SENSITIVITY OF EARLY LIFE STAGES OF FRESHWATER MUSSELS (BIVALVIA: UNIONIDAE) TO COPPER.

by

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(ABSTRACT)

Four life stages of freshwater mussels were tested for their sensitivity to copper and a metal-containing effluent. This permitted an assessment of the variability in sensitivity among the life stages in order to identify those stages most threatened in the wild from copper exposure. Glochidia, while held within the marsupia of the adult, the released or isolated glochidia, the encysted glochidia, and the gravid mussel were tested.

Little effect on glochidia was detected following 30-day artificial stream exposures of gravid adults to 19.1 ug Cu/L and an effluent containing an average of 23.9 ug Cu/L. Isolated glochidia were killed by copper concentrations ranging from 20-80 ug/L in 24-hour exposures, with sensitivity increasing with hardness and temperature. Encysted glochidia were resistant to exposures up to 400 ug Cu/L. No significant effect on metamorphosis to the

stage was detected. This is likely due to the encapsulation of the glochidium by the host fish. Juvenile mussels reduced their activity during 24-hour exposures to copper concentrations as low as 17 to 24 ug/L and concentrations of 30 to 42 ug/L caused mortality.

Juvenile mussels and glochidia within the marsupia are probably the two most sensitive stages in the life cycle of the freshwater mussel. Copper pollution will have its greatest impact in the summer, during periods of high water temperature and low flow.

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CHAPTER 1 - INTRODUCTION

There are approximately two-hundred and fifty species of freshwater mussels (Unionidae) in the United States (Burch 1973). As biological filters and a wildlife food resource, they are important in both lotic and lentic aquatic communities. In addition, a small industry exports the valves, which are formed into pellets and used to seed commercial pearl oysters (Fritz 1985; Madson 1985; Van Horn 1985). In recent years, they have also been used as biological monitors of pollution, given their propensity to accumulate toxic materials from the ambient environment (Fuller 1974). Unfortunately, over the past century, declines in mussel populations have been reported at most locations around the United States.

Some of the first records were of losses due to intense commercial harvesting. In 1889, the first pearl button factory was opened on the Mississippi River at Muscatine, Iowa. Mussels were harvested from the river for their valves from which circular blanks were cut for the buttons. Within 10 years, serious depletions of the mussel beds in the Mississippi River were being reported (Oesch 1984). In response to these declines, as well as those reported from

other rivers, the United States Bureau of Fisheries opened the Fairport Biological Station at Fairport, Iowa, in 1908. This laboratory was assigned the task of studying the reproductive biology of freshwater mussels. It was hoped that this would lead to the artificial rearing and stocking of mussels. From the opening of the lab in 1908 until it burned to the ground in 1917, significant information was gathered on the reproductive patterns of freshwater mussels (Coker et al. 1921).

Although mussels were successfully raised and stocking was possible, loss of the laboratory ended this period of intense activity in mussel biology. It was not until the end of World War II, with the advent of plastics, that the pearl button industry began to decline.

In the early 1960's, it was discovered that a pearl oyster, seeded with a small piece of freshwater mussel nacre, could produce a gem-quality pearl in significantly less time than possible with conventional techniques (Madson 1985). Thus, the freshwater mussel continued to be exploited commercially, and still is today (R.J. Neves personal communication).

With expanding human populations, rural development, and continued industrialization of the United States, freshwater mussels face an even more serious threat. As early as 1858, die-offs of mussels due to anthropogenic causes, unrelated to

harvesting, were reported. Higgins (1858) speculated that a decrease in species of Unionidae in Ohio streams was due to pioneers bringing more and more wilderness land under cultivation. Lewis (1868) reported declines of Anodonta lewisii in the Erie Canal, New York, and implicated chemical pollution as the cause.

As development around the United States progressed, more declines in mussel populations were reported. These were attributed to numerous factors, including commercial collecting, channel modifications, agricultural runoff, sedimentation, industrial pollution, and recently, competition from the introduced Asian clam, Corbicula fluminea (Ortman 1909; Baker 1922; Ellis 1931, 1936; van der Schalie 1938; Athearn 1967; Isom 1969; Stansbery 1970; Dineen 1971; Fuller 1974, 1978, 1980; Kraemer, 1979; Strayer 1980; Gordon 1982; Clarke 1986; Neves 1987).

Of particular concern to malacologists were declines in mussels of the Cumberland Plateau region of the southeastern United States (Ahlstedt 1984). The rivers of the Cumberland Plateau are a center of freshwater mussel diversity and endemism. This region contains approximately 45 endemic or "Cumberlandian" species (Ortmann 1924). The rivers of the area include the Buffalo, Cumberland, Duck, Elk, and the headwaters of the Tennessee River, to include the Clinch, Holston, and Powell rivers. The Clinch River, originating in

Tazewell County, Virginia, and flowing southwesterly through the state into Tennessee, is the stronghold for the "Cumberlandian" mussel fauna of the region's rivers (Ahlstedt 1984). Nearly 50 species, or 20% of all North America's freshwater mussels, are found within its watershed (Table 1). A high level of diversity is also found among the fish fauna of the river (Masnik 1974).

Attention was focused on the Clinch River in the early 1970's when two catastrophic chemical spills occurred, originating from the American Electric Power Corporation's power plant, located in Carbo, Virginia, at CRM 266.1. In 1967, a fly-ash holding pond dike collapsed, sending a highly alkaline slug of metal-contaminated water into the river. Fish were reportedly eliminated as far as 40 kilometers beyond the Tennessee border. Mussels were reportedly eliminated for 28.3 kilometers below the plant. In 1970, a spill of sulphuric acid from the plant again impacted the fauna for 30 kilometers downstream (Cairns et al. 1971).

Ahlstedt (1984) found mussels virtually absent from CRM 255.6 upstream to CRM 268.2, just above the power plant at Carbo, Virginia. This stretch was impacted by the spills of 1967 and 1970. Only six live mussels of four species were found in the reach. Stansbery et al. (1986) reported similar

Table 1. Mussels of the Clinch River. (Ahlstedt 1986; Neves personal observations).

Actinonaias ligamentina	Leptodea fragilis
Actinonaias pectorosa *	Lexingtonia dolabelloides *
Alasmidonta marginata	Ligumia recta
Amblema plicata plicata	Medionidus conradicus *
Cumberlandia monodonta	Pegias fabula * +
Cyclonaias tuberculata	Plethobasus cyphyus
Cyprogenia irrorata	Pleurobema cordatum
Dromus dromas * +	Pleurobema oviforme *
Elliptio crassidens	Pleurobema plenum +
Elliptio dilatatus	Pleurobema rubrum
Epioblasma brevidens *	Potamilus alatus
Epioblasma capsaeformis *	Ptychobranchus fasciolaris
Epioblasma t. gubernaculum * +	Ptychobranchus subtentum *
Epioblasma triquetra	Quadrula cylindrica *
Fusconaia barnesiana *	Quadrula intermedia * +
Fusconaia cor * +	Quadrula pustulosa
Fusconaia cuneolus * +	Quadrula sparsa * +
Fusconaia subrotunda	Strophitus rugosus
Hemistena lata * +	Truncilla truncata
Lampsilis fasciola	Villosa iris *
Lampsilis orbiculata +	Villosa perpurpurea *
Lampsilis ovata	Villosa v. vanuxemensis *
Lasmigona costata	
Lemiox rimosus * +	

* Cumberlandian Species (21)
+ Endangered Species (11)

results for this same reach of the river. He assumed that recovery had occurred by CRM 255.4 where ten species were found, compared to the twelve species found immediately above the plant (CRM 268.2).

Considerable debate has focused on whether the diverse mussel fauna above CRM 268 and below CRM 256 ever occurred within this 20 kilometer stretch (Stansbery et al. 1986). If it had, the 1967 and 1970 spills presumably eliminated it, and recovery has not occurred. It is possible that mussels have been unable to recolonize this stretch of the river because of continued discharges from the plant. It was therefore of interest to know how sensitive mussels are to releases from the plant.

Adult mussel sensitivity to a number of pollutants has been well-studied (Havlik and Marking 1987). Based on these findings, concentrations of metals measured in the plant's effluents are high enough to negatively affect adult mussels (Van Hassel and Gaulke 1986; P.J. Jacobson personal observations). There is little doubt that the discharges could be affecting adult mussels immediately downstream of the plant. It is unknown however, whether they could be affecting adults as far as 28.3 kilometers downstream. Since the early life stages of an organism are often more sensitive to a toxicant than adults, it may be that releases from the plant prevent recolonization by affecting early stages.

Little is known of the sensitivity of early life stages of freshwater mussels to pollution. In contrast, the early lifestages of marine bivalves have been well studied (Hidu 1965; Granmo 1972). There is even a standard method for conducting static acute toxicity tests with marine bivalve larvae (A.S.T.M. 1980). Few such methods have been developed for freshwater mussels.

Varanka (1977, 1978, 1979) investigated the effects of various pesticides on freshwater mussel larvae. Goudreau (1988) examined the larval sensitivity to monochloramine and ammonia. Recently, several researchers have tested the sensitivity of juvenile mussels to various pollutants, such as heavy metals (Keller and Chrisman 1989; Wade et al. 1989). These studies indicated that early life stages of freshwater mussels are quite sensitive to various toxicants. Therefore, a series of protocols allowing testing of the various life stages was needed. This would allow collection of data from which comparisons of sensitivity among the life stages and various species could be made. These data are critical if water quality criteria, protective of freshwater mussels, are to be successfully developed.

