal concentrations. A group of clams was transplanted from an area closed to shellfishing due to bacteria to an area that was open for shellfishing. While the clams were depurating bacteria the researchers measured the metal concentration in the clams and reported no significant decrease in body concentrations of Cu, Cd, Cr, Ni, Pb, and Zn after 50 days.

Rule and Alden, 1996, reported on the relationship of copper and cadmium in geochemical fractions and their bioavailability. *Palaemonetes pugio*, *M. edulis*, and *M. mercenaria* were exposed to sublethal concentrations of copper and cadmium and respiration, metal uptake, and interactions assessed. They emphasized the importance of multiple element testing when studying the ultimate fates of toxins in natural systems.

Embryos and larvae of *M. mercenaria* and *C. virginica* were exposed to 52 different compounds including insecticides, herbicides, solvents, bactericides, fungicides, algicides, and a nematocide. Generally embryos were more sensitive to the compounds than were larvae but in some cases larval growth was affected at concentrations that had little effect on embryos (Davis and Hidu, 1969).

The toxicity of mercury, silver, zinc, nickel, and lead to *M. mercenaria* embryos was reported by Calabrese and Nelson, 1974. Tests were initiated with one hour old fertilized eggs and terminated after 42 to 48 hours of exposure, the time normally required for development from embryos to straight hinge larvae. The order of toxicity based on LC50s was Hg>Ag>Zn>Ni>Pb with LC50s ranging from 4.8 µg/l to 780 µg/l metal added to synthetic seawater (Calabrese and Nelson, 1974).

Calabrese *et al.*, 1977(a), reported toxicity data for larval (straight hinge) *M. mercenaria* and *C. virginica* to mercury, copper, silver, zinc, and nickel. The order of toxicity for *M. mercenaria* was Hg>Cu>Ag>Zn>Ni with LC5, LC50, LC95 for copper after 8 - 10 day exposure of 4.9, 16.4, and 28.0 µg/l Cu added to background natural seawater. Growth in organisms surviving at the LC50 concentration was markedly reduced compared to controls. The natural seawater had 13.4 µg/l Cu ambient concentration.

The toxicity of cadmium, mercury, and silver to 10 different marine organisms, including finfish, molluscs, and crustaceans, as well as the effects on different developmental stages of each organism was reported by Calabrese *et al.* 1997(b). This summary of research included static, static renewal, and continuous flow testing methods. An extensive review of literature reports on toxicity to *M. mercenaria* at various life stages was prepared by Funderburke *et al.*, 1991, including petroleum products, polynuclear aromatic hydrocarbons, pesticides, TBTO, surfactants, syndets, inorganic compounds, and heavy metals. Additional reports were summarized regarding bioconcentration factors, depuration rates, accumulation rates, and clearance rates.

Algae Culture

Parsons, 1984 discusses methods and confidence in collection and counting of organisms such as bacteria, phytoplankton, and zooplankton. The preparation of artificial seawaters including recipes and organisms for which they are appropriate is also reported. Micro Algae Grow, formulated by Florida Aqua Farms Inc., is a modified Guillard's f/2 solution designed for commercial application in aquaculture. The formula includes Sodium Molybdate, Manganese Chloride, Cobalt Chloride, Zinc Sulfate, Cupric Sulfate, Thiamine, Biotin, B12, Sodium Nitrate, Sodium Ferric Ethylenediaminetetraacetate, Sodium Phosphate, and Sodium Metasilicate (Florida Aqua Farms). Carolina Biological Supply's publication, Culturing Algae, describes methods of culturing, media types, water recommendations, culture maintenance, requirements of specific algae, and methods of establishing and maintaining unialgal cultures (Carolina Biological Supply, 1978). Recipes and formulas of several commercial artificial seawaters are reported by Bidwell and Spotte, 1985. Recommendations for appropriate media for specific organisms were made.

Sub-Organism Toxicity

Dioxin binding proteins were detected in cytosols prepared from the gill and gonad in *Maya arenaria*, *C. virginica*, and *M. mercenaria*. While vertebrates contain proteins that mediate the toxic effects of halogenated aromatic hydrocarbons, no similar system has been identified in invertebrates. No proteins found in the invertebrates were

of similar size to the dioxin mediating proteins in vertebrates and the function of the invertebrate dioxin binding protein was unknown (Brown, *et al.*, 1997).

Mercenaria mercenaria contains glands that are composed of brown cells (Zaroogian *et al.*, 1992). These brown cells play a role in accumulation of foreign materials, excretion, detoxification, and disease defenses. The individual and combined toxic effects of Cd, Cu, and Ni to brown cells of *M. mercenaria* was reported by Zaroogian *et al.*, 1992. The assay utilized a dye that was incorporated into viable lysosomes in cells. Cytotoxicity was reported to be linear relative to Cu^{+2} and Cd^{+2} over ranges of 10 to 100 μM and 0.1 to 1.5 mM respectively. Copper was found to lessen the cytotoxicity of cadmium while nickel had no effect on the cytotoxicity of cadmium or copper (Zaroogian *et al.*, 1992). Another study utilizing the same neutral red assay by Zaroogian and Voyer, 1995, investigated the interactive cytotoxicities of organics and inorganics to *M. mercenaria* brown cells. Binary mixtures of copper, cadmium, benzo(a)pyrene, and N-ethylmaleimide (NEM) were tested and the interactions of copper and NEM, cadmium and B(a)P, and cadmium and NEM were shown to significantly alter brown cell survival compared to controls. A comparison of observed toxicity with a model of independent joint action indicated that the toxicity of these components was less than additive. Zaroogian and Anderson, 1995, reported curvilinear uptake of cadmium and nickel in brown cells of *M. mercenaria* with increasing metal concentration. In contrast, benzo(a)pyrene uptake was reported to be linear in relation to solute content. Cadmium, nickel, and B(a)P uptake was significantly decreased by buthionine-(S,R)-sulfoximine, a glutathione synthesis blocker.

Zaroogian, 1997, reported that glutathione heterogeneously distributed in the brown cells may be responsible for the partial kill occurrences in metal toxicity to

bivalves. Copper concentrations as high as 320 µg/l Cu reduced glutathione concentrations but did not deplete them and the reduction was not in a dose dependant manner.

Copper Chemistry and General Toxicity

A plan to determine a correction for dissolved organic carbon effects on copper toxicity was developed by AScl, 1997(a). The plan addressed technical issues, policy and regulatory issues, and recommended test methods for both acute and chronic tests. Prior to the research plan, AScl, 1997(b), prepared a literature review on copper toxicity in the presence of organic carbon. Conclusions from the review included that very little data existed relating DOC and copper toxicity, there were no data relating DOC to dissolved copper, TOC appeared to reduce total copper toxicity unlike DOC which only slightly decreased total copper toxicity, and that there were no data to determine the effects of dissolved or total organic carbon on dissolved copper toxicity. Gruber and Rasnake, 1997, reported similar data with TOC reducing toxicity by 1 to 2 orders of magnitude over their test range. Edwards, 1997, also stated that natural organic matter (NOM) can reduce the toxicity of metals. The importance of differentiating between strongly bound, weakly bound, and bioavailable copper is also mentioned as well as methods for determining these. In addition, the sorption of copper to filters used for soluble copper determination was measured.

Laboratory assays with copper and simulated natural dissolved organic carbon (DOC) were used to determine copper bioavailability and toxicity to young salmonids. DOC concentrations of greater than 8 mg/l were reported to reduce copper toxicity

slightly while concentrations of DOC less than 8 mg/l were reported to delay but not mitigate mortality. Inorganic copper was reported as more accurate at predicting toxicity than total copper in the presence of DOC (Marr, 1995).

Chapman *et al.*, 1996, reported that single bioaccumulation factors for organisms were inappropriate as toxicant bioaccumulation is dependant on environmental concentrations. Additionally, many metals such as copper are essential to health and many organisms have the ability to mediate internal concentrations of these potential toxicants thus making a single bioaccumulation factor too general. Renner, 1997, discussed the factors affecting toxicity of metals in natural waters and the relevance to regulatory efforts. Speciation, hardness, pH, and other factors affecting bioavailability were discussed in general terms regarding their impact on toxicity. Deaver and Rodgers, 1996, reported that bioavailable copper, as measured by differential pulse anodic stripping voltammetry of labile copper, explained toxicity to *H. azteca*. while measurements of total copper did not. Their 10 day experiments spanned a range of factors controlling speciation of copper: pH, alkalinity, hardness, and conductivity were varied in each test. Cathodic stripping voltammetry and adsorptive cathodic stripping chronopotentiometry were employed to determine the lability of nickel, copper, and titanium in estuarine environments. Nickel was reported to occur in both stable and unstable complexes that were not in equilibrium with each other on the time scale of an estuarine environment. Copper complexes however, were reported to be in equilibrium with each other (Van den Berg, 1993).

The chemistry of copper in natural waters was discussed by Leckie and Davis, 1979. Coordination chemistry, copper speciation, and interactions in natural aquatic systems were presented with extensive references. Hodson *et al.*, 1979 presented an

overview of copper toxicity to aquatic biota. Toxic effects were grouped by organism levels of algae, invertebrates, and fish. Within the categories issues of acute and chronic effects, value as indicator organism, mode of action, physiological effects, and experimental approaches were discussed.

Algae uptake and transfer of metals in the food chain.

Wikfors and Ukeles, 1982, reported extensive inhibition of growth in algae *Isochrysis galbana* (*I. galbana*) and *Monochrysis lutheri* (*M. lutheri*) at copper concentrations of 47.3 ppm in enriched natural seawater. After conditioning to high copper concentrations however, cultures were developed that were adapted to living in copper concentrations of 47.3 ppm copper. These cultures were fed to 48 hour old grazing larval *C. virginica*. High mortality and poor growth was observed in the grazing larval oysters fed the algae conditioned to high (47.3 ppm) copper concentrations.

Lipophilic ligands, diethyldithiocarbamate (DDC) and 8-hydroxyquinoline (Ox) were reported to enhance the transfer and accumulation of copper into the algae *Thalassiosira weissflogii*. They proposed that the natural copper complexes in South San Francisco bay water were relatively hydrophilic and therefore were not directly transported across the algae plasmalemma. The lipophilic copper complexes Cu(DDC)_2 and Cu(Ox)_2 were more readily transported across the membrane than natural complexes (Phinney and Bruland, 1997).

Algae, *I. galbana*, *Phaeodactylum tricornutum* (*P. tricornutum*), and *Duanaliella tertiolecta* (*D. tertiolecta*) were cultured to tolerate high (15 to 60 mg/l) concentrations of cadmium. Diets of these algae were fed to young post-set oysters (*C. virginica*) and

clams (*M. mercenaria*). Oysters fed contaminated *D. tertiolecta* experienced mortality and weight loss while those fed contaminated *P. tricornutum* experienced no significant deleterious effects. No growth was observed in clams fed any of the contaminated algae but only those clams fed contaminated *P. tricornutum* demonstrated mortality. These results were indicative of the role that nutritional factors play in cadmium toxicity. When readily digested diets were presented to oysters the expression of cadmium toxicity increased (Wikfors *et al.*, 1994).

In a study of cadmium transfer from phytoplankton to oyster to mouse Hardy *et al.*, 1984, reported that plankton accumulated 70% of all cadmium supplied and oysters filtered out 85 to 95% of the phytoplankton. Approximately 59% of the oyster accumulated cadmium came from food sources with the remainder coming from dissolved sources.

Light induced oxygen production by 7 unicellular marine algae was measured during exposure to copper, mercury, cadmium, and zinc. Oxygen production by *I. galbana* in a 15-min incubation period was reported to decrease at concentrations from 2×10^{-6} M Cu to 2×10^{-5} M copper where it then leveled off to approximately 50% of controls and began to increase slightly up to 3×10^{-4} M. Observations after a 24 hour incubation period found that oxygen production did decrease to near 0 (Overnell, 1976).

Copper, zinc, and cadmium effects on growth, toxicity, and metal accumulation by *I. galbana* were reported by Zhihong, *et al.*, 1989. The effects of EDTA, fulvic acid, cysteine, and oxine on the growth, toxicity, and accumulation of the three metals was also reported. A 96 hour EC50 (growth) of $1.4 \mu\text{mol Cu/l}$ was reported for *I. galbana*. Edding and Tala, 1996 reported the effects of copper to *I. galbana* cultures as well as several other algae cultures. Growth of *I. galbana* was significantly greater than controls

at 1.6 $\mu\text{g Cu} / \text{l}$ at 24 hours. Between 1 and 25 mg Cu / l growth was significantly inhibited. Copper accumulation by *I. galbana* was demonstrated after 24 hours of exposure to 40 $\mu\text{g/l Cu}$.

In a personal communication with Dr. Bruce Parker, 1998 (Parker, 1998) he mentioned that *I. galbana* is a naked flagellate and that high vacuum or pressure, such as those in typical filtration techniques for soluble copper sampling, can strip the algae of their exterior and release the interiors of the cells thus elevating the measured soluble copper. He commented that in the 1950s researchers investigating Penicillin observed a "U" shaped response due to competitive binding sites in the bacteria. At intermediate concentrations the penicillin interfered with itself. In addition he commented that not all biological actions are gradual or proportional to stimuli. Some actions are more binary in nature, much like a switch that once is turned on may take a great energy input to reverse.

Data Analysis

The Spearman-Kärber method is a nonparametric analysis for estimating LC50 and 95% confidence intervals for a data set when the data do not fit the probit model but partial mortalities occur in test solutions. Data that does not monotonically progress must be smoothed and adjusted using Abbott's procedures. Limitations of Spearman-Kärber require that the smoothed adjusted mortality for the lowest concentration (not the control) must be equal to zero and the smoothed adjusted mortality proportion for the highest concentration must equal 1. When these limitations are not met the Trimmed Spearman-Kärber method should be used (Chapman *et al.*, 1995).

The Trimmed Spearman-Kärber method modification of the Spearman-Kärber method estimates the trimmed mean of the distribution of the \log_{10} of the tolerance. This trim consists of the maximum of factors calculated from either the lowest-non control test concentration data or the highest test concentration data (Chapman *et al.*, 1995). The extensive calculations required for the Trimmed Spearman-Kärber method are best handled by a computer program available through EMSL-Cincinnati (EMSL).

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Copper Toxicity to Larval *M. mercenaria*

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Abstract

Pre-set *Mercenaria mercenaria* (*M. mercenaria*), larval clams, were exposed to copper concentrations from 4 to 500 $\mu\text{g/l}$ and monitored for mortality, activity, development, and metamorphosis in a simple though novel experimental container. Approximately 10 individual larval clams were held in sealed 30 mm petri plates which were filled with 1.5 ml of artificial seawater or toxin solution. The larval clams were observed directly with a light microscope for up to two weeks. This approach permitted meaningful comparisons of responses at low levels of copper concentration. For mortality observations, a repeatable non-standard dose response curve resembling an “M” was observed for clams exposed to 4 to 29 $\mu\text{g/l}$ Cu concentrations and fed the algae *Isochrysis galbana* (*parke.*) (*I. galbana*). The “M” dose response curve was characterized by survival similar to or better than controls at doses of 4 and 15 $\mu\text{g/l}$ Cu, while doses of 7 and 29 $\mu\text{g/l}$ Cu exhibited mortality greater than controls. In contrast to mortality, activity, as judged by swimming, was observed to follow an exponential dose response at these same concentrations. A comparison of larval clam responses to copper, dosed as either a commercial fungicide or copper nitrate, indicated the fungicide had no clear antagonistic or ameliorative effects relative to similar copper only exposures. Experiments on the uptake of soluble copper by *I. galbana* confirmed literature reports to the copper concentrating ability of these algae. A theory is proposed that relates the copper concentrating effect of algae to the “M” shaped dose response observed in these experiments.

Five keywords:

Mercenaria, copper, toxicity, *Isochrysis*, larvae

Introduction:

During the 1990s, as farms on the Eastern Shore of Virginia increasingly employed plasticulture, an increase in larval *M. mercenaria* mortalities in commercial hatcheries was observed. Plasticulture covers crop mounds with plastic, which physically prevents the growth of weeds and therefore reduces the need for herbicides (Garnaud, 1994). The plastic increases the imperviousness of the field, and thus runoff is accelerated and less water enters the soil (Scott *et al.*, 1990). While less water enters the soil naturally, irrigation lines placed under the plastic allow more control over soil water content, which can reduce the need for pesticides that control hydrophilic organisms. The irrigation lines are also used for distributing fertilizer directly to the soil surface, and in combination with the protection of the plastic can reduce nutrient losses from runoff (Aylsworth, 1997). Some chemicals, such as leaf surface applied copper based fungicides, chemical herbicides, and pesticides will readily be washed off leaves by rain, and without appropriate preventative measures, concentrations in runoff may be increased compared to traditional growing techniques.

Shellfish hatchery operators downstream of plasticulture fields alleged that extensive mortality and/or developmental defects in larval clams occurred during and immediately after significant rain events. Typically, those larval clams that survived beyond the rain event would fail to pass through metamorphosis from the straight hinge or prodissoconch stage to the pediveliger stage (Wilson *et al.*, 1997; Parks, 1997).

Monitoring of runoff from watersheds where plasticulture was employed in Virginia and South Carolina found high levels of copper, up to 1450 µg/l total Cu, and azinphosmethyl, fenvalerate, endosulfan, and chlorothalonil in concentrations from <1 to

> 100 µg/l in tidal creeks bordering on plasticulture fields following rain events (Scott *et al.*, 1990 and Dietrich *et al.*, 1996). For reference, the Virginia Department of Environmental Quality standard for endosulfan in saltwater is 0.0087 µg/l, 0.01 µg/l for azinphosmethyl in salt water, and 2.9 µg/l copper in saltwater.

Hatcheries are dependent on the watershed for water and food. Larval clams are held in large tanks containing water from nearby tidal streams or creeks and fed algae that is cultured in water from the streams. The use of artificial seawater would be prohibitively expensive so hatcheries must use the natural water available to them. It was theorized that runoff from fields entered the hatcheries causing increased mortality, deformation, and delayed development in the clams (Klawiter, 1998; Wilson *et al.*, 1997; R. G. Parks, 1997).