The reproductive cycle of freshwater mussels can be divided into four basic stages (Figure 1); gravid adult, isolated glochidia, encysted glochidia, and juvenile mussel (Figure 2). A number of early studies have detailed the

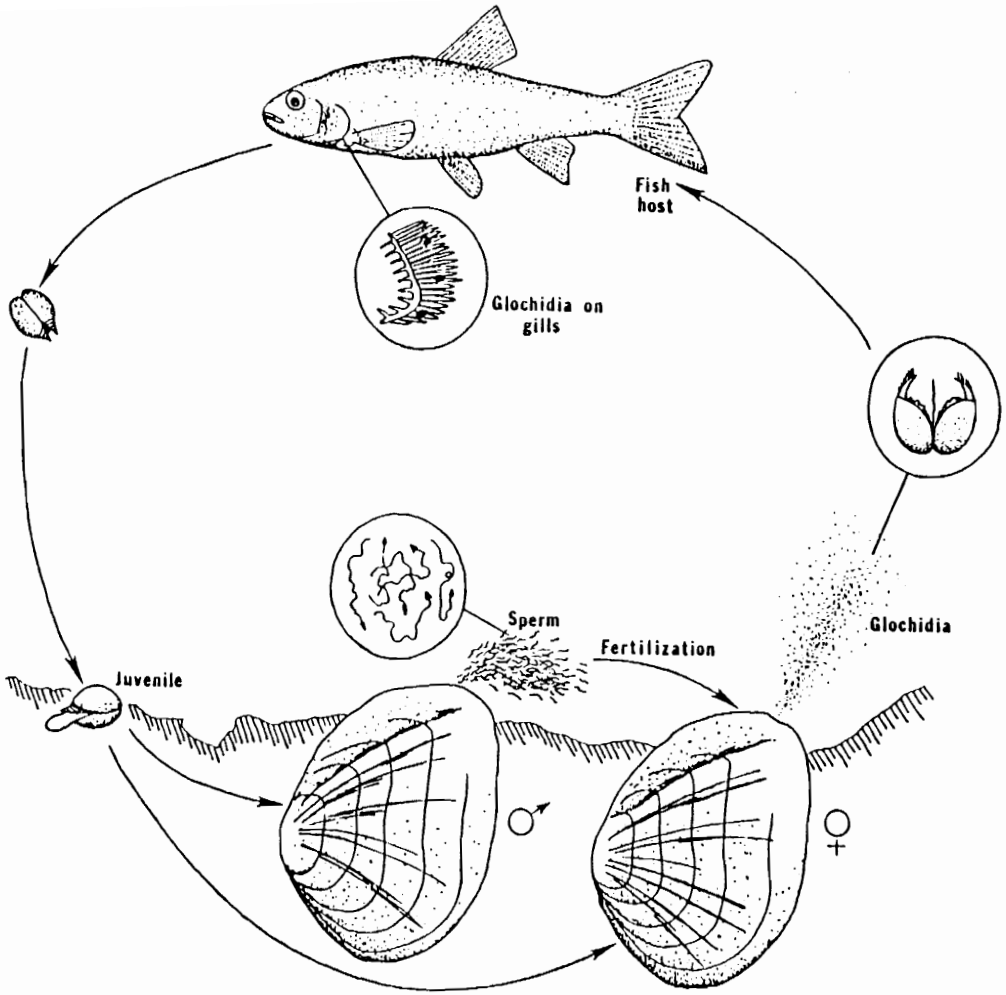

Figure 1. The life cycle of the freshwater mussel.

Figure 2a. Gravid Lampsilis ovata with mantle flap extended and marsupia visible.

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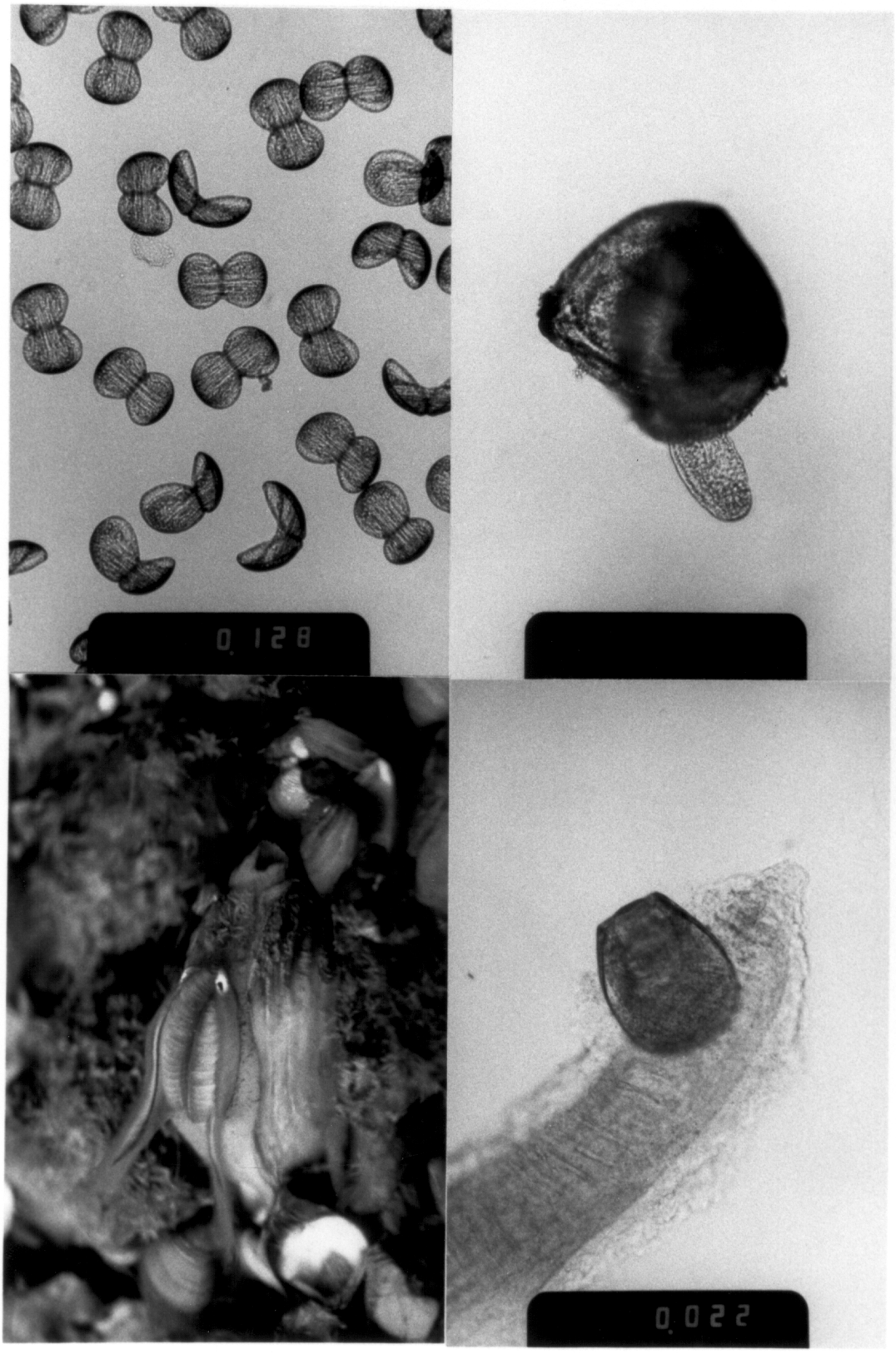
Figure 2d. Juvenile Anodonta grandis.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reproduction of freshwater mussels (Lefevre and Curtis 1910a, 1910b, 1912; Isely 1911; Howard 1914, 1915, 1922; Arey 1921; Coker et al. 1921).

After gametogenesis, ova are released into the suprabranchial chamber of the female and fertilized. The sperm, previously released into the water column by the male, reach the ova via the incurrent siphon of the female. The resulting zygotes are then deposited in the gills of the female, which act as marsupial brood pouches. While held within the marsupia of the female, embryos develop into the larval stage known as the glochidium.

Glochidia may be released almost immediately after maturation, or they may be held in the marsupia for an extended period. Glochidia of most species of freshwater mussels require a period of parasitism on fish to metamorphose to the juvenile stage. This parasitic period typically lasts 2-3 weeks, although it can vary significantly with both water temperature and species (Young 1911; Howard and Anson 1922; Zale and Neves 1982). Following metamorphosis on the fish host, juvenile mussels excyst and drop to the substratum to become free-living members of the benthic community.

The objective of this study was to determine the sensitivity of the four basic life stages described, in exposures to copper or the APCO plant's effluent. Copper was

chosen because it is one of the primary toxic constituents of the effluent, and it is known to be highly toxic to invertebrates (Van Hassel and Gaulke 1986; U.S.E.P.A. 1985). My goal was to assess the extent of variability in sensitivity among the life stages in order to identify those life stages most threatened in the wild from copper exposure.

Chapters 2 through 5 describe the studies of each of the four life stages previously described. Chapter 2 covers the work with glochidia within gravid females. Chapter 3 details studies on isolated glochidia. Chapter 4 describes experiments with encysted glochidia, and Chapter 5 focuses on tests involving juvenile mussels. Chapter 6 summarizes results of these studies, compares life stages, and concludes with a discussion of some of the major problems and protection measures for freshwater mussel populations today.

CHAPTER 2 - GLOCHIDIA HELD IN GRAVID ADULTS

Introduction

Freshwater mussels can be divided into two basic groups, bradytictic and tachytictic (Lefevre and Curtis 1912). The terms tachytictic and bradytictic (quick breeding and slow breeding), proposed by Ortmann (1911), are misleading, in that the spawning or breeding period does not vary substantially between the two groups (Coker et al. 1921). Rather, it is the period in which the mature glochidia are held in the marsupia that varies significantly between the two groups. A more appropriate description would be short-term and long-term brooders (Neves and Widlak 1988).

The bradytictic mussels typically spawn from late summer to early fall. Glochidia mature within a month after fertilization, and are then held in the marsupia of adults through the winter. Most glochidia are discharged in spring, presumably in response to rising water temperature (Neves and Widlak 1988). Tachytictic species, in contrast, spawn in spring to early summer and release glochidia almost immediately after maturation in mid-summer (Neves and Widlak 1988).

In the case of Villosa nebulosa, a bradytictic species, glochidia mature as early as mid-September and can be retained until the following June or July (personal observation). Some glochidia may be held in the marsupia for as long as 8-10 months before release. During this period, glochidia could be exposed to contaminants brought in through the incurrent siphon of the adult during normal feeding and respiration. This is a substantial period of possible exposure. It was thus suspected that copper pollution could exert an affect upon glochidia in the marsupia at very low levels, given the duration of possible exposure.

The objectives of this phase of the study were: (1) to determine the copper sensitivity of glochidia within the marsupia of the gravid adult; (2) to examine whether such exposure could affect the ability of the glochidia to encyst on a host fish; and (3) to assess whether the exposure affected sensitivity of glochidia to future exposures.

Materials and Methods

Artificial Stream Exposures

Thirty-day exposures were conducted in a series of artificial streams located on the site of the American Electric Power Corporation's plant at Carbo, Russell County,

Virginia, on the Clinch River. These outdoor streams were oval in shape (90 x 20 x 15 cm) and received Clinch River water drawn from just upstream of the plant (Farris 1986). Flow was established in the streams by motor-driven paddlewheels. Peristaltic pumps dripped stock solutions of $CuSO_4$-$7H_2O$ into the streams from 160 L carboys. Additional streams received a continuous flow of the plant effluent. Although there are several discharges from the plant, the cooling tower blowdown discharge was used. It is a major source of metals released into the river (Van Hassel and Gaulke 1986). By measuring the inflow of river water into the streams and then varying the inflow of copper stock or effluent accordingly, I controlled the concentration of copper and effluent in the streams.

Water in the artificial streams was sampled frequently (every 3-5 days) for copper concentration, pH, conductivity, temperature, hardness, and alkalinity. Copper stock solutions were renewed at the same time. Mussels were held in two streams containing 8 and 17 ug Cu/L, respectively, and a third stream containing both 17 ug Cu/L and 47 ug Zn/L. A fourth stream contained 10% effluent and a fifth received only river water as a control. These levels reflect the "chronic" USEPA national water quality criteria for Cu and Zn, 17.9 ug/L and 47 ug/L, respectively, in Clinch River water (USEPA 1985; Belanger et al. 1989).

Mussel Collection and Handling

Gravid Villosa nebulosa were collected from Copper Creek Mile (CCM) 50 in Scott County, Virginia. Gravid individuals were identified by gently opening the valves and inspecting for the presence of swollen gills, filled with glochidia. Gravid mussels were transported to the plant site in a chilled cooler containing Copper Creek water.

At the plant site, five mussels were randomly transferred into a rock-filled basket in each stream. They remained in place through the duration of the 30-day exposure. Following exposures, mussels were removed from the streams, placed into labeled coolers, and transported to the lab in chilled water from their artificial stream. Three additional mussels were collected from Copper Creek (CCM 50) on the last day of the exposure, and these served as a comparison to the mussels held in the control stream.

At the laboratory, glochidia were removed from the mussels by cutting the posterior and anterior adductor muscles, excising the marsupia and placing them in a petri dish filled with stream water. The valves were cleaned, labeled, and saved for later reference. Glochidia were isolated from the marsupia by gently teasing them apart with forceps and dissecting needle. After removing the remnants of gill tissue, the glochidia were examined.

Viability Determinations

After removal of glochidia from the adults, six subsamples of glochidia from each mussel were tested for viability. The glochidia were handled using a wide bore (1.5-2.0 mm I.D.) pasteur pipette which had previously been flamed about the tip to smooth sharp edges. Each subsample of approximately 50-75 glochidia was added to a separate well in a 12-well tissue culture plate. Each well had been previously filled with 3.5 ml of artificial stream water.

Glochidia respond to saturated NaCl solution with a contraction of their adductor muscle, closing their valves. Glochidia which did not exhibit closure in response to NaCl were recorded as dead. Those which did respond were assumed viable (Zale and Neves 1982b).

Glochidia in each well were counted, using a Zeiss stereomicroscope, and the number open and closed was recorded. A drop of saturated NaCl solution was then added. Each well was immediately recounted, and the number open and closed was again recorded. Glochidia open prior to the NaCl addition and closed afterward were recorded as viable. Those remaining open after NaCl addition or closed prior to it were recorded as dead. For example, in the first count of a well, 60 glochidia were open and 5 were closed. After NaCl addition, all were closed. This would be recorded as 60 viable and 5

dead or 92% viability. If five glochidia had remained open after the NaCl addition, I recorded 10 dead; the five closed prior to the NaCl addition and the five remaining open, or 85% viability. Although glochidia closed prior to the NaCl addition may have been alive, they were recorded as dead. A glochidium must be open in order to encyst upon a fish.

Encystment Ability Tests

Three subsamples of 100 open glochidia were taken from three mussels in each artificial stream. Each subsample was added to a 1 L beaker containing 400 ml of water from the stream in which they had been held. Five 4-5 cm largemouth bass, Micropterus salmoides, were selected at random from a holding tank and added to the beaker. The beaker, containing 100 glochidia and 5 fish, was aerated for 15 minutes. At the end of the exposure period, the contents of the beaker were poured through a sieve into an empty 1 L beaker. The sieve consisted of a 8-10 cm piece of 5 cm I.D. polyvinyl chloride pipe with an end covered with a mesh (0.75 cm) of silicone sink matting. The sieve retained the fish but passed unencysted glochidia into the empty beaker. The sieve, still containing the 5 fish, was rinsed into the beaker with an additional 400 ml of water.

The beaker with approximately 800 ml of water and

unencysted glochidia, was drained through a second sieve of 100-micron mesh, and rinsed with a wash bottle to remove any remaining glochidia. The sieve was then backwashed into an empty petri dish which was examined for unencysted glochidia.

Acute Exposures of Isolated Glochidia

After isolating the glochidia from the 30-day exposures of females in each of the five streams, 24-hour static toxicity tests were conducted. A copper stock solution, using 40 mg of $CuSO_4$-$7H_2O$ and 1 L of filtered Clinch River water, yielded a stock solution of approximately 10 ug Cu/ml. A series of concentrations, ranging from 20 to 200 ug Cu/L, were made by diluting aliquots of this stock in filtered river water. Hardness, alkalinity, pH, dissolved oxygen, and conductivity were measured in each dilution. A 50 ml sample, acidified with 150 ul of 50% HNO_3, was saved for metal analysis. The total concentration of copper in the solutions was determined by atomic absorption or inductively-coupled argon plasma emission spectroscopy.

Glochidia were exposured in 12-well, polystyrene tissue culture plates containing 3.5 ml of toxicant solution. Concentrations were replicated three times and randomized between and within plates using a random number table (Zar 1984). After addition of the solutions, glochidia were added

row by row to the trays using a flamed, wide-bore pasteur pipette. Approximately 50 to 75 glochidia were added to each well. The trays were then transferred to an incubator for the 24-hour exposure. Temperature was maintained at 20 C, and photoperiod was a 16:8 light-dark cycle.

At the end of the exposure period, trays were removed from the incubator, one at a time, and the glochidia were examined. A count of open and closed glochidia was made before and immediately after the addition of a drop of saturated NaCl solution. Glochidia closed before and those remaining open afterwards were recorded as dead. Glochidia closing in response to the NaCl addition were recorded as live.

Results from these exposures were analyzed using the Spearman-Karber method (Finney 1971; Hamilton et al. 1977; Stephan 1977; Buikema et al. 1982). The three replicates of each concentration were pooled for analysis, yielding the exposed and those affected for each concentration.

Results

Throughout the 30-day exposure, 6/14/89-7/18/89, the Clinch River was running at abnormally high levels due to excessive rains. This resulted in large amounts of suspended particulate matter entering the artificial streams. This

material covered the bottom of the streams and was also kept suspended to a degree by the turning paddle-wheels. Copper levels reported for the period were total copper, as measured by atomic absorption spectroscopy. Measured concentrations were very close to the target levels, and temperatures ranged from 16.0-25.5 C (Table 2).

The various treatments had little effect on viability of glochidia (Table 3). The mean percent viability of the glochidia ranged from 95 to 98% for all treatments, excluding the 10% effluent. This compares favorably to the viability of glochidia removed from mussels freshly collected from the stream (personal observations). However, the 10% effluent stream was significantly different (90% viability) from the other treatments ($p<0.001$).

The encystment ability of glochidia was unaffected in all treatments. No significant differences ($p=0.96$) were detected between the control stream, the three treatments tested, and the glochidia from mussels freshly collected in Copper Creek (Table 4). Glochidia from the 17 ug Cu-47 ug Zn/L exposure were not tested.

As can be seen from the data in Appendix A, there were unusually high levels of control mortality in some acute tests. The control stream exhibited mortality from 8 to 11% among replicates while the 17 ug Cu/L-47 ug Zn/L stream had from 4 to 9%. Mortality in the 10% effluent treatment

Table 2. Artificial stream water chemistry, 6/14-7/15/89. (Standard deviation in parentheses).

	Stream				
	Control	10% Eff.	8ug Cu/L	17ug Cu/L	17ug Cu + 47ug Zn/L
n	26	9	27	26	9
Cu (ug/L)	3.2 (1.5)	23.9 (11.5)	10.6 (3.7)	19.1 (5.1)	17.0 (4.3)
n	24	8	29	24	8
pH	8.35 (0.10)	8.21 (0.08)	8.39 (0.09)	8.39 (0.07)	8.37 (0.06)
Conductivity (umhos)	278 (34)	397 (84)	279 (30)	281 (29)	282 (31)
Alkalinity (mg/L)	132 (12)	125 (14)	132 (12)	133 (12)	132 (13)
Hardness (mg/L)	155 (13)	237 (38)	152 (17)	157 (16)	156 (16)

Table 3. Percent viability of isolated Villosa nebulosa glochidia after 30-day artificial stream exposures.