Although extensive literature exists in metal toxicity to embryonic, larval (Ringwood *et al.*, 1995; Ruiz *et al.*, 1995; Beiras and His, 1994; Spangenberg *et al.*, 1996), and adult bivalves (Behrans *et al.*, 1981 (a); Behrans *et al.*, 1981 (b); Zamuda and Sunda, 1982; Riedel *et al.*, 1995; Rule *et al.*, 1996), there is little recent literature relevant to larval *M. mercenaria* and copper toxicity (Davis and Hidu, 1969; Calabrese *et al.*, 1977 (a); Calabrese *et al.*, 1977(b)). One of the most frequently cited references, Calabrese *et al.*, 1977(a), reported an LC5, LC50, and LC95 of 4.9, 16, and 28 µg/l respectively for two day old larval clams exposed to copper for eight to 10 days in a static renewal dosing scheme (Calabrese *et al.*, 1977 (a), (b)). These experiments were initiated with 48 hour straight-hinge clams that had just passed beyond the embryonic state. Larval clams, (10,000 to 12,000) were placed in one liter beakers containing 1 µm filtered seawater at 24 ± 2 ppt salinity and $25^{\circ} \text{C} \pm 1^{\circ} \text{C}$ and fed *Isochrysis galbana* (*I. galbana*), and *Monochrysis lutheri* (*M. lutheri*). Copper - containing toxins were prepared from CuCl_2 and changed daily except on weekends. After 8 to 10 days larvae

were sieved and resuspended in 200 ml filtered seawater. A subsample was taken from the concentrated larvae, preserved in 5% neutral formalin, and survival counts made with an IIMC Particle Measurement Analyzer. Growth, based on the greatest width of shell along a line parallel to the hinge, was measured on a sample of 100 preserved larval clams from each dose. Effects of copper on metamorphosis to pediveligers or sublethal effects, other than growth, were not reported.

Preliminary research in this project included reproduction of methods similar to Calabrese *et al.*, 1977b. Results from those experiments were inconclusive, demonstrated a need for less variability in the results, and a less labor intensive method of assessing larval condition. The ideal approach would be a direct observation method to replace subsampling where all organisms included in the test would be accounted for on a daily basis whether live or dead.

The speciation and bioavailability of copper and its relation to toxicity has been discussed at length (Hodson *et al.*, 1979; Leckie and Davis, 1979; Van Den Berg, 1993; Breault *et al.*, 1996; Chapman *et al.*, 1996; Deaver *et al.*, 1996; Renner, 1997). In addition to the role of the copper species, the active role of the organism's dietary needs must be considered. Algae commonly fed upon by larval clams (*I. galbana*, *M. lutheri*) have been shown to accumulate metals in excess of their metabolic needs (Overnell, 1976; Zhihong *et al.*, 1989; Wikfors *et al.*, 1994; Edding and Tala, 1996; Phinney and Bruland, 1997). Zhihong reported a copper / cell ratio of $1.1 * 10^{-8}$ and $3.8 * 10^{-8}$ $\mu\text{g Cu} / \text{cell}$ for *I. galbana* cultures in equilibrium with 40 and 80 $\mu\text{g/l}$ copper respectively and 200 $\mu\text{g/l}$ EDTA. In the absence of EDTA copper / cell ratios of $3.04 * 10^{-8}$ and $6.55 * 10^{-8}$ $\mu\text{g Cu} / \text{cell}$ in equilibrium with 40 and 80 $\mu\text{g/l}$ copper were reported.

Edding and Tala, 1996, reported total copper to algae dry weight ratios over time in comparison to Cu^{++} concentrations in cultures of *I. galbana*. After 24 hours the weight to weight ratio of copper in algae was 37 times the weight to weight ratio of Cu^{++} in water. The calculated weight wet of an *I. galbana* cell based on cell diameter of 2 μm and length of 5 μm was $1.5 * 10^{-5}$ μg . Several studies have shown that algae contaminated with copper do transfer copper to bivalves and can cause significant mortality (Wikfors and Ukeles, 1982; Wikfors *et al.*, 1994; Edding and Tala, 1996). Doherty, 1986, discussed the dual modes of metals toxicity in *Cobiculara flumenia* (*C. flumenia*), the asiatic clam, from particulate - associated metals and soluble metals. Metals affected and accumulated in different organs depending on whether the exposure was of soluble or particulate nature. In *C. flumenia*, exposure to dissolved cadmium resulted in higher gill, mantel, and adductor concentrations of cadmium. Exposure to particulate (algae) associated cadmium resulted in higher concentrations of cadmium in visceral mass. Hardy *et al.*, 1984, concluded that bivalves accumulate more metal in the presence of phytoplankton than in the absence.

Thomann *et al.*, 1995, developed a steady state model relating the ratio of metal concentrations in *C. virginica* and *Mytilus edulis*, (*M. edulis*), the blue mussel, to sediment metal concentrations. Relevant components included sediment-water column partitioning, bioconcentration factor, depuration rate, metal assimilation efficiency from food, the bivalve feeding rate, and the growth rate of the organism. Thomann *et al.* 1995, reported that the model, calibrated with data from the NOAA Mussel Watch, indicated the food route of exposure was significant for metals but particularly so for Zn, Cd, Cu, and Hg.

A complicating factor in embryonic and larval toxicity assays is high natural mortality. Survival from embryos (0.080 mm) to a size that can be removed from a

hatchery and placed in the field (4 - 6 mm) is generally less than 1%. Much of this mortality occurs during early stages of development (Castagna and Kraeuter, 1982). The standard embryogenesis assay as presented by ASTM E724-94 provided methods for the static testing of the embryos of four species of saltwater bivalve mollusks including *M. mercenaria*. ASTM presented a minimum control survival for 48 hour embryogenesis assays of 60% for *M. mercenaria* and 70% for oysters such as *Crassostrea gigas* (*C. gigas*), the pacific oyster, for test acceptability. A simplification of the ASTM methods was presented by His *et al.*, 1996, that reduced the time and cost of testing including features such as: induced natural spawning for gamete quality, earlier test exposure, and direct observation without subsampling. His reported 91-93% normal development of *C. gigas* and *Mytilus galloprovincialis* (*M. galloprovincialis*), the Mediterranean mussel, embryos to D-larvae in tests conducted in Coulter Counter accuvettes. His proposed a minimum normal development of 80% in *C. gigas* as acceptable with the simplified technique but he also stated that the acceptable level of survival and deformation is more a matter of convention and is largely dependent on the assay design.

Objectives

The objectives of this research were: 1) to assess the effects; mortality, activity, development, and metamorphosis; of copper on larval clams; 2) develop a compact test method with low variability to permit evaluation of the effects of copper without sacrificing the organisms; and 3) to gain insight into low level toxicity effects and the role of algae (as food) in the toxicity of copper to larval clams through comparisons of fed and unfed clams.

Methods

Apparatus preparation

All experimental equipment, with the exception of petri plates which were purchased sterilized and sealed by the manufacturer, was nitric acid washed (10%) and rinsed with Nanopure™ water. All equipment that contacted algae and clams was autoclaved following acid washing and rinsing with Nanopure™ water.

Artificial seawater

Artificial Seawater (ASW) was prepared in volumes sufficient to last through each experiment by adding Instant Ocean™ to Nanopure™ water until a salinity of 26.5 ppt was measured. This solution was continuously mixed for at least two weeks prior to use by dried house air that was passed through a 35 µm screen and a sterile 0.2 µm filter. Minor salinity adjustments were made as needed. ASW was filtered through a 0.45 µm Nanopure™ washed filter prior to use to remove any particulates. Total copper measurements for 26.5 ppt ASW ranged between 1 and 2 ppb which was comparable to published values of 3 ppb for Instant Ocean™ prepared at 32 ppt (Bidwell and Spotte, 1985).

Toxicant solutions

Copper nitrate solutions, 500 µg/l, were prepared from FisherBrand copper standard solutions by dilution with ASW. Soluble copper was then measured by Flame

Atomic Absorption Spectrophotometry (FAAS) to confirm concentrations. Immediately prior to test initiation a serial dilution from the 500 µg/l stock solution was made to produce toxicant solutions at each concentration. Each dilution was checked for copper content by Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS). For the 5 - 495 µg/l experiment, dilutions were divided into two bottles, one for algae fed testing and one for unfed testing and water chemistry.

Water Quality

Artificial seawater (ASW) was checked for its suitability to maintain *M. mercenaria* cultures prior to use. Ideal larval development conditions were: salinity 26 to 27 ppt, temperature between 22.5 and 26.6° C, pH between 7.5 and 8.5, and dissolved oxygen above 5 mg/l (ASTM, 1994; Funderburk *et al.*, 1997). Dissolved oxygen was measured with a YSI Model 58 dissolved oxygen meter that could compensate for salinity. All solutions had an initial dissolved oxygen concentration greater than 7 mg/l. A Fisher Model 620 Accumet meter was used to measure pH. The pH of ASW and toxicant solutions was between 8 and 8.5. Total ammonia was determined with Dry Tab® Ammonia NH₃ / NH₄ test kits from Aquarium Pharmaceuticals. A temperature slightly below the midpoint of the range of optimum temperatures was selected to minimize bacterial growth. Temperature was maintained in a walk-in constant temperature room at 24° C with a continuous recording monitor. Temperature plots were inspected regularly to confirm the temperature had not deviated significantly from 24° C. Salinity was measured by a hydrometer.

Counting and observation

An Olympus CH-2 light microscope was used for algae and clam counting in conjunction with a Bausch and Lomb WP 7854 stereoscope for confirmation of counts as needed. An Olympus BH-2 light microscope was used for photography with an Olympus PM-6 35 mm camera. A Sony Model DXC-151A video camera was used for recording activity observed with the Olympus BH-2. A 0.02 mm calibrated slide was used throughout photography and videography.

Algae culture

I. galbana (parke.) was obtained from Carolina Biological Supply and was cultured continuously in 26.5 ppt ASW with Florida Aquafarms Microalgae Grow at 24° C without aeration. A light cycle of 16 hours on and 8 hours off from cool white florescent bulbs, ~ 230 lux, was used for established cultures while new cultures were started in reduced light, ~130 lux. Typical maximum density of 1,500,000 cells per ml was reached in 10 to 14 days. Algae density was determined with a Hausser Hy-lite Ultra Plane Improved Neubauer Hemacytometer. Algae were concentrated on a Beckman model J21-C centrifuge at 3500 rpm for 20 minutes for feeding. Concentrated algae were added to toxicant solutions and mixed to bring the algae to a density of 100,000 cells / ml.

Copper uptake by algae

A stock test solution was prepared from ASW, MicroAlgae Grow™ fertilizer, and CuNO₃. Test cultures were prepared in one liter Erlenmeyer flasks filled with stock

solution and inoculated with *I. galbana*. Control solutions were made with stock test solution only and were not inoculated. Controls and test cultures were maintained in the same environment previously described for algae culture. Soluble copper samples were taken and algae counts made at 0.5, 1, 2, 4, 8, 16, and 32 hours then daily thereafter until cultures matured. Accumulation of copper by algae was measured as the difference of soluble copper concentrations between the control and test cultures. Tests were also performed on mature cultures of *I. galbana* that were no longer in the growth phase, typically cultures 2 to 3 weeks past initial inoculation. Mature cultures were dosed with CuNO_3 then soluble copper and algae densities were measured over time.

Metal Analysis

A Perkin Elmer HGA-600 GFAAS with an AS-60 autosampler and Zeeman 5100 background correction unit and a Perkin Elmer 703 Atomic Absorption Spectrophotometer were used for copper determinations. The use of standards prepared in ASW of the same salinity as the samples were employed for FAAS measurements.

Soluble and total copper was measured in accordance with USEPA Methods 3005, 3010, and 3020 (USEPA, 1997). Soluble copper was also measured by centrifugation. As shown by Hidmi and Edwards, 1998, 0.45 μm membrane filters commonly used in the determination of soluble copper will sorb soluble copper at a pH greater than 8. Instead of filtration, samples were centrifuged @ 14,000 rpm for 30 minutes to remove particulate associated copper from solution. Also shown by Hidmi and Edwards, 1998, centrifugation provided similar soluble copper measurements when compared to membrane filtration at conditions where no sorbtion was detected. Further

review of Hidmi and Edwards, 1998, indicated lower centrifuge speeds would be acceptable such as 4000 rpm for 20 minutes.

Organism transport and acclimation

Prodissoconch (straight hinge) clams were transported in natural seawater at approximately 13°C from a coastal hatchery to Virginia Tech. Clam densities in transfer were approximately 25 clams per ml. Total time in transit was a maximum of 10 hours. Clams were transferred to two 1 liter flasks and were acclimated over a 24 hour period by siphon addition of *I. galbana* at 100,000 cells / ml in ASW immediately upon arrival. The clams were fed daily to maintain flask concentrations of 100,000 algae cells / ml and ASW changes every 48 hours were made by sieving clams on a 35 µm polyester screen followed by resuspension with fresh ASW.

Experiment setup

Larval clams were sieved from acclimation flasks on a 35 µm screen and then gently washed into a petri dish with the appropriate toxicant or control solution. 10 ± 1 clams were gently pipetted to clear 30 mm plastic petri dishes using an Eppendorf pipette in 10 to 20 µl volumes. Impingement stress was minimized by not repeatedly placing and removing clams. A 1.5 ml volume of toxicant or control solution was pipetted to the petri dish and tilted slightly to spread the solution completely over the petri bottom. The dish was sealed by dispensing a ring of petroleum jelly to the interior perimeter of the petri dish top with a syringe and gently placing the cover on the dish. The initial weight was then recorded. Daily observations of live, dead, and swimming clams as well as their

development were made using light microscopy, and dish weight was monitored to keep track of salinity rise via evaporation.

Control Survival

While His *et al.*, 1997, did not report data on *M. mercenaria* with the simplified methods, based on ASTM differentiation between species, it is likely that normal development in *M. mercenaria* would exceed the 60% minimum of ASTM, but less than reported 91-93% normal development in *C. gigas*. Calabrese *et al.*, 1977(a) reported survival data as a percentage of controls but did not report a control survival percentage or report any minimum acceptable survivorship in methods. Based on these criteria a minimum survivorship of 60% in controls was established for test validity in tests of survival through metamorphosis.

Statistical Analysis

LC50 and EC50 calculations were performed by Trimmed Spearman-Kärber (TSK) Program Version 1.5 (USEPA Ecological Monitoring Research Division). Further analysis was performed by Dunnett Program Version 1.5 (USEPA Ecological Monitoring Research Division). Dunnett Program Version 1.5 conducts an ANOVA which is used to obtain an error value for Dunnett's Procedure, a comparison of treatment means to determine which means are different from the control at the 5% level of significance, and the magnitude of difference required for a sample to be statistically different from the control mean. Comparisons of survival data at single doses were performed by ANOVA: single factor with the respective p-statistic noted.

Results

Copper uptake by algae

The first measured difference in soluble copper between the control and test solutions occurred after algae densities had increased to 17 times the initial population from 57,500 to 992,500 cells/ml in 15 days. Soluble copper concentration in the controls was 101 $\mu\text{g/l}$ Cu while copper concentrations in algae solution cultures had decreased to 70 $\mu\text{g/l}$ Cu. The copper to cell ratio based on 992,500 cells/ml and an uptake of 31 $\mu\text{g/l}$ Cu was $3.12 * 10^{-8}$ $\mu\text{g/cell}$. A concentration factor (relative to the weight to weight concentration of soluble copper in water) of 29,700 was calculated based on the copper to cell ratio and a cell wet weight of $1.5 * 10^{-5}$ μg . Repetition of the experiment found similar results with the first measurable difference occurring after densities had risen from 27,500 to 430,000 cells / ml, a 16-fold increase in four days. Soluble copper decreased from 72 $\mu\text{g/l}$ Cu in the control to 66 $\mu\text{g/l}$ Cu in the algal solutions which resulted in a copper to cell ratio of $1.4 * 10^{-8}$ $\mu\text{g} / \text{cell}$ based on sorption of 6 $\mu\text{g/l}$ Cu. A concentration factor (relative to the weight to weight concentration of soluble copper in water) of 14,100 was calculated based on the copper to cell ratio and a cell wet weight of $1.5 * 10^{-5}$ μg .

Note that the difference in growth times was a feature of the kinetics of growth. In the first test, the culture was initiated with 57,500 cells / ml, approximately twice the initial concentration in the second assay and the first measurable difference was near peak density. As the culture approached peak density the rate of growth tapered off which extended the assay time. In the second test, measurements were made in the period of rapid growth prior to peak density.

Algae in the stationary growth phase rapidly accumulated copper. An average density of 1,135,000 algae cells / ml, exposed to an initial 100 µg/l soluble copper, accumulated 30 µg / l soluble copper within 30 minutes. Stationary growth phase culture density ranged from 1,005,000 cells / ml to 1,207,500 cells / ml.

Evaluation of sealed 30 mm petri plate design

To evaluate the petri plate design, five day old larval clams were placed in 10 replicate, 30 mm petri dishes, initially containing 100,000 cells / ml *I. galbana* in 26.5 ppt ASW and monitored for mortality, setting, and activity. No enumeration of algae densities in the petri dishes was made during the assay but cultures did not visibly vary greatly over the assay duration. Control survivorship exceeded 90% through 48 hours and was above 80% at 120 hours. Coefficients of variance (CV) remained less than 10% through 96 hours and less than 20% through 168 hours. Greater than 60% survival was observed beyond 400 hours. Settling and metamorphosis was first observed at 240 hours and reached a final value of 51.5 % of initial clams set at 480 hours (Fig. 1). Evaporation from containers was linearly related to time and evaporative losses remained less than 3.5% through 288 hours (3.5% evaporative loss is equivalent to 1 ppt salinity increase from an initial salinity of 26.5 ppt).