	# Examined	# Nonviable	% Viability	Mean*	S.D.
Control	359	16	96		
	312	12	96	95[a]	1.2
	911	19	94		
Copper Creek	361	10	97		
	316	9	97	97[a]	0.0
	284	8	97		
17ug Cu + 47ug Zn/L	289	15	95		
	367	12	97		
	400	9	98	96[a]	2.0
	500	37	93		
	443	14	97		
10% Effluent	466	52	89		
	359	42	88		
	447	47	89	90[b]	2.4
	324	21	94		
	428	46	89		
8ug Cu/L	310	15	95		
	276	6	98	97[a]	1.4
	297	7	98		
	302	10	97		
17ug Cu/L	310	7	98		
	358	8	98	98[a]	0.0
	246	5	98		

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD (Koopman's, 1987, p.352)

Table 4. Encystment ability of isolated Villosa nebulosa glochidia after 30-day artificial stream study.

Treatment	Mean* (# of encysted glochidia)	Standard Error (n=3)
Control	55[a]	14.0
Copper Creek	55[a]	7.0
10% Effluent	58[a]	3.8
8ug Cu/L	52[a]	2.3
17ug Cu/L	51[a]	5.2

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD (Koopmans 1987, p.352)

ranged from 12 to 15%, while the 8 ug Cu/L treatment was from 9 to 23%. Glochidia from Copper Creek exhibited 11 to 31% control mortality, while the 17 ug Cu/L treatment ranged from 26 to 91%. Mean mortality and coefficients of variation for control mortality in each treatment are shown in Table 5. The Copper Creek glochidia, tested on 7/15/89, and the 8 ug Cu and 17 ug Cu/L treatment glochidia, tested on 7/20/89, are distinct in exhibiting both high mean control mortality and coefficients of variation (Table 5).

Results from acute exposures, presented in Appendix A, were analyzed using the Spearman-Karber method, as previously described. To facilitate this, control data were deleted from analysis. When the controls were excluded, remaining data exhibited a normal dose-response relationship (Appendix A). No trends were discernible among the LC_{50} values calculated, which ranged from 25 to 51 ug Cu/L (Table 6). Water chemistry from these tests is presented in Table 7.

Discussion

Although little effect was detected on glochidia following the 30-day exposures, I am unable to draw any firm conclusions on the sensitivity of glochidia held within marsupia for two reasons: (1) the unusually high river stage during the test, and (2) the possible response of the female

Table 5. Mean mortalities and coefficients of variation for controls in 24-hour acute exposures of isolated Villosa nebulosa glochidia from 30-day artificial stream study (n=3).

Date	Stream	Mean*	Standard Deviation	Coefficient of Variation
7/15/89	Control Stream	0.090	0.016	17.8
7/15/89	Copper Creek	0.198	0.104	52.5
7/15/89	10% Eff.	0.129	0.015	11.6
7/15/89	17ug Cu + 47ug Zn/L	0.063	0.022	34.9
7/20/89	8ug Cu/L	0.150	0.072	48.0
7/20/90	17ug Cu/L	0.643	0.342	53.2

* A value of 1.0 = 100% mortality.

Table 6. Twenty-four hour LC_{50}* values (ug Cu/L) of isolated Villosa nebulosa glochidia from 30-day artificial stream study. (95% confidence interval in parentheses).

	Mussel #1	Mussel #2	Mussel #3
Control	44 (42-46)	30 (29-31)	33 (31-36)
Copper Creek	26 (25-28)	47 (45-49)	50 (47-53)
10% Effluent	25 (24-27)	27 (26-28)	43 (41-46)
17ug Cu + 47ug Zn/L	30 (28-31)	26 (25-27)	36 (33-38)
8ug Cu/L	51 (50-53)	46 (44-47)	34 (33-35)
17ug Cu/L	41 (40-42)	45 (44-47)	44 (40-45)

* Spearman-Karber Method (control relicates excluded from analysis).

Table 7. Water chemistry for twenty-four hour acute exposures of isolated Villosa nebulosa glochidia from 30-day artificial stream study (Temp. 20 C).

Target (ug/L Cu)	Actual (ug/L Cu)	pH	DO (mg/L O_2)	Cond. (umhos)	Alk. (mg/L)	Hard. (mg/L)
7/15/89						
0	5	8.57	8.5	280	140	173
20	22	8.57	8.5	280		
40	34	8.58	8.5	281		
60	58	8.58	8.5	281		
80	82	8.58	8.5	282		
100	93	8.58	8.5	282		
150	136	8.58	8.5	284		
200	183	8.58	8.5	285	140	171
7/20/89						
0	26	8.37	8.0	295	129	160
20	30	8.37	8.0	295		
30	38	8.37	8.0	295		
40	51	8.37	8.0	297		
50	65	8.37	8.0	297		
60	71	8.37	8.0	297		
80	90	8.37	8.0	300		
100	112	8.36	8.0	300	129	160

to exposure.

The average copper concentration was 19.1 ug Cu/L in the 17 ug Cu/L stream and 23.9 ug Cu/L in the 10% effluent stream. These numbers represent the total copper, and they give no indication of the amount biologically available. The free cupric ion is the principal copper species responsible for acute toxicity (Andrew et al. 1977; Engel and Sunda 1979). Concentration of the free cupric ion can vary widely in natural waters relative to other physico-chemical forms. Copper can exist as the free aquated metal ion (Cu^{2+}); as metal-inorganic complexes ($CuCO_3^{\circ}$); as metal-organic complexes (Cu-fulvate); adsorbed on inorganic colloids (Cu-clay); adsorbed on organic colloids (Cu-humics); as metal-biota associations (Cu-algae); and as metal-particulates (Daly et al. 1986; Florence and Butley 1980; Hart 1982a, 1982b; Leppard 1983; Nelson et al. 1986).

The results of the 30-day study are therefore suspect. Although copper was present in the streams at levels that have affected isolated glochidia in 24-hour static exposures, it is possible that a large fraction of it may not have been in the highly toxic, free cupric ion form. Under summer river conditions, with low river flow and higher temperatures, the ratio of free cupric ion to the other forms should be higher. A significant affect might then be observed at the concentrations tested.

The possibility that the adult mussel responded to the metals and altered its behavior cannot be discounted. Salanki (1979) reported that adult Anodonta cygnea reduced the frequency and duration of siphoning in response to copper. This would in turn, reduce the exposure of glochidia within the gills. In addition, other metals, such as zinc, iron, and aluminum could have affected glochidia.

Results of the encystment trials show that no sublethal effects, which would impair encystment ability, occurred. It appears that if a glochidium responds to NaCl, it is capable of attachment to a host fish. The LC_{50} values calculated for the acute exposures, following the 30-day treatments, did not exhibit detectable differences. There was no measurable effect on the sensitivity of glochidia to subsequent copper exposures.

High control mortalities in acute exposures may be partly explained by the condition of dilution water. Although the Clinch River water used in the 7/15/89 tests appeared normal and contained only 5 ug Cu/L, the water collected and used for the 7/20/89 tests was turbid and contained 26 ug Cu/L. This does not explain, however, the drop in the percent affected in the first toxicant concentration, shown in Appendix C.

As judged by the effects observed in the 10% effluent treatment, glochidia can be affected by contaminants while held within the marsupia. Although the 30-day exposure did

not have a detectable impact on glochidia, a longer exposure may be deleterious. As discussed previously, a 6-month or longer brooding period is not unusual for many species of freshwater mussels. Tests of longer duration, including periods of low river flow, are needed. The potential significance of this route of exposure cannot be discounted.

CHAPTER 3 - ISOLATED GLOCHIDIA

Introduction

Glochidia develop from embryos in several weeks within the marsupial demibranch (gill) of female mussels (Zale and Neves 1982b; Yeager and Neves 1986). After development, the glochidia are either released almost immediately, or they are retained within the marsupia, often for long periods, before release.

When released from the adult, the glochidium must contact and attach to a suitable host fish. This parasitic relationship is obligatory for most species to develop to the juvenile stage. To facilitate this, some species possess unique behaviors or structures to enhance the probability of the glochidia achieving successful encystment.

In some species, glochidia are released from adults in small packets called conglutinates. Glochidia within the conglutinates are held together by a gelatinous matrix. Conglutinate shape is a function of marsupial structure and varies between species. Most however, resemble a small worm or some other invertebrate. When a fish is attracted and

feeds on a conglutinate, glochidia may contact the gills and achieve encystment.

Most species release their glochidia singly or in small groups. In lotic habitats, these glochidia are transported downstream, thereby increasing the probability of contact with a host fish. Glochidia attaching to the gills could be brought into the mouth of the fish during respiration or feeding. Neves and Widlak (1988) reported a high frequency of infestation among drift-feeding fish following peaks in glochidia release. In lentic habitats, however, glochidia likely remain within a short distance of the adult.

All reproductive strategies rely on survival of the isolated glochidia for a short period of time before encystment. In fact, the longer this period of survival after release from the adult, the greater the chance of encystment. Tedla and Fernando (1969) found that glochidia of Lampsilis radiata siliquoidea did not survive beyond 9 days at 10 C or 1 day at 20 C. They defined survival as the ability to infest a fish host, and noted that the ability of a glochidium to survive outside the marsupia will improve its chance of finding a host. However, by the time the glochidium completes development, the limited yolk content of the embryo has been depleted. Therefore, isolated glochidia have few energetic reserves, and their continued survival is dependent upon external inputs. Gordon and

Layzer (1989) noted that it seemed unlikely that mature glochidia, particularly those of long-term brooders, would be able to survive the brooding period without some sort of energetic input.

Lefevre and Curtis (1910b) were the first to speculate that glochidia might obtain nutrients from the adult mussel, while held within the marsupia, although no experimental evidence was presented. Wood (1974) attempted to test whether glochidia were "feeding" while in the marsupia. Adult mussels were fed algae which had been radioactively-labeled. The glochidia were then examined to see if any incorporation of this radioactivity had occurred. Glochidia were found to contain elevated levels of radioactivity, suggesting that they were obtaining some form of nutriment while in the marsupia. It was assumed that mucous secretions from the epithelium lining of the gills was the source. Wood (1974) added that once released, glochidia must quickly become attached to a host. This suggested that "feeding" was assumed not to occur outside of the adult. Isom (1986) has demonstrated, by metamorphosis of glochidia in liquid media, that the isolated glochidium is able to absorb nutrients from its ambient environment.

The glochidia used in Wood's (1974) study were taken from Anodonta cygnea. Glochidia of the subfamily Anodontinae are unusual in that they possess lateral pits

(Wood 1974). These structures are located at the posterior end of the glochidium, near the hinge and adjacent to the adductor muscle. Microscopic observation reveals that these pits are heavily ciliated around their margins. The cilia can be seen to beat rapidly, creating a flow of water circulating about the pits. Particulates are swept into these pits by the induced current (personal observations). Wood (1974) found radioactivity concentrated in the cells of the mantle and the lateral pits but did not state whether glochidia could have ingested algae via the lateral pits.

Freshwater mussels of the subfamily Anodontinae are characteristic of lentic habitats, being found in lakes, ponds, or pools in slow-moving rivers. Thus, glochidia released from the adult would probably not be distributed by currents, as mentioned previously. Encystment would depend upon a suitable host fish entering the area near the gravid adult. A longer lifespan outside of the adult would confer an obvious advantage to the glochidia.

Isolated glochidia of anodontids do have a relatively long lifespan in comparison to lampsilid glochidia. At 10 C, complete mortality of isolated Villosa nebulosa and Medionidus conradicus glochidia occurs within 14 days (personal observation). In contrast, glochidia of Anodonta grandis and Anodonta cataracta exhibit virtually no mortality over the same period (personal observation).

Whether or not the presence of the lateral pit offers any explanation for these observations is unknown.

Because the probability of encystment would increase with prolonged survival after release, any factor which reduced this survival time could negatively affect population recruitment. It was therefore of interest to determine the sensitivity of isolated glochidia.

Glochidia have been tested previously for sensitivity to various pollutants. One of the earliest studies was by Labos and Salanki (1963) in which they investigated the effect of alkalia metal ions on the glochidia of Anodonta cygnea. Glochidia of anodontids often exhibit a rhythmic contraction of the adductor muscle, producing periodic "snapping" of the valves. Varanka (1977, 1978, 1979) investigated the affects of various pesticides upon this rhythmic activity and found that most induced a slight increase in activity followed by a prolonged inhibition. This was also associated with a tonic contraction of the adductor muscle, resulting in long-term closure of the glochidium. The declines of mussel populations in many environments were attributed to these effects, which would negatively affect encystment ability and hence, population recruitment.

Goudreau (1988) investigated the affects of monochloro-amine and ammonia on the glochidia of Villosa nebulosa.

They exhibited a sensitivity equal to or greater than that seen for many species of fish and invertebrates. Mortality was determined by the lack of response of glochidia to a NaCl solution. Living glochidia would respond with a rapid contraction of the adductor muscle, resulting in closure of the valves. Although the glochidium closes in response to NaCl, following exposure to a toxicant, it cannot be assumed to be unaffected. It may be unable to successfully detect and encyst upon a host fish, rendering it functionally dead.

Varanka's work has clearly demonstrated that impairment can occur at sublethal levels. Therefore, if the post-exposure condition of glochidia is going to be assessed using the NaCl response, their encystment ability following sublethal exposures must be examined. In addition, the release of glochidia occurs nearly year-round. It was therefore of interest to examine the extent of variation in sensitivity with changing temperature. Finally, the toxicity of copper and other metals is known to vary with water hardness (Nelson et al. 1986). The degree to which this occurred in tests of isolated glochidia needed evaluation.

The principal objectives of this phase of the project were to: (1) determine the sensitivity of isolated glochidia to copper and APCO plant effluent, (2) assess whether sub-lethal effects impaired encystment ability, and (3) deter-

mine the effect of temperature and water hardness on sensitivity of glochidia.

Materials and Methods

Collection and Isolation of Glochidia

Gravid specimens of Actinonaias pectorosa, Lampsilis fasciola, Medionidus conradicus, and Villosa nebulosa were collected in Copper Creek and the Clinch River, Virginia. Collections in Copper Creek were made at or near Copper Creek Mile (CCM) 50, Scott County, Virginia. Mussels from the Clinch River were taken upstream of the plant at Clinch River Mile (CRM) 270.5, Russell County, Virginia. Gravid specimens of Anodonta grandis were collected from Claytor Lake, Pulaski County, Virginia.

Gravidity of mussels was determined in the field by partially opening the valves, using a pair of reversing pliers. Gravid individuals were characterized by the swollen appearance of the two outer gills. They were then transported to the laboratory in a chilled (5 C), water-filled cooler for removal of glochidia prior to testing.

Glochidia were removed from adults by cutting the anterior and posterior adductor muscles; marsupia were excised and placed in a petri dish containing filtered Clinch

River water. The valves were cleaned, labeled, and stored for later reference. Glochidia were separated from the gill by gentle teasing with a forceps and dissecting needle. Upon removal, a sample of glochidia was tested for viability. This was done by adding three subsamples of approximately 50 glochidia to 3 wells of a 12-well tissue culture plate filled with dilution water. The contents of each well were examined under a Zeiss stereomicroscope at 8-12 times magnification. The number of open and closed glochidia was recorded. A drop of saturated NaCl solution was then added to each well, followed by a second count of the open and closed glochidia. Glochidia which closed in response to the NaCl were recorded as the number viable. Those which were closed prior to the addition, or remained open afterwards, were considered dead. Glochidia were not used in a test if viability was below 90%.

Acute Exposures

After removing glochidia from the adult's marsupia, static exposures were conducted. The toxicant solutions consisted of various concentrations of copper in dilution water or dilutions of the APCO plant's effluent. The copper solutions were made by diluting an appropriate amount of stock solution, approximately 40 mg of $CuSO_4$-$7H_2O$ to 1 L of

the dilution water. This yielded a stock containing approximately 10 ug of copper per 1 ml of solution or 10,000 ug/L. A series of eight concentrations ranging from 20 to 200 ug/L were then made with dilution water obtained by filtering freshly collected Clinch River water through a Whatman No. 2 filter or using dechlorinated tap water. Eight concentrations of the plant's effluent, no more than 48 hours old, were also made by dilution. An appropriate amount of effluent and dilution water was mixed to achieve the desired effluent concentration. After preparation, the toxicant solutions were tested for water chemistry characteristics; hardness, alkalinity, pH, dissolved oxygen, and conductivity. An additional 50 ml sample, acidified with 50% HNO_3, was taken for later metal concentration analysis. This analysis was performed in the Virginia Tech Soils Testing Laboratory by inductively-coupled, argon plasma emission spectroscopy.

Exposures of glochidia were made in 12-well, poly-styrene tissue culture plates. Each well contained 3.5 ml of toxicant solution. Concentrations were replicated three times and randomized within and between plates by assignment using a random number table (Zar 1984). After the addition of solutions, glochidia were added row by row to each tray. A flamed, wide-mouth (1.5-2mm) pasteur pipette was used for the transfer of about 50 to 75 glochidia per well. The

trays were then transferred to an incubator, set at the desired temperature (10 to 25 C) for the duration of the exposure. Photoperiod was maintained in the incubator on a 16:8 light-dark cycle.

To determine whether a significant decrease in detectable metal concentration occurred during the exposure period, an additional set of trays was filled with the series of toxicant solutions. Upon completion of the exposure period, the solutions were stored for later metal analysis, as previously discussed.

At the end of the 24-hour or 48-hour exposure period, trays were removed, one at a time, and the glochidia were examined. A count of open and closed glochidia was made before and immediately after adding a drop of saturated NaCl solution from a pasteur pipette. Glochidia closed before and those remaining open after the addition of NaCl were recorded as dead. Those closing in response to the NaCl were recorded as alive. Results from these exposures were analyzed using the Spearman-Karber method and probit analysis (Finney 1971; Hamilton et al. 1977; Stephan 1977; Buikema et al. 1982). The three replicates were pooled for analysis to yield a total exposed and total affected number for each toxicant concentration.

Prior to NaCl addition, glochidia of Anodonta grandis were examined for the presence of "snapping." Each repli-

cate was observed for 1 minute, and the total number of snaps was recorded. Observations for the three replicates of each concentration were averaged and expressed as the mean number of snaps per 100 glochidia for 1 minute.

Encystment Success Tests

Glochidia used for encystment success tests were exposed in 6-well polystyrene tissue culture plates under conditions previously described for acute exposures. Each well contained approximately 500 glochidia. At the end of the exposure period, three subsamples of 100 open glochidia were removed from each well. Each group of 100 glochidia was added to a 1 L beaker containing 400 ml of filtered water (Clinch River) at 20 C. The beaker was aerated to keep glochidia suspended. Five largemouth bass, Micropterus salmoides, of approximately 4-5 cm in length, were selected at random from a holding tank and added to the beaker. The beaker containing 100 glochidia and 5 fish was aerated for 15 minutes. At the end of the exposure period, contents of the beaker were poured through a sieve into an empty 1 L beaker. The sieve consisted of a 8-10 cm long piece of 5 cm I.D. polyvinyl chloride pipe. The end of the pipe was covered with a piece of mesh-type, silicone sink matting. This sieve retained the fish but allowed the water and any

unencysted glochidia to pass into the empty beaker. The sieve, still containing the 5 fish, was rinsed into the beaker with an additional 400 ml of water. The fish were removed to a separate holding tank to await later release.