Range Finding Experiment

A range finding experiment was performed with 3 replicate sealed, 30 mm petri plates, containing 6 day old clams and 100,000 algae cells/ml ASW solution. Soluble copper concentrations that were evaluated were 29, 115, and 459 µg/l. After 24 hours, survival of clams exposed to 29 µg/l Cu was not significantly different from that of clams

exposed to 459 $\mu\text{g/l}$ Cu and both exhibited 97% survival. However, clams exposed to 115 $\mu\text{g/l}$ Cu showed a significantly lower survival of 74% ($p < 0.05$) (Fig. 2). While most clams exposed to 29 $\mu\text{g/l}$ Cu were actively feeding and swimming, clams exposed to 459 $\mu\text{g/l}$ Cu were not swimming, were not feeding, and appeared to be tightly closed with little internal organ and cilia activity apparent. The living clams exposed to 115 $\mu\text{g/l}$ Cu concentration demonstrated more internal activity than clams exposed to 459 $\mu\text{g/l}$ Cu but, like the 459 $\mu\text{g/l}$ Cu treated clams, were not swimming. This unusual pattern did not extend beyond 24 hours.

At 48 hours there was no significant difference in survival between the 115 and 459 $\mu\text{g/l}$ Cu doses ($p = 0.91$) but there was a significant difference between those two concentrations and the control and 29 $\mu\text{g/l}$ Cu groups ($p \leq 0.05$). The first significant difference between the 29 $\mu\text{g/l}$ Cu dose and control concentration was observed at 168 hours ($p \leq 0.01$) (Fig. 3). Complete (100%) mortality was observed after 144 and 120 hours for clams exposed to 115 and 459 $\mu\text{g/l}$ Cu respectively. After 480 hours 84% mortality was observed in clams exposed to 29 $\mu\text{g/l}$ Cu while at the same time only 46% mortality was observed in controls.

Although the control clams began to set at 168 hours and 95% of the surviving, 54% of (initial), control clams set by 480 hours, no setting was observed for clams exposed to 115 or 459 $\mu\text{g/l}$ Cu. Setting that did occur at 29 $\mu\text{g/l}$ Cu was delayed, the clams were lethargic, poorly developed, and in some cases deformed. After 10 days of exposure, 11% of the initial control clams had set while only 3% (1 out of 32) of clams exposed to 29 $\mu\text{g/l}$ Cu had set. Most common deformations were extruded organs and malformed siphon. Other abnormalities included clams that appeared to have set but their shell had not increased in size or shape beyond the "D" shape and failure to set after 6 weeks of exposure. Beginning at 48 hours, a small, approximately 30 x 10 μm , orange-

red area about the color of a new penny was observed about the perimeter of the shells of clams exposed to 115 and 459 $\mu\text{g/L}$. This feature did not appear in any of the control experiments, or in the lower dose of 29 $\mu\text{g/l}$, or in any succeeding experiments.

Evaporative losses remained below 3.5% through 264 hours and as in the control experiment were linearly related to time.

5 - 495 $\mu\text{g/l}$ range experiment

A dilution series of 5, 7, 14, 29, 57, 119, 240, and 495 $\mu\text{g/l}$ soluble Cu was tested with 3 replicates, each containing 10 to 11 7-day old larval clams in solutions prepared containing 100,000 cells/ml *I. galbana* in ASW. Five replicate controls were prepared with copper content of 1 $\mu\text{g/l}$. In addition to treatments containing algae, 5 replicate controls without algae and 3 replicates each at 14, 29, and 495 $\mu\text{g/l}$ without algae were tested to compare survival, development, and activity with clams that were fed and were not fed *I. galbana*.

A distinct “M” shape dose response was consistently observed for survival up to 120 hours. Clams exposed to a solution of 5 $\mu\text{g/l}$ Cu had slightly better survival than the control clams that were exposed to 1 $\mu\text{g/l}$ Cu (the copper concentration of controls). Clams exposed to 7 $\mu\text{g/l}$ Cu survived less well than both the controls and clams exposed to 5 $\mu\text{g/l}$ Cu but instead of survival continuing to decrease with increasing copper concentration, survival increased at 14 $\mu\text{g/l}$ Cu (Fig. 4). Survival then dropped again with higher copper concentration.

A survival of 60% or better was maintained through 144 hours for the control group of algae-fed clams and setting was first observed for these clams at 192 hours (8

days after first exposure to copper or 16 day old clams). By 408 hours, 21.2% of the initial control group of clams had set. The first complete mortality in algae-fed clams exposed to copper was observed at 72 hours for clams treated with 495 $\mu\text{g/l}$ copper (Fig. 4). Only one observation of swimming activity (48 hours) demonstrated the same bimodal response observed in the survival data. All other observations showed a brief increase in activity from the control concentration to 5 $\mu\text{g/l}$ but thereafter decrease (Fig. 4). Unlike the previous range finding experiment conducted with 6 day old clams, very few clams in the full range experiment showed any gross deformations. The few abnormalities observed included mild extrusion of internal organs and immature shells.

LC50 and EC50 values were calculated for algae-fed larval clams exposed to copper using the USEPA Trimmed Spearman Karber software; these are presented in Table 1. LC50 values of 142, 62.4, 21.2, and 11.7 $\mu\text{g/l}$ Cu were determined at 24, 48, 96, and 192 hours, respectively. EC50 values (based on active swimming) were considerably lower than the LC50 values; for example, at 24 hours the EC50 was 20.2 $\mu\text{g/l}$ Cu while the LC50 was 142 $\mu\text{g/l}$ Cu. Using the ANOVA function (alpha - 0.05, 1 sided t-test with Bonferroni adjustment) in the USEPA Trimmed Spearman Karber software for data at 288 hours, a LOAEL (lowest observed adverse effect level) of 14.4 $\mu\text{g/l}$ Cu and a NOAEL (no observed adverse effect level) of 7.4 $\mu\text{g/l}$ were calculated for mortality.

For the unfed larval clams, 60% or better survival was observed through 192 hours in the control group. Setting was first observed for the unfed control clams at 168 hours, but these clams had organs and shells that were visibly smaller than the fed controls. No noticeable differences were observed for active swimming between the algae-fed and unfed control groups (Fig. 5). Although the unfed clams did not receive *I. galbana*, some other microorganisms other than *I. galbana* were observed in solution. As it was not possible to completely rid the test solutions of accompanying microbiological

organisms, it is possible that the larval clams brought food organisms with them that propagated over the period of the assay.

Between days 5 and 9 of exposure, unfed clams had significantly higher ($p \leq 0.05$) survival in soluble copper solutions of 29 $\mu\text{g/l}$ compared to algae fed clams dosed at the same copper concentration. No significant difference in survival was observed between fed and unfed clams in 14 $\mu\text{g/l}$ soluble copper solutions (Fig. 6). At 495 $\mu\text{g/l}$ soluble copper dose, 100% of unfed clams were killed within 24 hours. Fed clams dosed at 495 $\mu\text{g/l}$ soluble copper demonstrated survival not significantly different from zero at 24 hours and were completely killed by 72 hours. Unfed clams were significantly more active than fed clams in soluble copper solutions of 14 $\mu\text{g/l}$ during days 1 and 3 ($p \leq 0.02$) and noticeably more active from day 1 through 9. Unfed clams were significantly more active than fed clams in soluble copper solutions of 29 $\mu\text{g/l}$ during days 1 and 2 ($p \leq 0.05$) and noticeably more active from day 1 through 4 (Fig. 6).

While in the 14 and 29 $\mu\text{g/l}$ copper doses the absence of algae generally enhanced survival to varying degrees of significance, no significant difference was observed in larval metamorphosis. No setting occurred in unfed or fed clams exposed to 29 $\mu\text{g/l}$ Cu. While the fed clams that were exposed to 14 $\mu\text{g/l}$ set one day earlier than unfed clams the difference in setting, 2.8% for unfed and 3.3% for fed clams, was insignificant. Similar to the unfed undosed group, however, the set unfed clams exposed to 14 $\mu\text{g/l}$ Cu were noticeably smaller than those that set at the same fed dose.

Copper Nitrate and Kocide 101

A low level dilution series in the range of what was likely to be found in Eastern Shore watersheds was tested to compare the responses of larval clams to CuNO_3 and a

copper based fungicide used in tomato plasticulture. Two replicates of 7 day old larval clams treated at 4, 8, 11, 15, and 29 $\mu\text{g}/\text{l}$ total Cu from CuNO_3 or Kocide 101™ were prepared with 4 controls. Kocide 101 is designed as a wettable powder in that it never totally dissolves but stays suspended in solution, and consequently total copper was chosen as a reference base instead of soluble copper. Unlike previous experiments, this experiment was performed without sealing the 30 mm petri dishes with petroleum jelly. The salinity increase remained within an acceptable range for larval development over the first five days with salinity rising from 24.5 ppt on day 0 to 27.5 ppt on day 5.

Copper Nitrate Response

As in the 4-500 $\mu\text{g}/\text{l}$ experiment the “M” dose response pattern emerged quickly and was persistent. Control survivorship remained above 60% through 96 hours. While clams exposed to 4 $\mu\text{g}/\text{l}$ Cu from CuNO_3 consistently survived less well than controls, clams exposed to 8 $\mu\text{g}/\text{l}$ survived better than controls. At both 48 hours and 96 hours survival of larval clams exposed to 15 and 29 $\mu\text{g}/\text{l}$ Cu from CuNO_3 were similar and both exhibited significantly greater survival than clams exposed to 11 $\mu\text{g}/\text{L}$ Cu from CuNO_3 ($p \leq 0.10$) which consistently demonstrated the worst survival of all CuNO_3 exposures (Fig. 7).

Kocide 101™ Response

A similar “M” pattern was observed in clams exposed to Kocide 101™. Survival of clams exposed to Kocide 101™ was substantially less than controls at 4 $\mu\text{g}/\text{L}$, but unlike clams exposed to CuNO_3 , mortality was consistently greatest at 15 $\mu\text{g}/\text{L}$ Cu and least between 8 and 11 $\mu\text{g}/\text{l}$ Cu. ANOVA was applied to interpret the responses of larval

clams to Kocide 101™ and CuNO₃. While survival at 11 µg/L Cu was consistently different and less than controls for CuNO₃ exposures, the response of larval clams to Kocide at 15 µg/l Cu was consistently different from controls. Both CuNO₃ and Kocide exposures exhibited at least one response significantly different from controls at 4 µg/l (Table 2). A response observed in the Kocide 101™ exposures but not seen in the CuNO₃ exposures was a change in the general response curve at 96 hours. Survival of clams after 96 hours of exposure to 11 µg/L Cu from Kocide 101™ differed ($p = 0.11$) from survival at 8 µg/L Cu. In contrast to this during observations at 48 and 72 hours survival of clams exposed to 11 µg/l Cu from Kocide 101™ was not significantly different from 8 µg/L Cu.

Interchemical comparison

Based on this single experiment, the source of copper, CuNO₃ or Kocide 101, did not significantly alter survival of *M. mercenaria* larvae at concentrations of 4 or 7 µg/l Cu after either 48 or 96 hours of exposure. At 11 and 15 µg/l Cu the source did affect the survival of *M. mercenaria*. Larvae survived significantly better in solutions containing 11 µg/l Cu prepared from Kocide than larvae exposed to 11 µg/l Cu prepared from CuNO₃. At 15 µg/l Cu the responses were reversed. Larvae survived significantly worse in solutions containing 15 µg/l Cu prepared from Kocide than larvae exposed to 15 µg/l Cu prepared from CuNO₃.

Discussion

Chamber viability

Evaluation of the petri dish experimental design demonstrated the feasibility of the petri dish for examining the effect of toxins to larval *M. mercenaria* survival and metamorphosis. The very thin water layer produced by 1.5 mls of solution made observation of the entire water depth possible without refocusing the microscope. Observation without subsampling reduced the variability associated with test chambers of larger volume where subsampling is required. Evaporative water loss was less than 3.5% at 288 hours which allowed salinity to remain within acceptable limits during the experimental period. Addition of algae established a self-supporting microcosm where the algae reproduced and provided food for the clams. It is likely that bacteria and other microorganisms grew in the petri plates but these were not enumerated or evident by a cloudy or turbid appearance. Minimal organism handling reduced losses associated with impingement from seining and pipetting and reduced the total time required for maintenance relative to static-renewal systems. The small toxicant volume necessary for testing is advantageous in terms of minimizing waste after the experiment is complete. The small size permits an experiment with 50 chambers to be performed in only 2 square feet, which in turn would allow them to be held in an incubator, rather than in a large water bath or constant temperature room.

Algae uptake of copper

Experiments in algae uptake of copper concurred with published data regarding uptake of metals by algae (Overnell, 1976; Zhihong *et al.*, 1989; Wikfors *et al.*, 1994;

Edding and Tala, 1996; Phinney and Bruland, 1997). A decrease in soluble copper was measured in cultures that were in the exponential growth phase. Algae accumulated copper to a copper / cell ratio of 1.4 to $3.1 * 10^{-8}$ $\mu\text{g}/\text{cell}$. Algae in the stationary growth phase accumulated copper to a similar degree. Stationary growth phase algae accumulated copper to a copper / cell ratio of $2.64 * 10^{-8}$ $\mu\text{g}/\text{cell}$ when initially exposed to $100 \mu\text{g}/\text{l}$ soluble copper. Copper / cell accumulation rates by algae in both the stationary and exponential growth phases were similar to ratios reported by Zhihong *et al.*, 1989.

While the algae did sorb copper from solution, it is unlikely that soluble copper concentrations declined appreciably in the petri plates. The low algal density used in test containers, $100,000$ cells / ml, was too low to significantly alter the soluble copper concentration in the test solutions. Algae did not noticeably increase in density during the test duration. This was probably due to the absence of algae fertilizer. Had algae populations increased significantly then soluble copper concentrations would have decreased appreciably.

For example, if it was assumed that each algae cell accumulated copper to the ratio of $2 * 10^{-8}$ $\mu\text{g}/\text{cell}$ in a culture of $100,000$ cells / ml, then $2 \mu\text{g}/\text{l}$ Cu would be removed from solution. However, the algae were cultured in control water which had between 2 and $3 \mu\text{g}/\text{l}$ Cu. Therefore, the algae would not exert all the copper demand on a new toxicant solution of higher copper concentration than the controls.

Gallagher, 1988, reported a satiated rate of ingestion of 86 and 388 cells *I. galbana* per hour for 2 and 10 day old larvae respectively. At 388 cells / hour and an algae copper content of $1.1 * 10^{-8}$ μg Cu / cell, (Equilibrium concentration with $40 \mu\text{g}/\text{l}$ copper in solution and $200 \mu\text{g}/\text{l}$ EDTA; Zhihong *et al.* 1989), the organism would

consume $1.02 * 10^{-4}$ ug of copper per day assuming feeding was not inhibited by a soluble copper concentration of 40 $\mu\text{g/l}$. Based on conservative calculated wet larval clam weight of 0.18 μg , from a spherical object with diameter of 150 μm and density equivalent to water, a copper consumption rate of 57 $\mu\text{g Cu}$ per day per gram of larval clam tissue is determined. By volume the consumption rate was 57,000 $\mu\text{g Cu}$ per day per liter of tissue.

Mortality and impairment

All three dosing experiments demonstrated non-standard dose responses when larval clams were exposed to copper and fed. Initially this was met with skepticism by the authors but each new experiment added confidence to the authenticity of observations. While survival at the 459 $\mu\text{g/l}$ concentration in the range finding experiment was similar to control survival, activity was significantly less. The clams stopped swimming and did not appear to be feeding suggesting that they were protecting themselves from copper exposure. Similar behavior has been documented in adult clams and has been suggested in pediveliger clams (Cunningham, 1979). Similarly, in the 4-500 $\mu\text{g/l}$ experiment, activity was significantly reduced when clams were exposed to concentrations below those producing mortality: e.g. a 48 hour EC50 (swimming) of 15.6 $\mu\text{g/l Cu}$ versus a 48 hour LC50 of 62.4 $\mu\text{g/l Cu}$ and a 24 hour EC50 (swimming) of 20.2 $\mu\text{g/l}$ that was 7 times smaller than the 24 hour LC50 of 142 $\mu\text{g/l}$.

An eight day LC50 of 12 $\mu\text{g/l Cu}$ was determined in this research; this value is 4 $\mu\text{g/l}$ lower than the 8 to 10 day LC50 of 16 $\mu\text{g/l}$ reported by Calabrese *et al.*, 1977(a). Unlike experiments of Calabrese *et al.*, 1977(a) initiated with 2 day old straight hinge clams, these experiments began with 5 to 7 day old larval clams that were approaching

metamorphosis. The lower LC50 from this research suggests that this stage of development may be more sensitive to copper than the younger organisms possibly due to the impending metamorphic activity and accompanying metabolic activity.

Sublethal effects

Development interferences, such as deformations, declining activity, and reduced feeding, occurred at concentrations below lethal doses. Deformation development may be linked to age at initial exposure. Clams in the range finder experiment were 6 days old at initial exposure and exhibited many deformations while clams in the wide range experiment were 7 days old at initial exposure and exhibited fewer abnormalities. Reduction in activity and feeding was apparent regardless of age at initial exposure.

Kocide - CuNO₃ Comparison

A slight difference in response was observed between Kocide 101™ and CuNO₃. The general shape of the responses were the same with the Kocide exhibiting greatest toxicity at 15 µg/L in contrast to CuNO₃ at 11 µg/L. However, both exhibited toxicity significantly different from controls at the 4 µg/L concentration which suggests that there were only subtle differences, if any, in the toxic effect on larval clams between the two chemicals. The inert ingredients in the Kocide 101 formulation do not appear to have major ameliorating or synergistic effects on clams at these concentrations.

Toxicity mechanism

While larval clam survival typically expressed a non-standard dose response pattern, activity was observed to follow a more typical exponentially decreasing response with increasing copper dose. In addition, as copper concentrations increased clams that were not fed survived better than clams that were fed. Considering that algae uptake and concentrate copper in excess of their metabolic needs, it is theorized that both particulate associated copper (e.g., algae) and soluble copper control the mortality rate in copper exposed clams.