The beaker with approximately 800 ml of water and unencysted glochidia, was drained through a second sieve of 100 micron mesh. It was rinsed into the sieve with a wash bottle to remove any remaining glochidia and the sieve was then backwashed into an empty petri dish. The petri dish was examined under a dissecting scope for unencysted glochidia. Three replicates for each concentration were averaged, yielding a mean number of unencysted glochidia per concentration.

Results

Acute Exposures

Isolated glochidia of all species were sensitive to copper. Twenty-four hour LC_{50} values (ug Cu/L) ranged from 16 for Medionidus conradicus to 140 for Actinonaias pectorosa. These values varied with temperature, water hardness, and length of exposure. Actinonaias pectorosa exhibited LC_{50} values ranging from 40 to 140 ug Cu/L, while Lampsilis fasciola ranged from 26 to 48 ug Cu/L. Tests of

M. conradicus yielded LC_{50} values from 16 to 88, while LC_{50} values for Villosa nebulosa ranged from 36 to 88 ug Cu/L (Tables 8-12). Raw data used in the analyses are presented in Appendix B, and water chemistry measurements are presented in Appendix C.

Glochidia were also affected by exposures to the APCO plant's effluent. Medionidus conradicus was the most sensitive, with a 24 hour LC_{50} of 17% effluent at 20 C (Table 11). Actinonaias pectorosa was the most resistant, exhibiting only 23% mortality in 100% effluent (Table 8). LC_{50} values for L. fasciola ranged from 49% effluent to only 48% mortality in 100% effluent (Table 10). Villosa nebulosa exhibited a similar range, with values ranging from an LC_{50} of 43% to 30% mortality in 100% effluent (Table 12).

An increase in the length of exposure period decreased the LC_{50}. For example, a 24-hour exposure of M. conradicus to copper yielded an LC_{50} of 56 ug Cu/L at 20 C. In contrast, an LC_{50} of 23 ug Cu/L was calculated for a 48-hour exposure. A similar pattern was observed in effluent exposures. A 24-hour LC_{50} of 19% was calculated, dropping to 10% in a 48-hour exposure. Similar results were observed in tests of A. pectorosa, L. fasciola, and V. nebulosa (Tables 8,10, and 12).

The response of isolated glochidia also varied with changes in water hardness. At a hardness of 190 mg/L, a 24-

Table 8. Results of the exposures of isolated glochidia of Actinonaias pectorosa to copper and effluent solutions.

Toxicant	Duration (hr)	Temp. (C)	Hardness (mg/L)	Chemistry	LC_{50}* (95% C.L.)	
					Probit	Spearman-Karber
Copper	24	10	140	2/13/89	132 (121-144)	140 (135-145)
Copper	24	25	140	2/13/89	42 (34-58)	40 (38-41)
Effluent	24	10	140	2/13/89	No mortality	
Effluent	24	25	140	2/13/89	93 (82-120)	87 (86-89)
Effluent	24	25	140	2/16/89	62 (53-71)	62 (60-64)
Copper	24	15	150	3/22/89	93 (59-192)	97 (94-100)
Copper	24	20	170	5/23/89	67 (45-90)	68 (66-69)
Copper	48	20	170	5/23/89	51 (40-61)	53 (51-55)
Effluent	24	20	170	5/23/89	23 at 100%	
Effluent	48	20	170	5/23/89	63 (48-76)	63 (60-65)

* Units of ug/L for copper and % for effluent.

Table 9. Results of the exposures of isolated glochidia of Anodonta grandis to copper and effluent solutions.

Toxicant	Duration (hr)	Temp. (C)	Hardness (mg/L)	Chemistry	LC_{50}* (95% C.L.)	
					Probit	Spearman-Karber
Copper	24	10	170	None	347 (329-365)	364 (344-384)
Effluent[1]	24	10	170	None	No mortality	
Copper	24	10	170	None	26% mortality at 160 ug Cu/L	
Copper	24	20	50	None	46 (25-66)	56 (52-60)

[1] 100% Effluent contained 264 ug Cu/L.

* Units of ug/L for copper and % for effluent.

Table 10. Results of the exposures of isolated glochidia of Lampsilis fasciola to copper and effluent solutions.

Toxicant	Duration	Temp. (C)	Hardness (mg/L)	Chemistry	LC_{50}. (95% C.L.) Probit	Spearman-Karber
Effluent[1,2]	24	10	-	None	48% mortality at 100% effluent	
Effluent[3]	24	10	-	None	72 (61-85)	71 (69-70)
Copper	24	20	170	5/23/89	48 (45-50)	45 (43-48)
Copper	48	20	170	5/23/89	40 (38-42))	43 (41-45)
Effluent	24	20	170	5/23/89	69 (52-105)	67 (64-69)
Effluent	48	20	170	5/23/89	46 (38-55)	49 (47-50)
Copper	24	20	160	6/20/89	26 (19-36)	26 (25-28)
Copper	24	20	75	8/10/89	46 (41-52)	46 (45-48)

[1] 100% effluent contained 265ug Cu/L.
[2] Results at end of 24 hour exposure to effluent.
[3] Results at end of 24 hour postexposure in control water.

* Units of ug/L for copper and % for effluent.

Table 11. Results of the exposures of isolated glochidia of Medionidus conradicus to copper and effluent solutions.

Toxicant	Duration (hr)	Temp. (C)	Hardness (mg/L)	Chemistry	LC_{50}* (95% C.L.)	
					Probit	Spearman-Karber
Copper	24	20	170	5/23/89	46 (17-94)	56 (50-63)
Copper	48	20	170	5/23/89	16 (9-25)	23 (21-26)
Effluent	24	20	170	5/23/89	17 (14-22)	19 (17-20)
Effluent	48	20	170	5/23/89	9 (7-12)	10 (9-11)
Copper	24	20	160	6/20/89	41 (36-45)	40 (38-42)
Copper	24	20	150	7/21/89	81 (57-117)	88 (82-95)
Effluent	24	20	150	7/21/89	39% mortality at 100%	
Copper	24	20	185	10/5/89	37 (35-40)	32 (31-33)
Copper	24	20	185	10/5/89	40 (37-44)	40 (36-43)
Copper	24	20	185	10/5/89	69 (61-79)	73 (69-77)

* Units of ug/L for copper and % for effluent.

Table 12. Results of the exposures of isolated glochidia of Villosa nebulosa to copper and effluent solutions.

Toxicant	Duration (hr)	Temp. (C)	Hardness (mg/L)	Chemistry	LC_{50} (95% C.L.)[1]	
					Probit	Spearman-Karber
Effluent[2]	24	10	-	None	37% mortality at 100%	
Copper	24	20	170	5/23/89	75 (71-80)	76 (71-80)
Copper	48	20	170	5/23/89	66 (63-69)	65 (62-68)
Effluent	24	20	170	5/23/89	30% mortality at 100%	
Effluent	48	20	170	5/23/89	43% mortality at 100%	
Copper[3]	24	20	160	6/20/89	46 (36-59)	52 (47-57)
Copper[4]	24	20	160	6/20/89	36 (31-41)	36 (34-38)
Copper[3]	24	25	160	6/20/89	46 (43-50)	49 (46-53)
Copper	24	20	150	7/21/89	46 (28-60)	50 (48-53)
Copper	48	20	150	7/21/89	46 (33-57)	46 (44-47)
Effluent	24	20	150	7/21/89	30% mortality at 100%	
Effluent	48	20	150	7/21/89	38% mortality at 100%	
Copper	24	20	150	7/26/89	46 (37-55)	46 (45-48)
Effluent	24	20	150	7/26/89	43 (34-53)	39 (36-42)
Copper	24	20	155	8/10/89	37 (35-40)	38 (36-41)
Copper	24	20	155	8/10/89	39 (34-45)	40 (38-41)
Copper	24	20	185	10/5/89	63 (62-65)	63 (61-64)
Copper	24	20	185	10/5/89	65 (56-77)	70 (66-74)
Copper	24	20	185	10/5/89	46 (33-61)	47 (44-49)

[1] LC_{50} given as ug Cu/L or % effluent.
[2] 100% effluent contained 264 ug Cu/L.
[3] Freshly collected animal.
[4] Animal collected and held (flow-through tank; tap water) 30 days prior to test.

Table 12 (cont.). Results of the exposures of isolated glochidia of Villosa nebulosa.

Toxicant	Duration (hr)	Temp. (C)	Hardness (mg/L)	Chemistry	LC_{50}* (95% C.L.)[1]	
					Probit	Spearman-Karber
Copper	24	20	190	10/22/89	80 (71-94)	88 (83-92)
Copper	24	20	55	10/22/89	38 (37-39)	43 (42-44)
Copper	24	20	190	10/22/89	69 (65-72)	72 (68-76)
Copper	24	20	190	10/22/89	73 (49-107)	79 (73-85)
Copper	24	20	55	10/22/89	55 (32-88)	56 (54-58)
Copper	24	20	50	12/14/89	71 (60-89)	71 (68-74)

[1] LC_{50} given as ug Cu/L or % effluent.

* Units of ug/L for copper and % for effluent.

hour LC_{50} of 80 ug/L was calculated for glochidia of V. nebulosa at 20 C (Table 12). At a hardness of 55 mg/L, the 24-hour LC_{50} dropped to 38 ug/L. In a second test, a 24-hour LC_{50} of 79 ug/L was calculated at a hardness of 190 mg/L. At 55 mg/L, the LC_{50} dropped to 56 ug/L.

Temperature had a significant effect on the sensitivity of glochidia to both copper and effluent. A 24-hour LC_{50} of 132 ug/L was calculated for an exposure of A. pectorosa glochidia exposed to copper at 10 C. At 25 C, the 24-hour LC_{50} dropped to 42 ug/L. Glochidia exposed to effluent exhibited a similar pattern. No mortality was observed in concentrations up to 100% effluent during a 24-hour exposure at 10 C although a 24-hour LC_{50} of 93% was calculated for 25 C.

The length of time gravid mussels were held in the laboratory prior to testing also affected the sensitivity of the isolated glochidia. A 24-hour LC_{50} of 52 ug/L was calculated for glochidia from a freshly collected V. nebulosa exposed to copper at 20 C. In contrast, a 24-hour LC_{50} of 36 ug Cu/L was calculated for glochidia from a mussel which had been held for 30 days in the laboratory in dechlorinated tap water (Table 12).

Post-exposure periods, following 24-hour exposures of V. nebulosa glochidia to copper and effluent, did not result in any detectable changes. Table 13 shows the number of

Table 13. Mean number of open Villosa nebulosa glochidia in each replicate of the 24-hour exposure to effluent before and after a 24-hour post-exposure period in control water. Standard error is given in parentheses (n=3) (Temperature = 10 C). Water chemistry: 11/2/88.

% Effluent	Before Post-Exposure		After Post-Exposure	
0.00	74	(6.4)	78	(6.4)
6.25	74	(10.4)	77	(8.1)
12.50	70	(7.5)	73	(5.2)
25.00	66	(17.9)	69	(15.6)
50.00	54	(16.2)	56	(15.0)
100.00	44	(7.5)	45	(8.1)

open V. nebulosa glochidia before and after a 24-hour post-exposure period following a 24-hour exposure to effluent. No difference in the number of open glochidia before and after post-exposure was observed. A similar result was obtained following an exposure of V. nebulosa glochidia to copper. A third test with V. nebulosa also exhibited no difference before and after a 24-hour post-exposure, following a 24-hour exposure to effluent. In this test, mean mortality was recorded from the number of closed glochidia (Table 14). In contrast, there was a notable difference in mortality before and after the post-exposure period following an exposure of L. fasciola glochidia to effluent (Table 15).

Finally, post-exposure water samples contained a lower level of detectable copper. Copper solutions exhibited a decrease in detectable copper of approximately 15% in 48 hours (Table 16). Effluent solutions exhibited greater losses, dropping 34% in 48 hours. Declines in detectable copper occurred within 24-hours but were not as high, averaging 8% for copper solutions and 31% for effluent solutions.

Snap Rate Tests

The spontaneous adductor muscle contractions of

Table 14. Mean mortality (percentage closed) of isolated Villosa nebulosa glochidia, exposed to effluent, before and after 24-hour post-exposure period in control water. The standard error is given in parentheses (n=3). (Temperature = 10 C). No water chemistry.

% Effluent	Before Post-Exposure		After Post-Exposure	
0	9.7	(3.2)	10.7	(3.9)
10	7.3	(1.4)	9.3	(1.8)
20	19.3	(6.4)	21.3	(5.2)
40	16.0	(2.3)	18.0	(2.0)
80	26.3	(8.4)	28.0	(8.1)
100	56.7	(1.2)	43.3	(3.2)

Table 15. Mean mortality (percentage closed) of isolated Lampsilis fasciola glochidia in each replicate after 24-hour exposure to effluent and after 24-hour post-exposure period in control water. The standard error is given in parentheses (n=3). (Temperature = 10 C). No water chemistry.

% Effluent	Before Post-Exposure		After Post-Exposure	
0	1.0	(0.6)	2.3	(0.7)
10	1.0	(1.3)	2.0	(1.0)
20	2.3	(1.3)	3.7	(0.4)
40	4.7	(1.7)	12.6	(2.9)
80	20.3	(3.9)	59.3	(19.7)
100	48.3	(2.0)	91.0	(2.7)

Table 16. Decrease in detectable copper with time in 12-well tissue culture plates. (Water chemistry-7/26/89)

	Copper Solution		Effluent	
	Cu (ug/L)			
Time (hr)	Target	Actual	% Eff.	Cu (ug/L)
0	20	25	10	38
24	20	24	10	29
48	20	21	10	27
0	50	49	40	129
24	50	43	40	85
48	50	40	40	85
0	80	76	100	289
24	80	69	100	192
48	80	67	100	179

Anodonta grandis glochidia were suppressed at copper concentrations as low as 17 ug/L (Table 17). This decrease in "snapping" occurred at copper concentrations below those causing significant mortality (>10%). Glochidia exposed to copper for 24-hours at 10 C exhibited a 90% reduction in adductor muscle activity at 80 ug/L. Mortality, as determined by NaCl addition, was only 4% at this concentration. In another test, snap rate was reduced by 62% at 23 ug Cu/L after a 24-hour exposure at 15 C. Mortality at this concentration was 5%. In a third test, a 24-hour exposure to copper at 24 ug/L at 20 C reduced snapping by 80% . Mortality was 16% at this concentration These three tests were conducted in Clinch River water with a hardness of 170-190 mg/L. A fourth test, with a 94% reduction in adductor muscle activity at 17 ug Cu/L, was conducted at 20 C in dechlorinated tap water with a hardness of 50 mg/L.

Encystment Success Tests

Two encystment success tests were conducted with the glochidia of Villosa nebulosa following 24-hour exposures to copper at 20 C. In the first test, no significant ($p=0.23$) change in encystment ability was observed at concentrations up to 59 ug Cu/L (Table 18). Another test, conducted under

Table 17. Mean snap rate of isolated Anondonta grandis glochidia after being exposed to copper for 24 hours. Snap rate is expressed as snaps/minute per 100 glochidia (n=3). Dates reference water chemistry shown in Appendix C.

Cu (ug/L)	Mean Snap Rate	Standard Error	% Survival
	12/14/89 20 C		
2	17	1.7	
17	1	0.6	
30	0	0.0	
	No Water Chemistry 10 C		
0	45	2.5	100
10	44	5.5	100
20	41	0.67	100
40	35	3.0	100
80	4	3.1	96
160	0	0.0	74
	3/22/89 15 C		
5	42	4.0	100
23	16	2.9	95
41	3	2.5	61
87	0	0.0	9
	10/22/88 20 C		
5	24	1.6	98
24	5	1.4	84
37	1	0.2	76
42	0	0.0	74

Table 18. Results of the encystment ability test of isolated glochidia of Villosa nebulosa after 24 hour exposure to copper at 20 C. Dates reference water chemistry given in Appendix C.

Cu (ug/L)	Mean* (# of unencysted glochidia recovered)	Standard Error (n=3)
	10/22/89 20C	
5	48[a]	2.7
24	54[a]	5.0
42	46[a]	2.0
59	61[a]	8.1
	7/26/89 20 C	
6	42[a]	3.8
22	55[ab]	9.6
40	60[ab]	3.7
57	76[b]	6.3

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD. (Koopmans, 1987, p.352).

similar conditions, exhibited an increase in the number of unencysted glochidia recovered. A significant difference ($p=0.03$) was detected in the number of unencysted glochidia recovered at 6 ug Cu/L compared with 57 ug Cu/L.

Discussion

Glochidia Removal

Removal of the glochidia by dissection was easily accomplished and had no apparent effect on sensitivity. In previous studies, glochidia have been removed from gravid adults by flushing the marsupia with water injected from a hypodermic needle and syringe (Waller et al. 1985; Goodreau 1988; Waller and Mitchell 1989). While this technique is satisfactory for obtaining glochidia for study and fish encystments, it may not be suitable when the glochidia are to be used in studies of toxicity.

Goudreau (1988) reported that up to 50% of the glochidia removed by flushing had closed valves. This was attributed to the mechanical agitation of the syringe and the water, which was injected under pressure, forcing the glochidia from the marsupia. I found few closed glochidia after surgical removal of the marsupia, usually from 0 to 5%, and similar observations have been reported by Jerry

Farris (personal communication). However, in studies of rare or endangered species, dissection cannot be used. For some of these species, another non-lethal technique must be considered for removing glochidia.

Lefevre and Curtis (1912) noted that all species of the genus Quadrula tended to abort the contents of their marsupia shortly after collection. They attributed this to the fact that all four gills function as marsupia in the genus. It was suspected that the abortion was an attempt to maximize respiration, which was presumably hindered by the presence of glochidia in the gills. This speculation was supported by their observation that abortion did not occur in species using only two of their four gills as marsupia.

Coker et al. (1921) also suggested that glochidial abortion could be due to a deficiency of dissolved oxygen or other stresses affecting respiration. Again, it was assumed that discharging of the gills improved their efficiency for respiration. Stein (1971) noted that the genera Amblema and Fusconaia had the habit of aborting the contents of their marsupia in response to stress. This response could be useful in obtaining glochidia for testing without sacrificing the adult mussel. Although it was assumed that this habit was restricted to species using all four gills as marsupia, it had apparently not been tested.

I attempted to recover glochidia in mass from species

using only two of their four gills as marsupia. Gravid V. nebulosa and A. grandis were subjected to dissolved oxygen levels as low as 1 ug/L for as long as 2 days at temperatures of 25 C; however, no abortion of glochidia was observed.

The fact that some species are so apt to abort their marsupia is potentially a cause for concern. Abortion could be occurring in the wild in response to anthropogenic disturbances. Effluent discharges with elevated temperatures or high biological oxygen demands may trigger the premature release of glochidia. In addition, pollutants such as zinc, which are known to attack the gill surface and interfere with respiration, might also induce marsupial abortions (A.Heath personal communication).