In these experiments, as soluble copper concentration increased larval clam activity decreased and feeding decreased. Copper is known to associate with algae at weight to weight (W/W) concentrations greater than the W/W concentration of copper in water and therefore provided a concentrated form of copper to the clam. Food associated copper has been shown to affect different target structures in clams (Doherty, 1986).

The effective copper dose is more appropriate than soluble copper concentration when considering the impact copper may have on larval clams. Effective copper dose includes: 1) soluble copper exposure; effects of which have been demonstrated in exposures where clams were not fed, and 2) particulate associated copper; effects of which have been less reported in literature (Wikfors and Ukeles, 1982, Wikfors *et al.*, 1994, Edding and Tala, 1996).

Algae in the petri dishes did not increase to the density observed in the uptake studies, rather they maintained populations of approximately 100,000 cells / ml. Therefore, although these algae concentrated copper ($\sim 2 * 10^8 \mu\text{g Cu per cell}$), the soluble copper concentration in the petri dish changed very little. The algae were a

source of particulate associated copper that could be consumed in addition to the soluble copper to which the clams were exposed directly and could not avoid.

If the rate of algae consumption by clams was assumed to exponentially decrease with increasing soluble copper concentration, as the data for clam activity suggested, until the clams were no longer feeding, then there existed a range of copper concentrations where the effective copper dose at low soluble copper concentrations was greater than the effective copper dose at higher soluble copper concentrations where the clam was no longer feeding. The effective copper dose increase and range depended on the threshold of activity reduction, how much the algae increased toxicity relative to soluble copper exposure, and the concentration at which activity ceased. Figure 8 is a theoretical exposure model of this concept.

Effective copper dose is calculated from soluble copper and particulate associated copper and equates the two copper sources to a single soluble copper exposure that would have produced the same net effect as the two separate copper sources.

As figure 8 shows, at concentrations below the activity threshold, effective copper dose “X” is the sum of soluble copper “C” added to the product of soluble copper and the algae potentiation factor “M”. At concentrations greater than the activity threshold “A” but less than the zero activity threshold “Z”, effective copper dose is the sum of soluble copper and the product of soluble copper, algae potentiation factor, and a decreasing activity function $\{(Z-C)/(Z-A)\}^{(F)}$. The decreasing activity function accounts for the decreased feeding observed in larval clams that began at the activity threshold (3.9 $\mu\text{g/l}$) and declined until the zero activity threshold (29 $\mu\text{g/l}$) when essentially all feeding had ceased. At soluble copper concentrations greater than the zero

activity concentration effective exposure is equivalent to the soluble copper concentration as the larval clam had essentially stopped feeding.

When survival was plotted against effective copper dose a more traditional dose response curve was observed (Figure 9). Survival increased modestly at concentrations slightly greater than controls due to a copper micronutrient effect and then declined monotonically with increasing effective copper dose.

Thomann *et al.*, 1995, considered sediment to water partitioning, bioconcentration, depuration, metal assimilation efficiency from food, feeding rate, and growth rate in developing a model to relate the ratio of metal concentrations in adult *C. virginica* and *M. edulis*. Based on the NOAA Mussel Watch data set he concluded that the route of food ingestion was a significant route of exposure and particularly so for Zn, Cd, Cu, and Hg. For *C. virginica*, 94% of copper was accumulated via food ingestion. Thomann's model was not addressed towards larval clams, but the relevant components are the same. The larval clam has the same exposure routes as the adult clam with the exception that the free swimming larval clam has less contact with sediments.

Thomann's model established a fixed food ingestion rate for the model rather than a variable one dependant on copper concentration. The larval clam has the same exposure routes as the adult clam with the exception that the free swimming larval clam has less contact with sediments. The use of a fixed food ingestion rate for adults rather than a variable one dependent on copper concentration was appropriate, given that adults are much less sensitive to heavy metals than larvae (Funderburke *et al.*, 1991). However as reported here, larvae have been observed to decrease in activity with increasing copper concentration, therefore the model developed in this report was a very simple structure that included a component of decreasing consumption with increasing copper

concentration. To illustrate the large potential consumption of copper by larval clams, Gallagher, 1988, was combined with the work of Zhihong *et al.*, 1989, and a calculation made of daily uptake via food. Fifty-nine μg Cu per gram of tissue per day via *I. galbana* was determined for a solution of 40 $\mu\text{g}/\text{l}$ copper and 200 $\mu\text{g}/\text{l}$ EDTA assuming no decrease in activity due to copper exposure.

All observed data points in Figure 8 were from 96 hours in the 4 to 500 $\mu\text{g}/\text{l}$ Cu experiment. The first observed survival and exposure data points were control points. The increased survival and activity relative to controls at the second concentration, 5 $\mu\text{g}/\text{l}$, was presumed to be a micronutrient effect (Trefry *et al.*, 1983). At 7 $\mu\text{g}/\text{l}$ the metabolic requirements for the clam have been exceeded and toxic effects are observed. At 14 $\mu\text{g}/\text{l}$ soluble Cu, activity and feeding had decreased enough that the clams were consuming considerably less algae than clams exposed to 7 $\mu\text{g}/\text{l}$ soluble copper. Less algae consumption reduced exposure to particulate associated copper and therefore the effective copper dose was less than that of clams exposed to 7 $\mu\text{g}/\text{l}$ soluble Cu and so survival was greater at 14 $\mu\text{g}/\text{l}$. Beyond 14 $\mu\text{g}/\text{l}$ soluble Cu, activity and feeding were reduced such that exposure was primarily via soluble copper therefore each greater copper concentration corresponded with less survival.

Effective copper does not refer to an absolute measurable copper concentration but rather an exposure that has an effect equivalent to an all soluble exposure. The threshold and zero activity levels were based on experimental observations and represent the soluble copper concentrations at which activity began to decrease and had effectively ceased respectively. The algae potentiation factor combined the copper concentrating ability of *I. galbana* and the toxin efficiency via ingestion. The value of the algae potentiation factor was a grouped factor encompassing several variables and did not imply a particular W/W concentration ratio to water.

Conclusions

The sealed petri plate represents a viable alternative method for toxicity tests on larval *M. mercenaria*. Control survivorship in excess of 60% beyond 400 days was demonstrated. An 8 day LC50 of 12 $\mu\text{g/l}$, 4 $\mu\text{g/l}$ lower than the 8-10 day LC50 for 2 day old larval clams previously reported by Calabrese *et al.*, 1977 (a), was determined for larval clams treated at six days of age. Activity, as measured by swimming, was impaired at concentrations up to 7 times lower than lethal concentrations (at 24 hours). The activity or swimming ability of *M. mercenaria* larvae may serve as an early warning of water quality problems due to toxicity effects. Sublethal effects such as reduced growth, delayed metamorphosis, and deformations occurred at concentrations of 29 $\mu\text{g/l}$ and less. Neither soluble copper nor particulate (algae) associated copper appear to solely control toxicity in the larval clam. The combined toxic effect of soluble and particulate associated copper may produce unexpected dose responses at low copper concentrations.

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Figure Legends:

Figure 1: (Page 90) Survival and metamorphosis (setting) of larval *Mercenaria mercenaria* in artificial seawater containing no added copper in sealed plastic petri dish with 100,000 cells/ml *Isochrysis galbana* added. Clams were 5 days old at initial exposure. N = 10. Bars represent 1 standard error.

Figure 2: (Page 91) Average survival of 6 day old larval *Mercenaria mercenaria* exposed to copper in artificial seawater containing 100,000 cells/ml of *Isochrysis galbana* in a sealed 30 mm petri plate (N=3). Bars represent one standard error. Survival of controls (not shown) at 24 hours was not significantly different from those clams exposed to 29 or 459 $\mu\text{g/l}$ Cu.

Figure 3: (Page 92) *Mercenaria mercenaria* response to copper. Clams were contained in sealed petri plates in 1.5 mls of test solution and had an initial *Isochrysis galbana* density of 100,000 cells/ml. Clams were 6 days old at initial exposure. Bars represent one standard error.

Figure 4: (Page 93) Comparison of *Mercenaria mercenaria* response over time to copper in sealed petri plates with 100,000 cells/ml initial *Isochrysis galbana*. Bars represent 1 standard error.

Figure 5: (Page 94) Comparison of survival and setting (top) and active swimming (bottom) between larval *Mercenaria mercenaria* in control groups fed an initial *Isochrysis galbana* density of 100,000 cells/ml and control larvae that were not fed *I. galbana*. Clams were 7 days old at initial exposure. No significant difference was observed in survival or swimming. Artificial seawater without added copper was the matrix. Bars represent one standard error.

Figure 6: (Page 95) Comparison of larval *Mercenaria mercenaria* (initially treated when 7 days old) survival (top) and active swimming (bottom) at 14 and 29 $\mu\text{g/l}$ soluble copper with and without feeding of an initial 100,000 cells/ml *Isochrysis galbana*. Bars represent one standard error. No significant difference was observed between fed and unfed clams exposed to 14 $\mu\text{g/l}$ soluble copper.

Figure 7: (Page 96) Survival of *Mercenaria mercenaria* in artificial seawater with copper dosing with CuNO_3 and Kocide 101™. Clams were held in unsealed petri dishes in a total water volume of 1100 μl s with an initial density of 100,000 cells/ml *Isochrysis galbana*. Control copper content = 2.4 $\mu\text{g/l}$. Bars represent 1 standard error.

Figure 8: (Page 97) Theoretical relative clam exposure and clam activity. Activity impairment threshold 3.9 $\mu\text{g/l}$ Cu. Zero activity threshold 29 $\mu\text{g/l}$ Cu. Exponential decay rate $F = 3.9$ and algae potentiation factor of 3.9.

Clam exposure is given by:

$$\begin{aligned} \text{For } C \leq A : & \quad X = C + M \cdot C \\ \text{For } A < C \leq Z : & \quad X = C + M \cdot C \cdot [(Z - C) / (Z - A)]^F \\ \text{For } C > Z : & \quad X = C \end{aligned}$$

Where:

- C: Soluble copper
- A: Activity threshold, the soluble copper concentration where activity begins to decrease
- Z: Zero activity threshold, the soluble copper concentration where activity is essentially zero
- M: Algae potentiation factor
- F: Exponent characterizing activity decline
- X: Equivalent effective copper concentration

Figure 9: (Page 98) Observed Survival % vs. Effective Copper $\mu\text{g/l}$

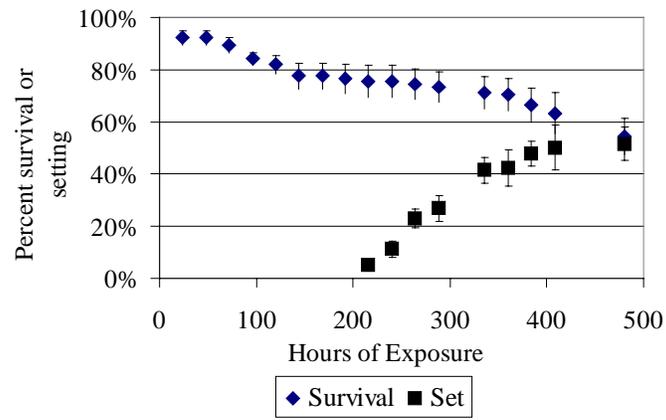


Figure 1

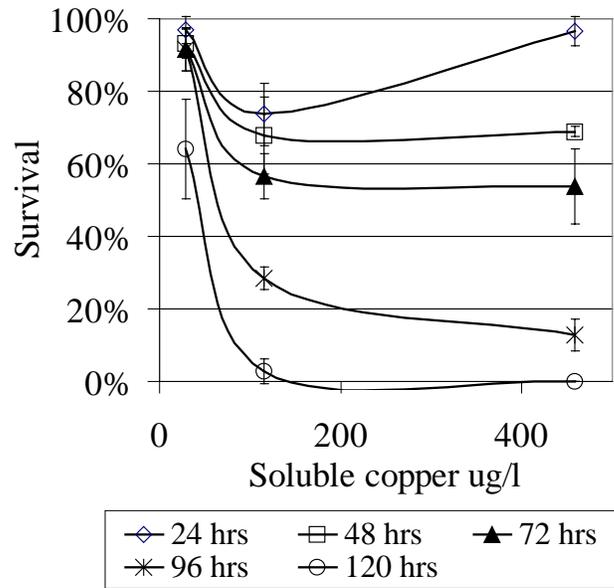


Figure 2

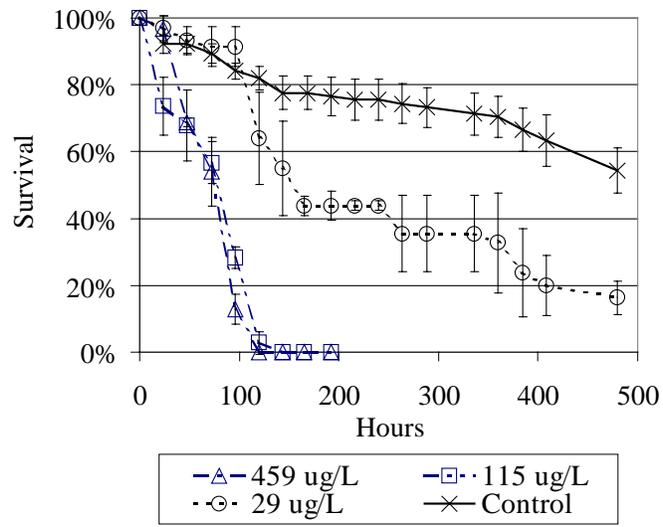


Figure 3

Mercenaria mercenaria copper dose response comparison over time

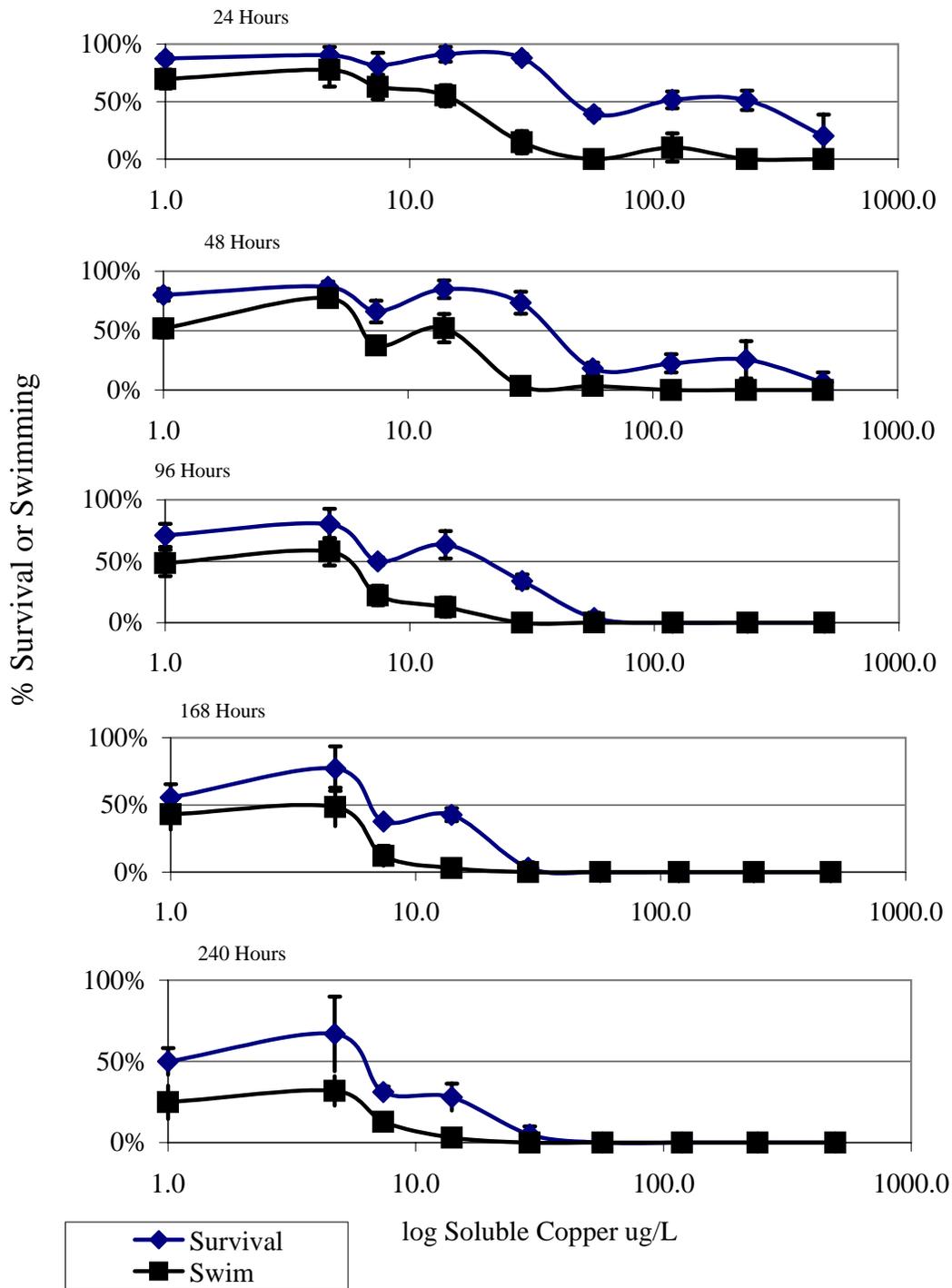


Figure 4

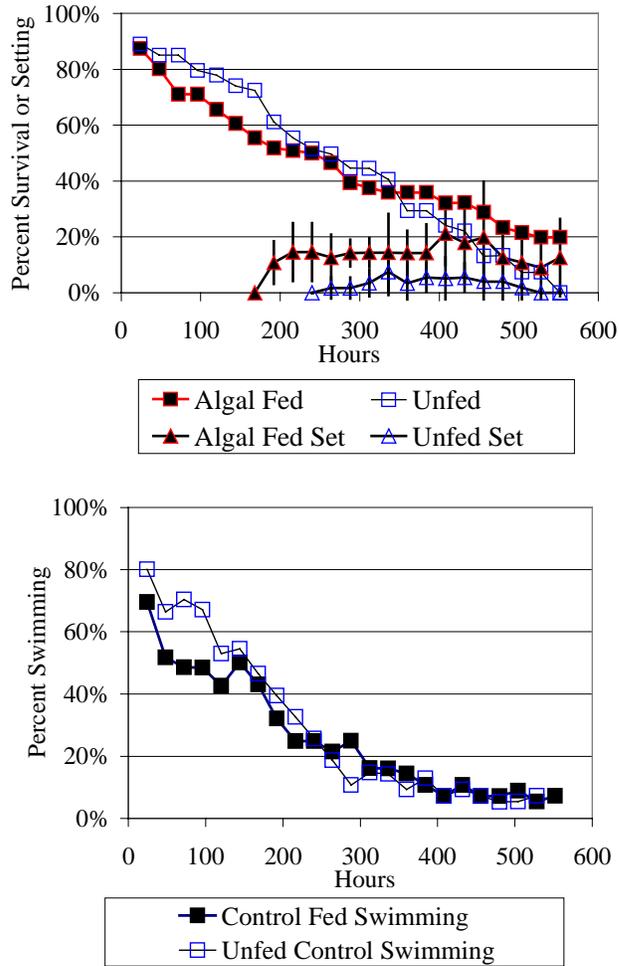


Figure 5

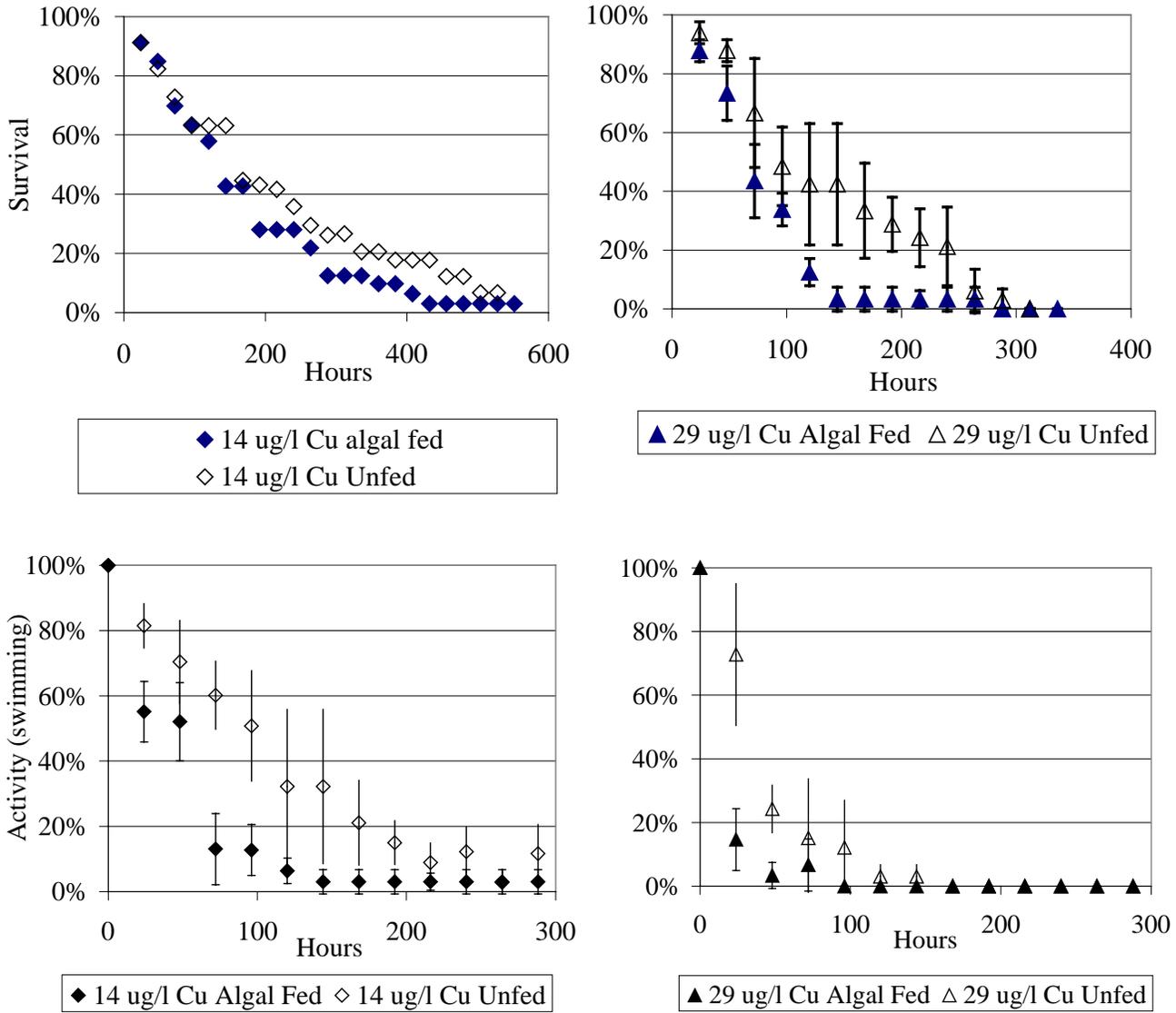


Figure 6

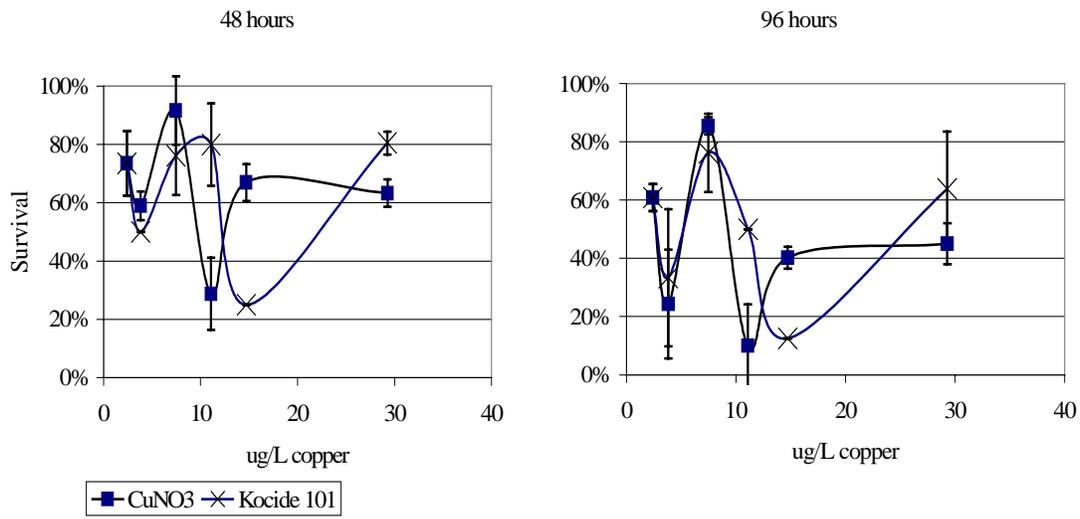


Figure 7

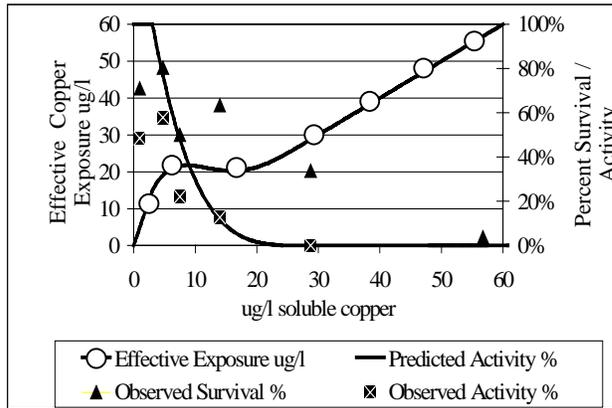


Figure 8

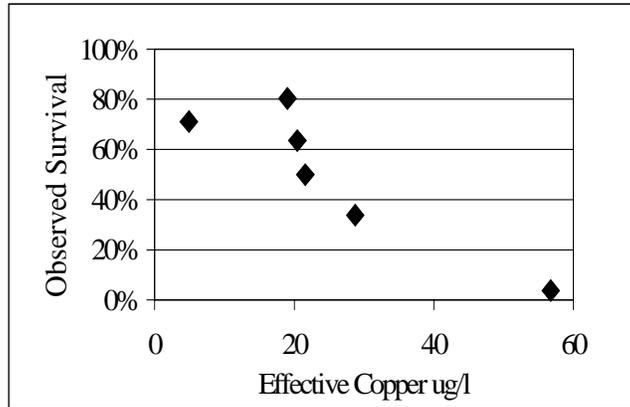


Figure 9

Table 1: Summary of ANOVA calculations of 7 day old *Mercenaria mercenaria* survival to copper exposure with initial *Isochrysis galbana* concentrations of 100,000 cells/ml. Control (artificial seawater) copper concentration was 1 ug/l.

| Time Hours | Soluble Copper ug/l | | | | | | | | | LC50 ^a | EC50 ^{a,b} |
|---------------|---------------------|---|---|----|----|----|-----|-----|-----|-------------------|---------------------|
| | Control | 5 | 7 | 14 | 29 | 57 | 119 | 240 | 495 | | |
| 24 | | | | | | X | X | X | X | 142 | 20.2 |
| 48 | | | | | | X | X | X | X | 62.4 | 15.6 |
| 72 | | | | | | X | X | X | X | 31.2 | 10.3 |
| 96 | | | | | X | X | X | X | X | 21.2 | 22.9 |
| 120 | | | | | X | X | X | X | X | 16 | 8.2 |
| 144 | | | | | X | X | X | X | X | 13.8 | 7.9 |
| 168 | | | | | X | X | X | X | X | 13.2 | 7.2 |
| 192 | | | | | X | X | X | X | X | 11.7 | |
| 288 | | | | X | X | X | X | X | X | 8.6 | |
| 336 | | | | | X | X | X | X | X | 10.3 | |

X = The mean for this concentration is significantly less than the control mean at an alpha = 0.05 (1-sided) by a t-test with Bonferroni adjustment of alpha level.

a - units, µg/l copper

b – EC50 based on swimming

| Table 2: Kocide and CuNO₃ ANOVA summary | | | | | | | | | | | | |
|---|--------------------|---|---|----|----|----|--------------------------------|---|---|----|----|----|
| μg/l Cu from CuNO ₃ | | | | | | | μg/l Cu from CuNO ₃ | | | | | |
| Time | Control = 2.4 μg/l | | | | | | Control = 2.4 μg/l | | | | | |
| Hours | Control | 4 | 8 | 11 | 15 | 29 | Control | 4 | 8 | 11 | 15 | 29 |
| 24 | | | | | | | | | | | | |
| 48 | | | | X | | | | | | | X | |
| 72 | | X | | X | | | | X | | | X | |
| 96 | | X | | X | | | | | | | X | |
| 120 | | | | X | | | | | | | X | |

X = The mean for this concentration is significantly less than the control mean at an alpha = 0.05 (1-sided) by a t-test with Bonferroni adjustment of alpha level.

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A Flow Through Test Chamber For Larval *Mercenaria mercenaria*

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Abstract:

A flow - through toxicity testing chamber was developed for observing larval *Mercenaria mercenaria* from free swimming to sedentary stages (approximately 80 to 150 μm). Organisms were held in 30 mm petri plates with 2 sections removed from the sides for toxicant solution drainage. These openings were covered by 35 μm polyester screening to retain the larval clams but permit algae (food) and waste products to pass through. This inner organism dish was housed in an outer catch dish (60 mm petri plate) that captured the effluent and routed it to a drain tube for collection and post chamber water quality measurements. Toxicant solution entered the chamber via a 0.8 mm id tube inserted into the chamber cover and dripped directly into the organism dish. Direct observation and enumeration of free swimming and set clams in the organism dish was possible without refocusing the microscope in this thin water layer environment. Water quality remained at optimum conditions throughout the assay due to five exchanges of test container volume per hour. Metamorphosis from free swimming to sedentary clams occurred with relatively low variability between replicates.

Five keywords

Mercenaria, copper, chamber, flow - through, larvae

Introduction:

While static and static-renewal testing methods have often been used for the determination of toxic effects to larval bivalves, flow - through methods for these organisms are lacking, in particular for *Mercenaria mercenaria* (*M. mercenaria*), the hard clam. Development of a flow - through method for *M. mercenaria* posed challenges due to the microscopic size of the clam and its high natural larval mortality. The goal of observing the organism at two different functional stages added additional challenges as well. Observation techniques had to be capable of observing both the swimming and sedentary stages of the organism. Table 1 categorizes the small size of *M. mercenaria* during the relevant period of the organisms life and Figure 1 is a typical free swimming larval clam.

Mercenaria mercenaria is much smaller than organisms from the *Cladoceran* family which forms the basis for most of the literature on flow - through testing of very small free swimming organisms. Mature *Ceriodaphnia dubia* (*C. dubia*) and *Daphnia magna* (*D. magna*), typically range from 1.4 to 8 mm in size respectively (Ferrando *et al.*, 1995; Pennak, 1953). While *M. mercenaria* eggs have a width between 60 and 85 μm when released, they have been observed to pass through a 35 μm screen but be retained on a 25 μm screen (Funderburk *et al.*, 1991).

M. mercenaria also has a very high natural rate of mortality in early life. While a mature healthy clam can release as many as 30,000,000 eggs in a given spawn, survival from the embryonic stage (0.080 mm) to a size that a hatchery can profitably place in the

field (4- 6 mm) is less than 1%. Much of the mortality occurs during the first few weeks following fertilization (Castagna and Kraeuter, 1982).

A test designed to evaluate the period from straight hinge through metamorphosis is desirable for assessing toxicity to larval clams. The metamorphosis from free swimming to bottom dwelling organism required observation and / or sampling techniques that could monitor both life stages equally and in a manner that permitted the enumeration of both life stages at the same time over a period of 7 to 10 days. The extended testing period made flow - through testing methods attractive as they could maintain dissolved oxygen levels and present a consistent toxic environment to the organism not possible with static and static-renewal techniques.

While numerous publications exist in metal toxicity to embryonic, larval (Beiras and His, 1994; Ringwood *et al.*, 1995; Ruiz *et al.*, 1995; Spangenberg *et al.*, 1996), and adult bivalves (Behrans *et al.*, 1981 (a); Behrans *et al.*, 1981 (b); Zamuda and Sunda, 1982; Riedel *et al.*, 1995; Rule *et al.*, 1996), literature relevant to the toxicity of copper to larval *M. mercenaria* is relatively old and few (Davis and Hidu, 1969; Calabrese *et al.*, 1977 (a); Calabrese *et al.*, 1977(b)). Calabrese *et al.* 1977 (a), determined an 8 to 10 day LC5, LC50, and LC95 of 4.9, 16.4, and 28 µg/l respectively for 48 hour old larval *M. mercenaria* in a static renewal dosing scheme.

Calabrese *et al.*(1977 (a), examined the clams only at the conclusion of the test and after preserving the organisms. Hatchery operators state that the larval clams often seem to be growing normally and then fail to set. Observing metamorphosis success and the developmental successes in the process is essential to gain a more comprehensive awareness of the effects of copper on larval *M. mercenaria*. Therefore a new method that assessed the toxicity of copper to larval *M. mercenaria* was initiated that provided for

observation of the development, survival, and activity from straight hinge clam up to and including metamorphosis to juvenile.

Eastern Shore Background

In the 1990s high levels of organic and inorganic pesticides found in agriculture runoff were suspected of producing excessive mortalities in shellfish hatcheries along the Eastern Shore of Virginia. A variety of organic and inorganic contaminants were found in agriculture runoff from these watersheds and similar watersheds in South Carolina including azinphosmethyl, fenvalerate, endosulfan, and chlorothalonil at concentrations from (<1 to > 100 µg/l) (Scott *et al.*, 1990; Dietrich *et al.*, 1996). Dietrich *et al.*, 1996, reported concentrations up to 1450 µg/l total copper in streams following rain events. The effects of agricultural runoff and its components were studied by Luckenbach *et al.*, 1996. Bioassays with caged *P. pugio*, an important link in the estuarine food web, stationed in agricultural runoff areas on the Virginia Eastern Shore showed that runoff could cause up to 100% mortality in the organism and that metal toxicity was a potential source of toxicity. Laboratory studies also demonstrated the toxicity of azinphosmethyl, fenvalerate, and endosulfan to *P. pugio*. This research was initiated to explore the relationship between copper and clam toxicity.

Acute, 48 hour static assays with bivalve embryos were described in ASTM Standard E 724-94 (ASTM, 1994). Standard practices, experimental conditions, control survivorship expectations, and analytical methods are described for tests with embryos (first exposure less than 4 hours following fertilization) of *Crassostrea gigas* (*C. gigas*), the pacific oyster, *Crassostrea virginica*, (*C. virginica*), the eastern oyster, *M. mercenaria*, and *Mytilus edulis* (*M. edulis*), the blue mussel. His *et al.*, 1997, reported a modified ASTM procedure for 48 hour embryogenesis tests using Coulter Counter

accuvettes that did not require subsampling. Control survival in excess of 90% was reported for *C. gigas* in 48 hour assays. While static techniques are generally the least technically demanding and least expensive they are frequently limited in duration due to oxygen depletion, toxin transformations, and water quality degradation.

In 1977 Calabrese *et al.* (a) reported heavy metal toxicity data for *M. mercenaria*. The test method placed 48 hour *M. mercenaria* larvae in beakers and exposed them to copper chloride solutions for 8 to 10 days with daily water changes except on weekends. At the conclusion of the experiment the organisms were seined, concentrated, mixed, subsampled, then preserved for counting and measurement.

The authors attempted to use a method similar to Calabrese *et al.* 1977 (a) to evaluate copper toxicity to larval *M. mercenaria* through metamorphosis. Larvae were placed in 1 liter flasks at a density of 15 organisms per ml and observed and counted at 48 and 96 hours. Initially it had been desired to assess vitality and metamorphosis daily but the process of triplicate counts with a Sedgewick Rafter cell with 40 test containers proved too time consuming. Moreover, data from the assay was too variable to provide conclusive results (LaBreche, 1998).

Previous research by the authors demonstrated that static petri plates techniques were compact, efficient, provided excellent control survivorship, and produced statistically significant discrimination between low (4 - 64 $\mu\text{g/l}$) copper concentrations. However, post experiment analysis of water quality was not possible in the 1.5 ml volume used. In addition, setting in controls was delayed to near the later end of the expected time to metamorphosis (clam age of 8 – 14 days). The petri dish was an excellent observation platform, for microscopic examination, and served as a starting

point for a flow - through chamber that would permit post exposure water quality monitoring (LaBreche, 1998 and LaBreche *et al.*, in submission).

An attempt to cage larval clams in 35 μm screened plastic 1 cm x 1 cm boxes and submerge them in flasks of gently circulated toxicants failed to produce a viable testing method. The optical properties of the screen were unacceptable for use with either a conventional microscope or inverted microscope. A reflecting stereoscope was used for observations but further difficulty in opening and closing the cages made the entire approach impractical. It was also determined that the flow through the 35 μm screen would be miniscule if the only driving force was the circulation of the toxicants around the chamber. A static pressure differential would be required to move a substantial volume of water across the screening.

Flowthrough methods

Many flow - through methods for a variety of organisms have been reported in the literature (Francis *et al.*, 1986; Knezovich and Harrison, 1987; Hatakeyama, 1987; Gallagher, 1988; Kersting and Wijngaarden, 1992; Kreutzweiser *et al.*, 1994; Lauth *et al.*, 1996; Diamantino *et al.*, 1997), but none exactly fit the needs of larval *M. mercenaria*. Many of the methods were designed for Daphnids with a general design theme being a bottle with screened entry and exit ports that allowed toxin to enter and exit but retained organisms. Gallagher, 1988, described a technique of tethering 2 and 10 day old larval *M. mercenaria* to a glass pipette. The tethered clams were then placed in a clear trough flowing with water and algae for observation of feeding mechanisms with high speed videography. While this had an advantage of easy observation of individual organisms, no data on long term survival following tethering was reported.

Lauth *et al.*, 1996 developed a flow-through apparatus for the testing of *C. dubia*. Two opposing 2.5 mm holes were bored in small (35 ml) polystyrene cups and covered with 120 μm Nitex screening. These cups were placed in troughs where toxicant solutions would flow in one end of the trough, through the screen, out the opposing screen, and on to the next cup in the trough. This method offered an interesting possibility of moving water across the static petri plate by screening the sides of the chamber. However, his method would have to be modified to produce a static head to drive toxicant across the smaller 35 μm screen as velocity heads safe for the enclosed organisms would be insufficient at producing adequate toxin circulation and waste elimination.

Mercenaria mercenaria is considerably smaller than any of the organisms examined by these test methods and required a microscope for assessment of development, activity, and life. Direct microscopic observation without subsampling required that the organisms be confined to a relatively shallow depth of field. This precluded the use of bottle techniques, as the depth of field would be too great for observation without refocusing.

Objective

The objective of this research was to develop a flow - through toxicity test system suitable for evaluation of larval *M. mercenaria* metamorphosis from free swimming to sedentary stage. The method was designed to permit direct observation of the microscopic clams without subsampling, allow simultaneous observation of both free

swimming and sedentary life stages, and be capable of maintaining uniform water quality.

Methods

Apparatus preparation

All experimental equipment was nitric acid washed (10%) and rinsed with Nanopure™ water. Glassware that contacted algae and clams was autoclaved following acid washing and rinsing with Nanopure™ water. The flow - through apparatus was not autoclavable therefore after acid washing and water rinsing each apparatus was washed with isopropyl alcohol (50%) and then rinsed with Nanopure™ water.

Toxicant Solutions

Artificial Seawater (ASW) was prepared in volumes sufficient to last through each experiment by adding Instant Ocean™ to Nanopure™ water until a salinity of 26.5 ppt was measured. This solution was continuously mixed for at least two weeks prior to use by dried house air that was passed through a 35 µm screen and a sterile 0.2 µm filter; minor salinity adjustments were made as needed. Artificial Seawater was filtered through a 0.45 µm Nanopure™ washed filter prior to use to remove any particulates. Total copper measurements for 26.5 ppt ASW ranged between 1 and 2 ppb that is comparable to published values of 3 ppb for Instant Ocean™ prepared at 32 ppt (Bidwell and Spotte, 1985).

A copper nitrate solution, 500 µg/l, was prepared from FisherBrand copper standard solutions by dilution with ASW. Soluble copper was then measured by Flame Atomic Absorption Spectrophotometry (FAAS) to confirm concentrations. Each time a toxicant series was prepared an 8 to 1 dilution from the 500 µg/l stock solution was made to produce a 62.5 µg/l Cu solution. A serial dilution was made from the 62.5 µg/l Cu solution to produce toxicant solutions at nominal concentrations of 31.3, 15.6, 7.8, and 3.9 µg/l Cu. Samples (500 ml) were taken at each dilution for water quality and metals analysis.

Water Quality

Prior to use, ASW was checked for its suitability to maintain *M. mercenaria* cultures. Ideal larval development conditions are: salinity 26 to 27 ppt, temperature between 22.5 and 26.6° C, pH between 7.5 and 8.5, and dissolved oxygen above 5 mg/l (ASTM, 1994; Funderburk *et al.*, 1997). Dissolved Oxygen was measured on a YSI Model 58 dissolved oxygen meter with appropriate compensation for salinity. All solutions had an initial dissolved oxygen concentration of greater than 7 mg/l. A Fisher Model 620 Accumet meter was used to measure pH. The pH of ASW and toxicant solutions was between 8.4 and 8.5. Total ammonia was determined with Dry Tab® Ammonia NH₃ / NH₄ test kits from Aquarium Pharmaceuticals. A temperature slightly below the midpoint of the range of optimum temperatures was selected to minimize bacterial growth. Temperature was maintained in a walk-in constant temperature room at 24° C with a continuous recording monitor. Temperature plots were inspected regularly to confirm the temperature had not deviated significantly from 24° C. Salinity was measured by a hydrometer.

Counting, observation, and growth measurement

An Olympus CH-2 light microscope was used for algae and clam counting in conjunction with a Bausch and Lomb WP 7854 stereoscope for confirmation of counts as needed. A calibrated reticule was used in combination with the Olympus CH-2 light microscope for size determinations. An Olympus BH-2 light microscope was used for photography with an Olympus PM-6 35 mm camera. A Sony Model DXC-151A video camera was used for recording activity observed with the Olympus BH-2. A 0.02 mm calibrated slide was used throughout photography and videography.

I. galbana (parke.) was obtained from Carolina Biological Supply and was cultured continuously in 26.5 ppt ASW at 24° C without aeration. Florida Aquafarms Microalgae Grow was used to fertilize cultures. A light cycle of 16 hours on and 8 hours off from cool white florescent bulbs, ~ 230 lux, was used for established cultures while new cultures were started in reduced light, ~130 lux. Typical maximum density of 1,500,000 cells per ml was reached in 10 to 14 days. Algae density was determined with a Hausser Hy-lite Ultra Plane Improved Neubauer Hemacytometer. To add to larval clams for feeding, algae were concentrated on a Beckman model J21-C centrifuge at 3500 rpm for 20 minutes. Concentrated algae were added to toxicant solutions and mixed to bring the algae to a density of 100,000 cells / ml.

Metal Analysis

A Perkin Elmer HGA-600 Graphite Furnace Atomic Absorption Spectrophotometer with an AS-60 autosampler and Zeeman 5100 background correction unit and a Perkin Elmer 703 Atomic Absorption Spectrophotometer were used for copper determinations. Standards prepared in ASW of the same salinity as the samples were

employed for FAAS measurements. Total and soluble copper was measured in accordance with USEPA methods 3005, 3010, and 3020 (USEPA, 1997). Following the completion of the experiment it was found that membrane filters used for soluble copper determination sorbed copper at pH greater than 8 and therefore soluble copper measurements were lower than actual. A different set of toxicant solutions identical to those used in this experiment were prepared and analyzed for soluble copper by centrifuge (Hidmi and Edwards, 1998) and showed that all copper in solution was present as soluble copper.

Organism transport and acclimation

Larval clams were transported in natural seawater at approximately 13°C from a coastal hatchery to Virginia Tech. Clam densities in transfer were approximately 25 clams per ml. Total time in transit was a maximum of 10 hours. Immediately upon arrival clams were transferred to two 1 liter flasks and were acclimated over a 24 hour period by siphon addition of *I. galbana* at 100,000 cells / ml in ASW. The clams were fed daily to maintain flask concentrations of 100,000 algae cells / ml and ASW changes every 48 hours were made by sieving clams on a 35 µm polyester screen followed by resuspension with fresh ASW.

Chamber Design

Two 8 x 12 mm sections were removed by a rotary grinding tool from the side of a 30 mm (i.d.) x 10 mm clear plastic petri dish (organism chamber). These holes were then covered by nylon screen with an opening size of 35 µm (organism chamber screen). This dish was placed in another larger (53 mm i.d. x 14 mm) plastic petri dish (catch

dish). A 1 cm hole was bored in the side of the outer catch dish and a 7 mm i.d. drain tube inserted. A hemispherical section was ground from the catch dish top to allow it to fit around the drain tube and neatly cover the entire assembly. A 1 mm i.d. filling tube was inserted through a hole drilled in the catch dish cover and the opposite end placed in the toxicant reservoir. The toxicant reservoir end of the filling tube was covered with a 35 μm nylon screen to minimize fouling of the organism chamber screen (Fig. 2).

Delivery tube diameter and differential water level (head) determined the rate of flow to the chamber. The catch chamber top prevented contamination from airborne materials and reduced evaporation. The organism dish retained the test organisms in an environment that permitted convenient observation. The catch dish retained sufficient water depth to keep the test organisms in water at all times even in the event that toxicant delivery was interrupted. This was possible because the catch dish was designed not to drain totally, but to leave enough water in the dish to keep the screens wet at all times. Keeping the screens wet at all times was necessary because in preliminary testing it was found that to initiate and maintain flow, both sides of the polyester screen had to be wet.

Approximately 12, 7 day old umboned clams were pipetted from acclimation flasks to the organism dish using an Eppendorf pipette in 10 to 20 μl volumes. Impingement stress was minimized by not repeatedly placing and removing clams. Control water, 1 ml, was then placed in the organism chamber and control water was added to the catch dish until water was in contact with both polyester screens. The toxicant filling tube was placed in its respective reservoir and a siphon initiated with a syringe. The delivery tube was then pushed through the hole in the top of the catch dish top and the catch dish top was then placed over the entire apparatus. The organism dish then began to fill with test solution and drain through the screen into the surrounding catch dish. Test solution drained intermittently from the catch dish, out the drain tube,

and into a catch bottle for water quality analysis. Three replicate chambers were used for each toxicant dose and controls. Each replicate chamber received toxicant solution from the same reservoir flask (Fig. 2).

Clams were observed after 24, 36, 48, and 72 hours of exposure. To count the organisms in a dish, the filling tube - cover assembly was placed aside and the organism dish was allowed to drain until approximately 1.5 mls of solution remained in the organism dish. The organism dish was removed from the catch dish, the base dried, and placed on a microscope for observation.

Statistical Methods

The Dunnett Program version 1.5 (EMRD 1.5) was used to perform EC50 calculations. Comparisons between individual toxicant concentrations were performed by a single factor ANOVA with respective p-statistic noted.

Results

Chamber Performance

The flow - through characteristics of the chamber were evaluated. Two tests of algae accumulation through the screen demonstrated little retention after 4 hours of algae exposure. Five hundred mls of algae solution at concentrations approximately three times the designed algae feed concentration were prepared and allowed to flow - through the apparatus. Algal densities higher than expected were intentionally selected to challenge the system. In test 1, there was no significant difference between the influent density and effluent density. In test 2, the influent initial density was 355,000 cells/ml and the effluent density was 305,000 cells /ml. In the second test some fouling of the

organism dish screen was observed. After this observation a screen of the same material as the organism dish screen was placed on the reservoir end of the filling tube. During toxicity testing *I. galbana* densities were not observed to increase and fouling of the organism dish screen was not observed.

Table 2 shows water quality parameters throughout the test. Average time weighted soluble and total copper concentrations in the influent and effluent are reported in Table 3. Note that while the influent and effluent soluble copper concentrations are similar, soluble copper concentrations are lower in general than the total copper concentrations. This is due to the use of membrane filtration for soluble copper measurements. As Hidmi and Edwards, 1998, demonstrated, at $\text{pH} > 8$ copper is sorbed to membrane filters commonly used for the measurement of soluble copper. Centrifuge methods were used to measure soluble copper in a later toxicant series identical to this and it was demonstrated that all copper as prepared in this assay was present in the soluble form.

The average flow was 420 mls / day per dish. This provided approximately 5 exchanges per hour in the organism dish. No clogging of screens was observed and no overflows of the organism dish occurred. Visual clarity of containers did not deteriorate over the test duration and observation remained as easy at 72 hours as at 0 hours. A single organism dish could be observed in 3 to 4 minutes.

Development and Mortality of M. mercenaria

No significant difference in survival was observed between 54, 26, 14, and 10 $\mu\text{g/l}$ soluble copper and controls at 24 hours. While a "U" shaped trend (survival at 54 $\mu\text{g/l}$ was similar to controls while the least survival was observed in intermediate doses) was

apparent in a plot of average survival, the lowest p-value determined was 0.12 when 14 μ g/l Cu was compared to controls. Survival of clams exposed to 14 μ g/l Cu was significantly lower than controls after 36 hours of exposure ($p = 0.04$). The "U" shape trend first observed at 24 hours continued and no significant difference in survival was determined between controls and any concentration other than 14 μ g/l. No significant difference in survival between controls and toxicant doses was determined at 48 hours. Unlike observations at 24 and 36 hours an "M" shaped trend emerged from a plot of the average survivals after 48 hours of exposure. The "M" shaped trend was characterized by decreased survival at 10 μ g/l Cu relative to controls, survival at 14 μ g/l greater than survival at 10 μ g/l, survival at 26 μ g/l less than survival at 14 μ g/l, and finally survival at 54 μ g/l greater than survival at 26 μ g/l Cu. After 72 hours of exposure, survival in clams exposed to 10 μ g/l Cu was significantly lower than survival in both controls ($p = 0.053$) and 54 μ g/l Cu doses ($p = 0.056$). No significant differences were observed between any other concentrations after 72 hours of exposure (Fig 3).

Metamorphosis was reported as the percentage of original organisms that were alive and set at the time of observation. Dead organisms were recorded but only live set organisms were included in comparisons of copper effects on metamorphosis. No metamorphosis was observed after 24 hours of exposure. Metamorphosis of clams exposed 14 and 26 μ g/l Cu for 36 hours was significantly lower than metamorphosis in controls ($p \leq 0.03$). Similarly metamorphosis of clams exposed to 14 and 26 μ g/l Cu was significantly lower than metamorphosis of clams exposed to 54 μ g/l Cu after 36 hours of exposure ($p = 0.03$, $p = 0.01$ respectively). No significant difference was determined between controls and the toxicant dose of 54 μ g/l Cu ($p = 0.49$) (Fig. 4)

Metamorphosis of clams exposed to 10 and 14 μ g/l Cu was significantly lower than controls after 48 hours of toxicant exposure ($p < 0.02$). Metamorphosis in the 14 μ

g/l Cu toxicant dose was significantly lower than that in the 54 µg/l Cu toxicant dose ($p < 0.05$) and metamorphosis of clams exposed to 10 µg/l Cu was substantially lower than that of 54 µg/l dose ($p = 0.07$). As in the 36 hour observations the 54 µg/l Cu dose and Controls were not significantly different from each other ($p = 0.60$) (Fig. 4).

Metamorphosis was significantly lower than controls in clams exposed to 10 and 14 µg/l Cu for 72 hours ($p < 0.01$, $p < 0.04$ respectively). Metamorphosis was significantly different between the two groups of clams exposed to 10 and 14 µg/l Cu as well ($p < 0.001$) with metamorphosis being significantly lower in clams exposed to 10 µg/l Cu than in clams exposed to 14 µg/l Cu. As observed at 48 hours, metamorphosis of clams exposed to 10 and 14 µg/l Cu was significantly lower than that observed in larval clams exposed to 54 µg/l Cu ($p < 0.02$, $p < 0.03$). As observed at both 24 and 48 hours no significant difference was observed between controls and larval clams exposed to 54 µg/l Cu ($p = 0.25$) (Fig. 4).

After 84 hours all live set clams were measured. Only the controls and 54 µg/l toxicant dose had sufficient live set clams for useful analysis. No significant difference in size, as measured along the set clams longest dimension, was determined between controls and clams exposed to 54 µg/l Cu. Set control clams averaged 147 µm in width while clams exposed to 54 µg/l Cu averaged 142 µm in width (Fig. 5).

Discussion

Flow - through chamber: Physical performance

The flow - through chamber performed according to design. Water quality measurements show that the chamber maintained a stable environment for the duration of the test. An average flow rate of 420 mls / day provided 5 water exchanges per hour in the organism chamber. Salinity, while it rose approximately 1 ppt from toxicant reservoir to the catch bottles, probably rose while in the catch bottles and not substantially while in the organism chamber . The organism chamber was completely covered while the catch bottles were partially covered to allow exiting toxin to drip into the catch bottle thus providing more surface area for evaporation. No accumulation of algae was observed in the organism chamber thereby providing the larvae with a consistent algae diet. Some initial difficulty with flow blockage by air bubbles in toxicant lines was remedied during startup and did not reoccur during the test. A larger toxicant reservoir with less height but broader dimensions would simplify dosing by providing longer duration between refilling of reservoirs.

Mortality and Impairment

The chamber demonstrated excellent potential for the determination of the effects of copper on metamorphosis. Clams began settlement at 8 days of age, which is comparable to the earliest typical day of settlement observed in the commercial hatchery from which these organisms came. Significant differences in metamorphosis were detected between very low (4 - 64 $\mu\text{g/l}$) concentrations of copper. The unexpected vitality of clams exposed to 54 $\mu\text{g/l}$ is not explainable with data from this experiment.

Nell and Holiday, 1986 reported that low levels (1.6 to 4.7 μM) of copper in solution enhanced settlement of the bivalve *Saccostrea commercialis* (Sydney Rock Oyster). While this is a different species it does suggest a possible reason for the enhanced survival. In contrast, survival of larval *M. mercenaria* exposed to 57 $\mu\text{g/l}$ copper was 18% and 3.7% after 48 and 96 hours exposure respectively in static petri plate experiments with no metamorphosis occurring during either period (LaBreche, 1998, LaBreche *et al.*, 1998). When the 54 $\mu\text{g/l}$ toxicant response was omitted, analysis yielded a 36 hour EC50 (metamorphosis) of 10.92 $\mu\text{g/l}$ with an upper 95% confidence interval of 12.41 $\mu\text{g/l}$ Cu and a lower confidence interval of 9.61 $\mu\text{g/l}$ Cu (EMRD, 1.5).

Survival in controls at the conclusion of the test was less than 40% and not significantly different from survival at 54 $\mu\text{g/l}$. While control survivorship was less than the 71 to 84 % observed in static petri plate experiments (LaBreche, 1998), this could be partially attributed to variability in robustness of organisms as well as to additional stress of being in a much more dynamic environment. Measurements showed that while the clams exposed to 54 $\mu\text{g/l}$ were slightly smaller on average, there was no significant difference in size between the controls and clams exposed to 54 $\mu\text{g/l}$ Cu.

Conclusions

The flow - through chamber demonstrated the ability to maintain the very small larvae of *M. mercenaria* in a practical observation environment. Water quality remained of high quality and consistent throughout the length of the assay and algae (food) density did not build up over time thus providing a uniform diet for the organisms. The method provided a convenient means of observing organisms without subsampling and without any additional manipulation following test initiation. Metamorphosis occurred at the

appropriate time and between replicate variability in metamorphosis was relatively low permitting statistical differentiation between 0 to 26 µg/l Cu, concentrations. Control mortality was in excess of what was desired but not completely unexpected as *M. mercenaria* naturally have a high rate of mortality and were placed in a more active environment than when tested in a static non-renewal or static renewal environment.

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List of Figures

- Figure 1:** (Page 130) Larval clams: Left; straight hinge, 48 hours. Right, umboned, 6 days.
- Figure 2:** (Page 131) Diagram of flow - through testing apparatus
- Figure 3:** (Page 132) Survival of *Mercenaria mercenaria* in flow - through chamber with continuous flow of test solutions and *Isochrysis galbana* at 100,000 cells/ml. Bars represent one standard error.
- Figure 4:** (Page 133) *Mercenaria mercenaria* metamorphosis from umbonate to pediveliger "setting" in flow - through petri plate apparatus. Organisms were continuously dosed copper and fed *Isochrysis galbana* at 100,000 cells/ml for the duration of experiment. Bars represent one standard error.
- Figure 5:** (Page 134) Comparison of size in set *Mercenaria mercenaria* after 84 hours of continuous exposure to copper in flow - through chamber. Control and toxin contained *Isochrysis galbana* at density of 100,000 cells/ml. Measurements are of the set clams greatest width. Bars represent one standard.

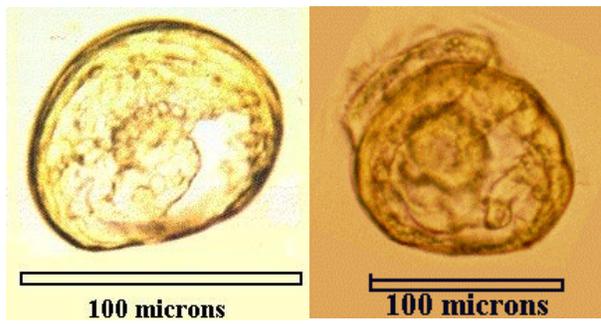
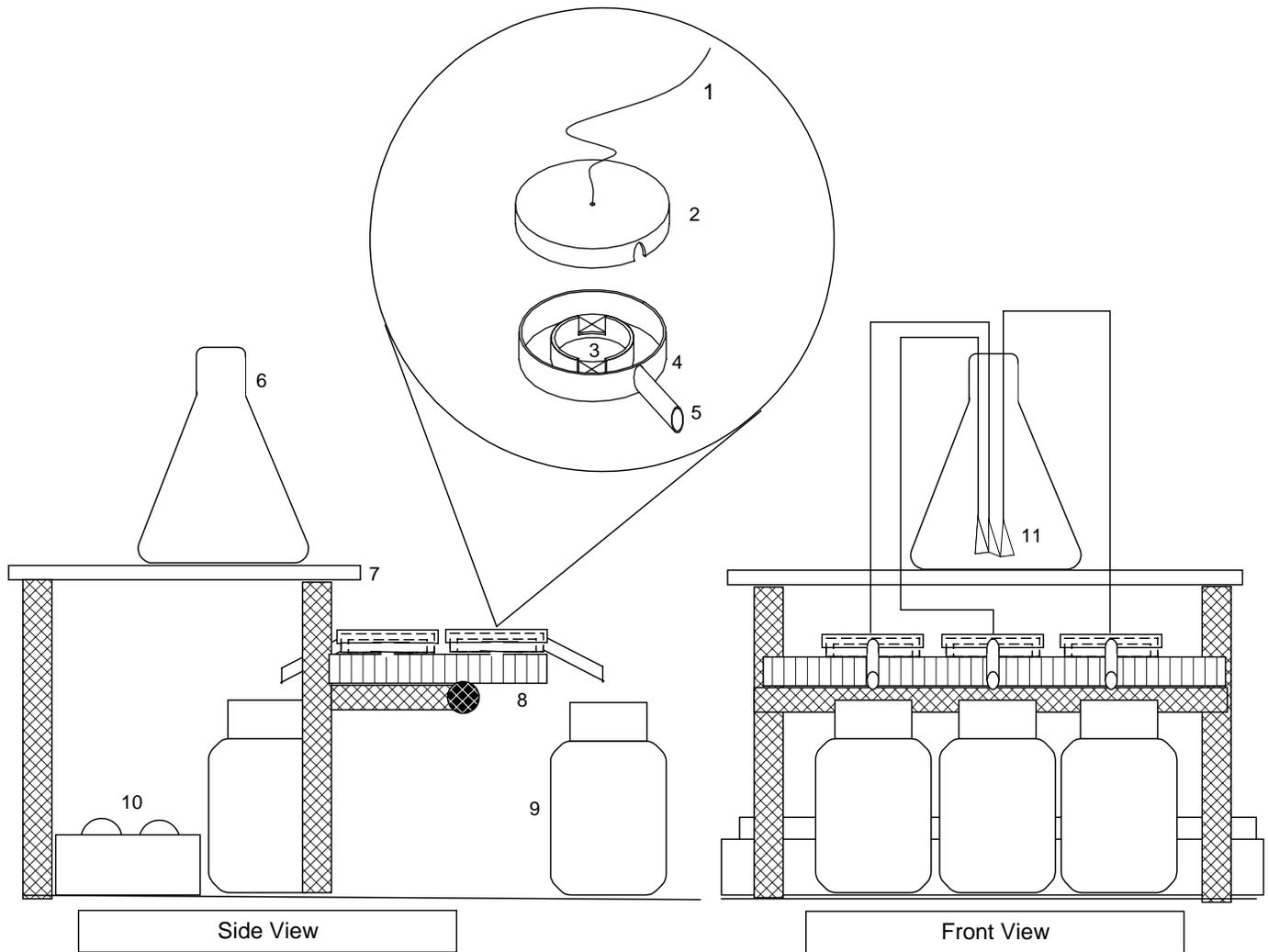


Figure 1



- | Legend | |
|--|-------------------------------------|
| 1. Fill Line | 6. Glass Erlenmeyer Flask Reservoir |
| 2. Cover | 7. Glass Table Top |
| 3. Organism Dish (Note "X" denotes Screened Areas) | 8. Plastic Light Diffuser |
| 4. Catch Dish | 9. Catch Bottle |
| 5. Drain Tube | 10. Florescent Light |
| | 11. Reservoir Screen |

Figure 2

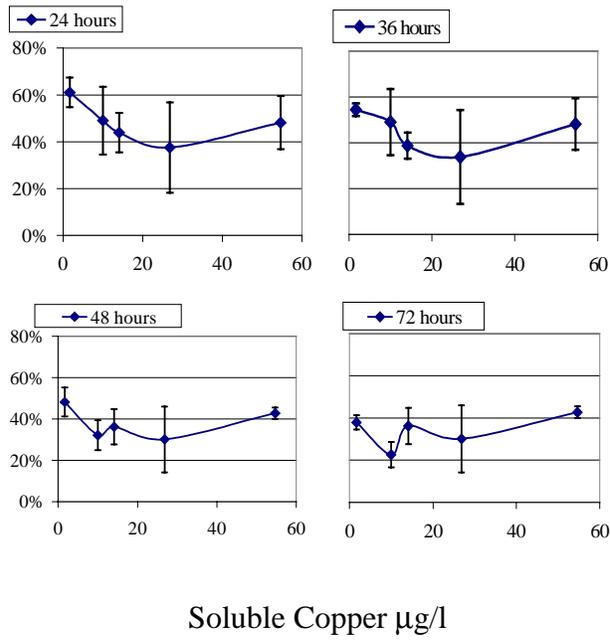


Figure 3

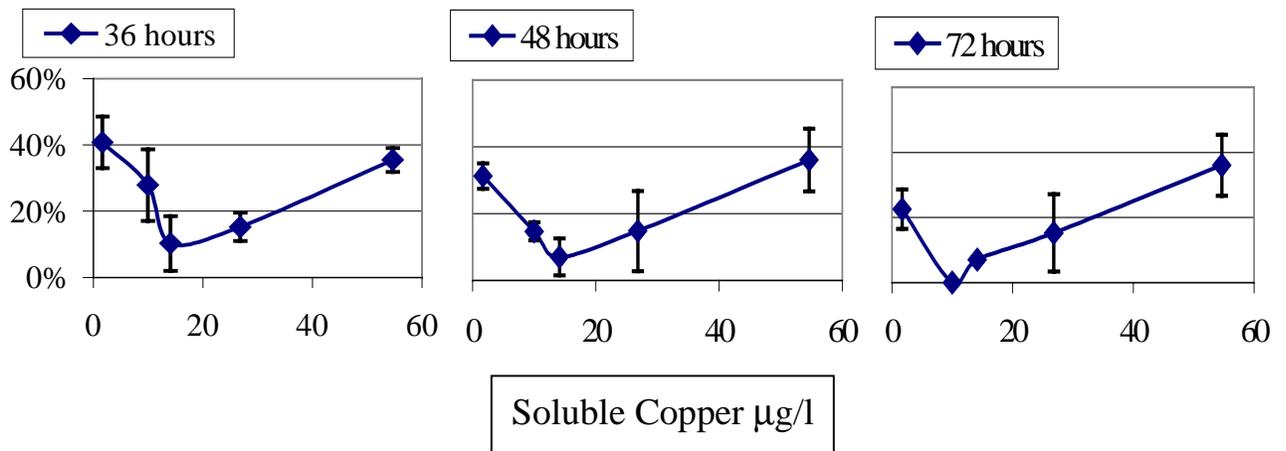


Figure 4

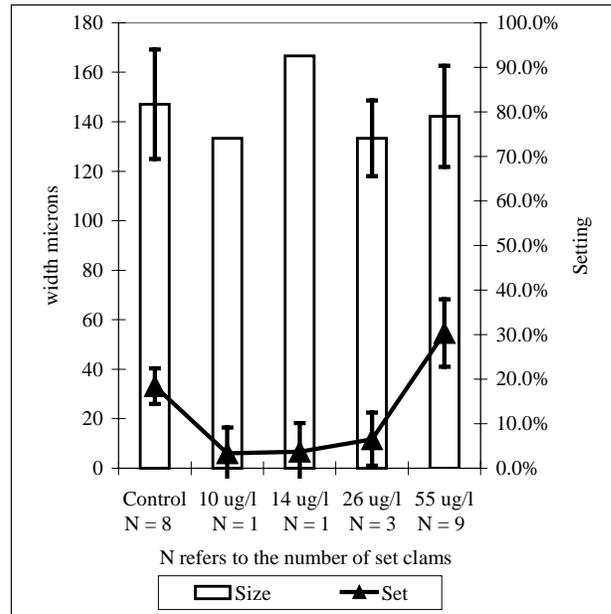


Figure 5

List of Tables:

Table 1: (Page 136) Typical size of *Mercenaria mercenaria* during first two weeks of life

Table 2: (Page 137) Summary of Water Quality Data

Table 3: (Page 138) Summary Of Influent and Effluent Time Weighted Copper Concentrations

| Table 1: Typical size of <i>Mercenaria mercenaria</i> during first two weeks of life. | | |
|--|-----------------------|---|
| 0 hours | 60-85 μm | Eggs at first release to the water |
| 0-fertilization | 163-179 μm | Gelatinous membrane expands in contact with water and expands the eggs diameter |
| 48 hours | 90-140 μm | Straight hinge, (prodissoconch I) |
| 48 hours to 8-10 days | 140-220 μm | Umboned |
| 8-10 days | 170-230 μm | Pediveliger |
| 8 days - and older | 200-210 μm | Metamorphosis from pediveliger to juvenile |
| Source: Funderburk <i>et al.</i> 1991, Castagna and Kraeuter, 1981 | | |

| Table 2: Water Quality Summary for Flowthrough Test | | | |
|--|---|--|--|
| Parameter | Initial Water Quality in Toxicant Reservoir | Water Quality in Toxicant Reservoir just prior to replenishment (12 to 15 hours) | Water Quality in Outwater |
| pH | 8.40 to 8.45 | 8.35 to 8.40 | 8.35 to 8.45 |
| Dissolved Oxygen | 7.3 to 7.8 mg/l | 7.0 to 7.3 mg/l | Rearation made measurement inappropriate |
| Salinity | 26.3 to 26.75 ppt | Not measured | 27 to 28 ppt |
| Ammonia | n.d. | n.d. to 0.35 mg/l | n.d. |

Table 3: Summary Of Influent and Effluent Time Weighted Copper Concentrations

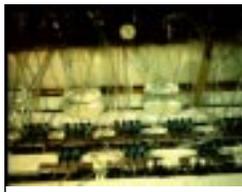
| | Influent | | Effluent | |
|---------|--------------------------------|------------------------------|--------------------------------|------------------------------|
| | Soluble $\mu\text{g}/\text{l}$ | Total $\mu\text{g}/\text{l}$ | Soluble $\mu\text{g}/\text{l}$ | Total $\mu\text{g}/\text{l}$ |
| Control | 1.3 | 1.9 | 2.3 | 5.1 |
| Dose 1 | 6.1 | 9.8 | 8.7 | 13.8 |
| Dose 2 | 10.0 | 14.2 | 11.4 | 18.5 |
| Dose 3 | 19.8 | 26.2 | 21.3 | 30.8 |
| Dose 4 | 42.3 | 54.1 | 51.6 | 52.4 |

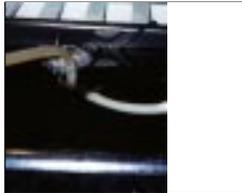
Note that the soluble copper concentrations were all measured with membrane filtration thus the soluble copper concentrations are lower than actual. Later measurements with identical solutions using centrifuge separation techniques demonstrated that all copper was present in the soluble form. The high effluent soluble concentration measured in dose 4 may represent a filter break.

Appendix A:

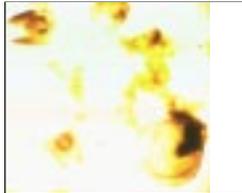
Photo Database Index

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|--------|--|---|--------------|---|
| 14 | LaBreche | | General Use | <i>Mercenaria mercenaria</i> swimmer close up from side | 600x, 1/50th |  |
| 15 | LaBreche | | General Use | <i>Mercenaria mercenaria</i> swimmer close up from side | 600x, 1/50th |  |
| 16 | LaBreche | | General Use | <i>Mercenaria mercenaria</i> swimmer close up from side | 600x, 1/50th |  |
| 45 | LaBreche | | Clams in General | Set Clam. Siphon is well extended and moving the body at such a rate to blur the image. | |  |
| 41 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | The Graduate Student (Timothy M. C. LaBreche) at work on the Eastern Shore of Virginia. | |  |

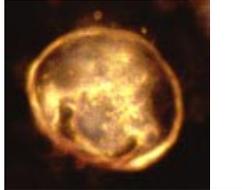
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|--------|---|---|----------------|---|
| 31 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Kegotank Bay Clam Company #1 | View From Dock |  |
| 32 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Kegotank Bay Clam Company #2 | View From Dock |  |
| 33 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Kegotank Bay Clam Company #3 | View From Dock |  |
| 34 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Aeration Manifold (gallery) | |  |
| 35 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Water Storage and Filtration (Bag filters: large bag is 25 um small bag is 1 micron filter) | |  |

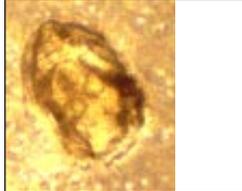
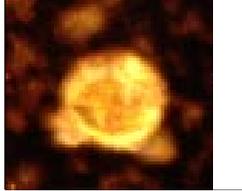
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|--------|---|---|---------|---|
| 36 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Pump and water bath for pump. Pump required continuous cooling to avoid thermal cutoff | |  |
| 37 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Heat Exchanger for Constant Temperature Bath. Aquarium Heaters are suspended in center of bucket. Coils of tubing circulate water back to the water bath. | |  |
| 38 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Front view of water bath, air lines, and flasks. 40 Flasks in total | |  |
| 40 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Bath and Flask arrangement. Black bands around neck of flasks are bungee cords. White dial is a thermometer. | |  |
| 39 | LaBreche | 3/1/97 | March 1997 Kegotank Bay Clam Company Static Renewal | Test Flask. White material is 35 um Polyester screening over the siphon drain tube. Blue object is an airstone. Not visible is a fill tube. | |  |

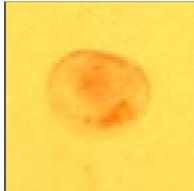
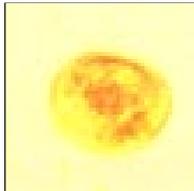
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|------------------|--|------------------------------------|---|
| 46 | LaBreche | 9/10/97 | Clams in General | Polyspermy. Too many sperm trying to fertilize one egg. The small flattend spheres around the periphery of the large spere are sperm. The inner sphere is the egg. | About 80 to 100 um wide |  |
| 48 | LaBreche | 9/10/97 | Clams in General | Straight hinge clam. 4 days old. Typically 100 um in width | |  |
| 49 | LaBreche | 9/10/97 | Clams in general | Normal Fertilization on the left: sperm no longer visible. Polyspermy on the right: many sperm still visible. | |  |
| 42 | LaBreche | 9/10/97 | Clams in General | Eggs and Sperm about 80 um across. <i>Mercenaria mercenaria</i> . 2 days old. | |  |
| 47 | LaBreche | 10/1/97 | Clams in general | 8 week old set clam. 0.8 mm wide. Raised in Erlenmeyer flask on diet of <i>Isochrysis galbana</i> . | Slide looks better than this image |  |

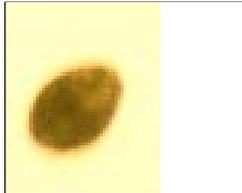
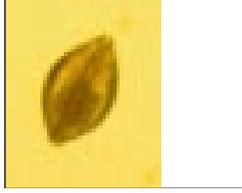
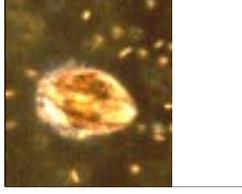
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|------------------|---------|------------------|--|--------------------|---|
| 43 | LaBreche | 10/1/97 | Clams in General | 8 weeks, Set clams up to 0.8 mm (largest) Raised in erlenmeyer flasks on diet of Isochrysis galbana. | |  |
| 44 | LaBreche | 10/1/97 | Clams in General | Stentor. A few of these organisms can clear out a dense algae culture (1 liter) in a day. Reference Dr. Bruce Parker Virginia Tech for more information. | |  |
| 70 | Natalie, Shepard | 4/20/98 | General | Algae growing chamber in constant temperature room. | |  |
| 79 | Shepard, Natalie | 4/20/98 | General | Improved Neubauer Hemacytometer. (Used for counting algae). | |  |
| 67 | LaBreche | 4/20/98 | Flow through | 29 ug/l Replicate K after 96 hours. Set / Near set | 150x magnification |  |

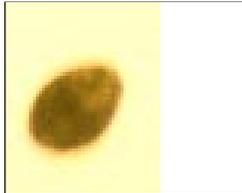
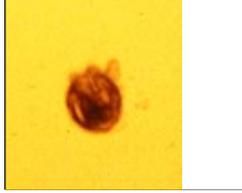
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|------------------|---------|--------------|--|---------|---|
| 69 | Shepard, Natalie | 4/20/98 | Flow through | Air filtration assembly. Cylinder is a 0.02 um filter. Not shown is a coars 35 um filter assembly. | |  |
| 72 | Shepard, Natalie | 4/20/98 | General | Artificial Seawater Carbouy. Continuously aerated. | |  |
| 71 | Natalie Shepard | 4/20/98 | General | Algae growing chamber with shroud down. | |  |
| 65 | LaBreche | 4/20/98 | Flowthrough | Replicate I. 55 ug/l Deformed Dead clam after 96 hours of exposure. | 150x |  |
| 73 | Shepard, Natalie | 4/20/98 | General | Constant temperature room view. Flowthrough rack on the right. Algae chamber on left | |  |

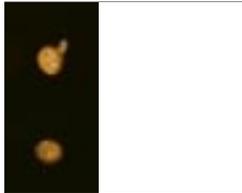
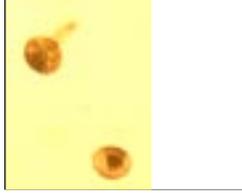
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|------------------|---------|--------------|---|----------------------|---|
| 75 | Shepard, Natalie | 4/20/98 | Flow through | Close up of flow through chamber. | |  |
| 76 | Shepard, Natalie | 4/20/98 | Flow through | Close up of flow through chamber. | |  |
| 77 | Shepard, Natalie | 4/20/98 | Flowthrough | Close up of reservoir filter screens | |  |
| 78 | Shepard, Natalie | 4/20/98 | Flow through | Flowthrough test stand. | |  |
| 68 | LaBreche | 4/20/98 | Flow through | 15 ug/l Cu replicate M after 96 hours of exposure | 150x phase contrast. |  |

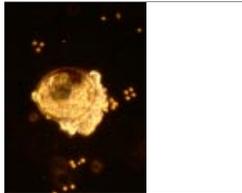
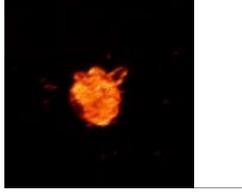
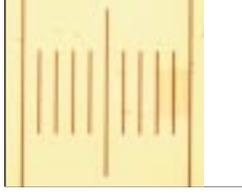
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|--------------|--|---------------------|---|
| 50 | LaBreche | 4/20/98 | General | Isochrysis galbana. A flagellated golden brown algae. Typical size is 5 to 8 microns | 1500x magnification |  |
| 64 | LaBreche | 4/20/98 | Flow through | 55 ug/l Copper. Replicate J set in profile after 96 hours of exposure | 150x magnification |  |
| 66 | LaBreche | 4/20/98 | Flow through | Control replicate H feeding. 96 hours of exposure. | 60x phase contrast |  |
| 62 | LaBreche | 4/20/98 | Flowthrough | 55 ug/l Cu. Replicate J afer 96 hours of exposure. Mixture of set and dead. | 60x |  |
| 63 | LaBreche | 4/20/98 | Flow through | 96 hours 55 ug/l Cu rReplicate I. 3 clams, two live and one dead. 96 hour | 150x Magnification |  |

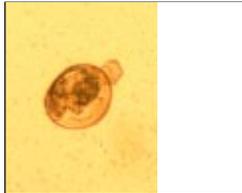
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|------------------|---------|--------------|--|-------------------|---|
| 74 | Shepard, Natalie | 4/20/98 | Flow through | View of Reservoir, chamber and catch bottles. | |  |
| 51 | LaBreche | 5/15/98 | Range Finder | 500 ug/l copper. Clam at 96 hours. Dead. Notice the tinge at the bottom right of the photo. Under normal exposure this was a bright copper color | 60x magnification |  |
| 52 | LaBreche | 5/15/98 | Range Finder | 500 ug/l copper. Clam at 96 hours. Dead. Notice the tinge at the bottom right of the photo. Under normal exposure this was a bright copper color | 60x magnification |  |
| 53 | LaBreche | 5/15/98 | Range Finder | 500 ug/l copper. Clam at 96 hours. Dead. Notice the tinge at the bottom right of the photo. Under normal exposure this was a bright copper color | 60x magnification |  |
| 58 | LaBreche | 5/18/98 | Range Finder | 29 ug/l Cu 168 hours exposure Swimming clam. | 60x magnification |  |

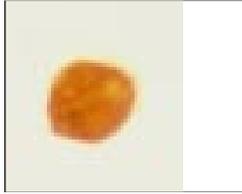
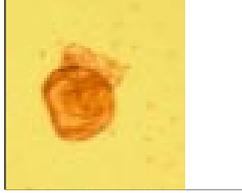
| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|--------------|---|--------------------|---|
| 61 | LaBreche | 5/18/98 | Range Finder | Control dish H swimmer. 168 hours. | 60x |  |
| 59 | LaBreche | 5/18/98 | Range Finder | 168 hours 27 ug/l (small set clam notice similarity to "D" shaped larvae). | 60x |  |
| 57 | LaBreche | 5/18/98 | Range Finder | 29 ug/l Cu 168 hours exposure Swimming clam. | 60x |  |
| 56 | LaBreche | 5/18/98 | Range Finder | 500 ug/l cu 168 hours of exposure. Dead in profile. | 60x magnification |  |
| 55 | LaBreche | 5/18/98 | Range Finder | Control replicate C swimmer 168 hours of exposure. Clam is 12 days old in photo. Blurred spots around clam are algae being swept by the clam. | 60x phase contrast |  |

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|----------------------|---|--------------|---|
| 54 | LaBreche | 5/18/98 | Range Finder | Control replicate C swimmer 168 hours of exposure. Clam is 12 days old in photo. | 60x |  |
| 60 | LaBreche | 5/18/98 | Range Finder | Control replicate H. 168 hours. Three stages. Top dead, middle slow development, bottom set. | 60x |  |
| 4 | LaBreche | 5/22/98 | Control Petri Dishes | Set clams with foot out dish I of 10 After 264 hours of exposure. Clam was 5 days old at initial exposure | 60x 1/50th |  |
| 17 | LaBreche | 5/22/98 | General Use | 600x Calibration slide. Distance between bold lines = 0.1mm | 600x, 1/50th |  |
| 18 | LaBreche | 5/22/98 | General Use | Calibration Slide 300x. Distance between bold lines = 0.1mm | 300x, 1/50th |  |

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|--------------------------|---|-----------------------------|---|
| 12 | LaBreche | 5/22/98 | Control Petri Dishes | Two Set clams. One with foot siphon extended one without. 264 hours of exposure. Clams were 5 days old at initial exposure. | 60x, 1/50th, phase contrast |  |
| 11 | LaBreche | 5/22/98 | Control Petri Dishes | Two Set clams. One with foot siphon extended one without. 264 hours of exposure. Clams were 5 days old at initial exposure. | 60x, 1/50th |  |
| 10 | LaBreche | 5/22/98 | Control Petri Dishes | Two Set clams. One with foot siphon extended one without. 264 hours of exposure. Clams were 5 days old at initial exposure. | 60x, 1/50th |  |
| 9 | LaBreche | 5/22/98 | Range Finding Experiment | Deformed Set Clam. 29 ug/l Copper dosed clam after 240 hours of exposure. Clam was 6 days old at initial exposure. Note Extruded internal mass. | 60x, 1/50th |  |
| 8 | LaBreche | 5/22/98 | Range Finding Experiment | Deformed Set Clam. 29 ug/l Copper dosed clam after 240 hours of exposure. Clam was 6 days old at initial exposure. Note Extruded internal mass. | 60x, 1/50th |  |

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|--------------------------|---|------------------------------|---|
| 7 | LaBreche | 5/22/98 | Range Finding Experiment | Deformed Set Clam. 29 ug/l Copper dosed clam after 240 hours of exposure. Clam was 6 days old at initial exposure. Note Extruded internal mass. | 60x, Phase contrast, 1/50th |  |
| 13 | LaBreche | 5/22/98 | General Use | <i>Mercenaria mercenaria</i> 600x. Untested, from stocks. 16 day old swimmer. | 600x, 1/50th |  |
| 5 | LaBreche | 5/22/98 | Control Petri Dishes | Set clam #2 from dish I (eye) of 10. Foot extended After 264 hours of exposure. Clam was 5 days old at initial exposure | Phase 100, 60x, light 6 |  |
| 3 | LaBreche | 5/22/98 | Control Petri Tests | Dish I of 10 dishes. Set clams with foot out After 264 hours of exposure. Clam was 5 days old at initial exposure | Phase contrast, 60 x, 1/50th |  |
| 1 | LaBreche | 5/22/98 | General Use | Calibration slide @ 60x magnification | The end lines = 1 mm |  |

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|--|--|-------------|---|
| 6 | LaBreche | 5/22/98 | Control Petri Dishes | Normal Set Clam #2 from Dish I (eye) of 10. Foot extended After 264 hours of exposure. Clam was 5 days old at initial exposure | 60x, 1/50th |  |
| 24 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | 3.9 ug/l C Fed Set clam. 456 hours of exposure. Clam was 7 days old at initial exposure. | 60x |  |
| 19 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | 15 ug/l Dish A Set unfed 456 hours of exposure. Immature set Clams were 7 days old at initial exposure. | 60x |  |
| 20 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | 15 ug/l Dish A Unfed. Set with small immature shell. 456 hours of exposure. Clams were 7 days old at initial exposure. Innards out of small shell. | |  |
| 21 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | 15 ug/l B Fed Swimming Clam after 456 hours of exposure | 60x |  |

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|---------|--|---|---------|---|
| 23 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | Control Fed B 2 set clams. 456 hours of exposure. Clams were 7 days old at initial exposure. | 60x |  |
| 25 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | 7 ug/l Fed Set with cilia odd swimming patterns. 456 hours of exposure. | 60x |  |
| 26 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | 7 ug/l A Fed Set but deformed and immature. 456 hours of exposure. Clams were 7 days old at initial exposure. | 60x |  |
| 22 | LaBreche | 6/29/98 | 4 - 495 Range Experiment Static Petri Plates | Control Fed B Swimmer after 456 hours of exposure | 60x |  |
| 27 | LaBreche | 7/2/98 | 4 - 495 Range Experiment Static Petri Plates | 14 ug/l copper dish C July 2, 1998 Deformed and immature set | 60x |  |

| ID | Photo Credit | Date | Experiment | Subject | Comment | Photo |
|----|--------------|--------|--|---|---------|---|
| 28 | LaBreche | 7/2/98 | 4 - 495 Range Experiment Static Petri Plates | 4 ug/l dish B "D-shaped" set clam. 528 hours of exposure. Clams were 7 days old at initial exposure | 60x |  |
| 29 | LaBreche | 7/2/98 | General Use | Unknown Creature found in unused algae / clam solution. Was not found in any test container. | 60x |  |
| 30 | LaBreche | 7/2/98 | General use | Unknown Creature found in unused algae / clam solution. Was not found in any test container | 60x |  |

Appendix G:

Video Recording Index

Index (Tape Time)

Subject:

Note, calibration slide is on tape at index 44:14 most clams were observed at 60x due to petri dish height.

16:35

Control A static petri dish 72 hours of exposure: Demonstration of counting technique. Small moving dots are algae, *Isochrysis galbana*. The video camera was not regularly used for counting due to the reduced field of view. 4 x 15 (60x) magnification

25:18

Control E static petri dish 72 hours of exposure: A very large swimming clam. Note the algae streaming into the clam. Near the end of this segment a few other clams are shown for proportion. 4 x 15 (60x)

29:30

Unfed A Control static petri dish 192 hours of exposure: Close to set clam? Note the very small size compared to other clams, the shell that still appears "D" shaped, and the translucence of the visceral mass.

31:01

Unfed A Control static petri dish 192 hours of exposure: Close to set clam? And a swimmer nearby. Notice the dramatic absence of algae compared to video of fed organisms. Video is switched over to phase contrast later on. A few sporadic small objects other than the clams are noticable in this mode. Do not confuse the grain in the background for food on the base of the dish.

34:28

Control D static petri fed dish 192 hours of exposure: Fast rotary swimmer. Algae is very obvious in this segment.

37:11

Control D static petri fed dish 192 hours of exposure: Spinning clam shown then a dead clam followed by 3 clams: 2 swimming and one stationary.

39:59

Control D static petri fed dish 192 hours of exposure: Almost set clam

40:39

15.6 ug/l Cu B static petri 192 hours of exposure: Small poorly developed but nearing set? Note internal movement during segment similar to a siphon extending.

41:36

15.6 ug/l Cu B static petri 192 hours of exposure: Note features of a set clam but the clam is swimming. A protrusion (siphon ?) is visible at one end of the clam and the swimming is very eccentric

43:42

31 ug/l Cu Unfed B replicate, 192 hours of exposure: Very translucent and not very mature clam.

44:14

Calibration slide: 4 x 15 (60x) magnification. A latter portion of this segment does include the inscribed dimensions on the slide. (mm and inches)

47:44

3.9 ug/l Cu A static petri 192 hours of exposure: Set clam.

50:12

Algae, *Isochrysis galbana* at 40 x 15 (600x) magnification. Isochrysis are a flagellated plant, the flagellum is not visible. Yes a swimming plant. Note the different shapes of algae. The algae that appear more like dumbbells, each end enlarge are about to split into two algae.

52:50

Algae, *Isochrysis galbana* at 100 x 15 (1500x) magnification. Flagellum is still not visible. At 54:40 a nice view is briefly recorded.

55:20

Clam, untested from 3.9 ug/l stock solution after 192 hours of exposure 600x magnification.in the 3.9 ug/l stock solution. Note circulating algae in gut of clam. "eye spot" is clearly visible in this clam. A variety of exposures and phase contrasts are explored for expressing different details. Clam was set but apparently camera shy about extending its foot. Note that cilia is not completely gone at this stage. Loss of cilia is a gradual process.

59:33

Unfed 15 ug/l Cu A replicate: Very small set with little activity. 360 hours of exposure

1:01:11

Calibration slide 60x magnification. 360 hours of exposure

1:01:46

Control A big swimmer 60x magnification. Note there are several cuts in this segment. 360 hours of exposure

1:02:47

Control A fed recently set, foot not out fully. 360 hours of exposure

1:03:23

Unfed control set clam. Note the "D" shape. 360 hours of exposure

1:04:34

Unfed control clam still swimming after 360 hours

1:05:26

Unknown creature found in left over clam shipping solution after 360 hours.

Vitae

Timothy Merrick Clark La Breche was born on November 18, 1972 in Pike County, Kentucky. He grew up in Pinson Holler and graduated from Johns Creek High School in 1990. He earned his Bachelor of Science degree in Civil Engineering from Purdue University in 1995 and completed his Master's of Science in Environmental Engineering from Virginia Polytechnic Institute and State University in 1998. His expertise includes ecotoxicology, air pollution, and hydrology. An active musician, his bass trombone accomplishments are recorded on three compact disks produced of the jazz ensembles he played with at Purdue and Virginia Tech. In the future Tim plans to return to air pollution consulting and continue his musical pursuits.