After the surgical removal of glochidia, exposures were conducted for 24 or 48 hours as previously described. Exposures of longer duration were attempted but were unsuccessful for several reasons. First, the condition of glochidia began to decline after 48 hours and appeared to be temperature-dependent. Glochidia held for 96 hours at 20 C exhibited a slow and delayed response to NaCl addition; the valves closing very slowly. In contrast, glochidia held at 12 C for 96 hours exhibited a rapid response to NaCl. The valve closure was immediate and rapid, upon addition of the NaCl.

In addition, glochidia held for more than 48 hours at 20-25 C were susceptible to attack by fungi and unidentified ciliated protozoans. The fungus was identified as a species of Saprolegnia. Glochidia in the controls and the lower toxicant concentrations were particularly susceptible to attack by these organisms. They were apparently inhibited by the higher Cu concentrations however, being absent from concentrations in excess of 100 ug Cu/L. Finally, the decline of detectable copper in the toxicant solutions made tests longer than 48 hours unsuitable.

At the end of the exposure period, the open and closed glochidia were counted, and their response to NaCl addition recorded. In these tests, all glochidia within each well were counted and recorded. In preliminary tests however, a subsample of 50 glochidia was counted from each well, rather than a complete count. This latter method was unsuitable because it was not possible to arbitrarily count 50 glochidia without obtaining a biased ratio of open to closed. The open glochidia were distributed throughout the well, whereas the more streamlined closed glochidia tended to clump in the center or along the sides. This resulted in an uneven or nonrandom distribution of open and closed glochidia in the well. Thus, the contents of the entire well were counted to ensure accurate results.

Glochidia Response to Copper

There was no apparent interspecies variation in sensitivity to copper or effluent among the five species tested. All species were affected by copper in the range of 20-80 ug/L, and sensitivities increased with increasing temperature and hardness. This is similar to observations made on other organisms, including fish and invertebrates. (Cairns et al. 1975; Nelson et al. 1986; Belanger et al. 1989).

Neves and Widlak (1988) found that lampsiline glochidia were present in stream drift year-round, peaking during June and July. Amblemine glochidia, in contrast, were found only from June to mid-August. River conditions during this summer period are characterized by the yearly peak in water temperature and low flow. Under these conditions, the amount of inorganic and organic materials which could bind the cupric ion and reduce its toxicity would be low. In addition, the low flow conditions would result in limited dilution of any toxic discharges to the river. At the same time, because of high ambient air temperatures, this period represents a time of peak power demand. This association of low river levels, high water temperatures, and peak power demand coincides with the major period of glochidial release.

The inhibition of the spontaneous adductor muscle contractions of A. grandis glochidia occurred at levels below the calculated LC_{50}. This observation is not of biological significance however, unless it is associated with an impairment of encystment ability. Thus the LC_{50} calculated for the isolated glochidia could lead to an underestimation of the effects of an exposure. In addition, after encystment, the glochidia must successfully metamorphose to the juvenile stage, an aspect not examined.

The encystment success tests with the glochidia of V. nebulosa suggest that there was little effect on encystment ability at sublethal concentrations. Therefore, the assumption that a glochidium is biologically functional, or able to encyst upon a fish host if it closes in response to NaCl, appears to valid.

However, I am also assuming, in the calculation and interpretation of the LC_{50}, that no latent responses occurred. Results of the 24-hour post-exposure periods, following exposures to copper or effluent, suggest that in some cases this may not be true. The glochidia of L. fasciola exhibited substantial post-exposure mortality following a 24-hour exposure to effluent. Similar tests with glochidia of V. nebulosa failed to exhibit any post-exposure effects however. It seems unlikely that this latent response would be unique to the glochidia of L.

fasciola; thus, the extent of these latent responses needs to further investigation. Hansen and Kawatski (1976) called for the use of a 24-hour post-exposure period in any toxicity study involving invertebrates. This technique may be warranted in future studies investigating the response of isolated glochidia to toxicants.

My observations suggest that once glochidia close in response to a toxicant, they remain closed. There was no evidence that the glochidia were closing their valves to avoid exposure as has been reported for adult freshwater mussels (Salanki 1979; Salanki and Varanka 1976; Millington and Walker 1983). Counting the closed glochidia as dead, prior to NaCl addition, was therefore appropriate.

CHAPTER 4 - ENCYSTED GLOCHIDIA

Introduction

Glochidia can be divided into three basic types; hooked, unhooked, and axe-headed glochidia (Arey 1921). Axe-headed glochidia are found only in the genus Potamilus. Hooked glochidia are characteristic of the subfamily Anodontinae, whereas unhooked glochidia are found within the family Margaritiferidae and the subfamilies Lampsilinae and Ambleminae. This morphological distinction has some functional significance in that the hooked glochidia encyst primarily upon the fins and other external surfaces of host fish (Figures 3a and 3b). In contrast, unhooked glochidia attach to the gills (Figures 2c, 3c, and 3d), although there are occasional exceptions (Lefevre and Curtis 1910b, 1912; Young 1911; Howard 1914; d'Eliscu 1972; Zale and Neves 1982b; Neves and Widlak 1988).

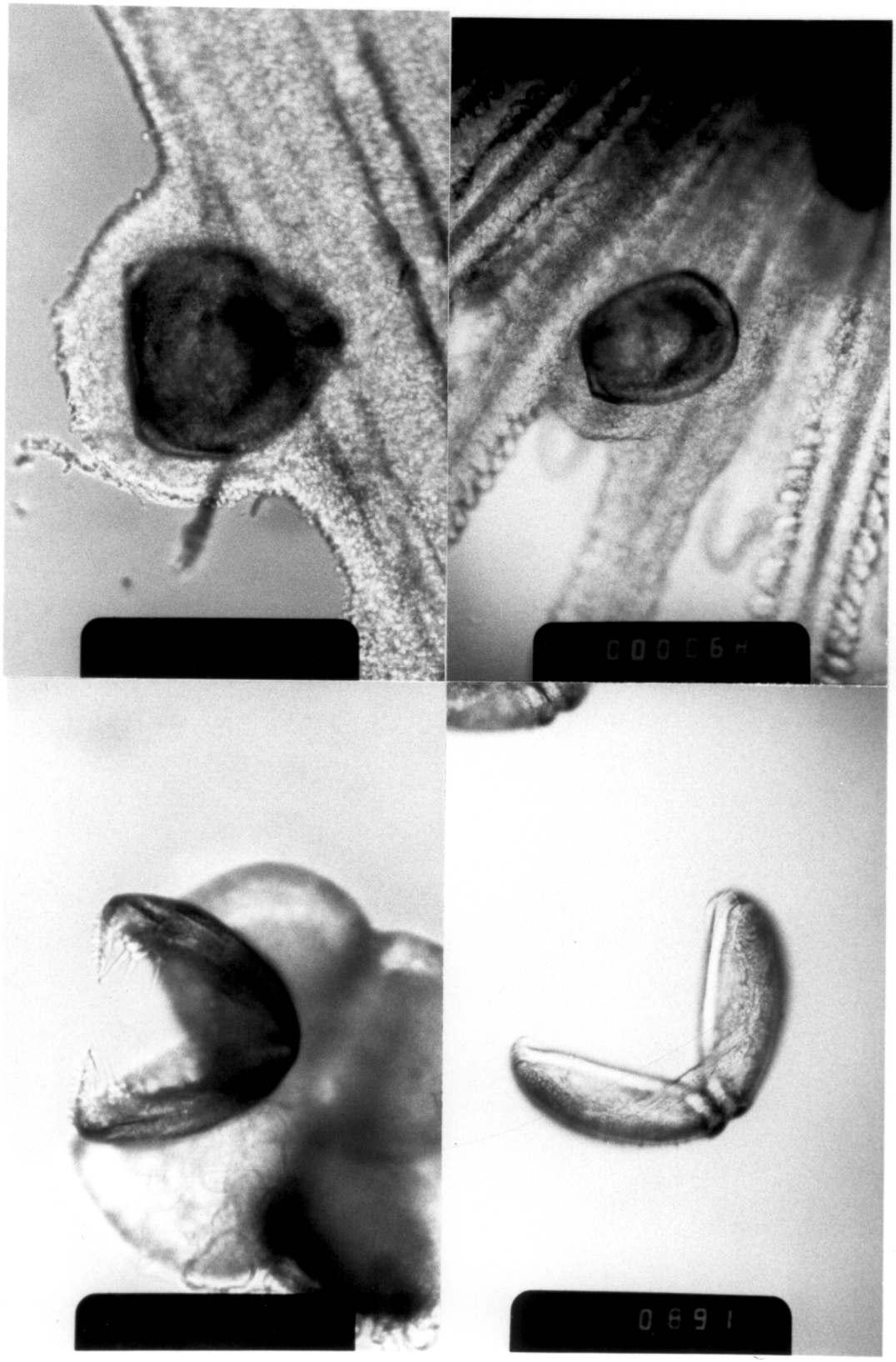
When a hooked or unhooked glochidium attaches, the host fish responds by encapsulating it in a layer of tissue (Figures 3b and 3d). Arey (1921, 1932) reported that encapsulation was accomplished by the proliferation of host cells as well as host cell migration to the site. He noted

Figure 3a. Isolated glochidia of Anodonta grandis.

Figure 3b. Encysted glochidia of Anodonta grandis on pectoral fin of bluegill.

Figure 3c. Isolated glochidia of Villosa nebulosa.

Figure 3d. Encysted glochidia of Villosa nebulosa on gill filament of largemouth bass.



that the process was quite rapid, with complete encapsulation occurring within 2-3 hours. This agrees with d'Eliscu's (1972) observations of Anodonta californiensis encysting upon mosquito fish, Gambusia affinis, within 3-4.5 hours at 20 C. Waller and Mitchell (1989) recently reported their observations of Lampsilis radiata siliquoidea on the gills of walleye, Stizostedion vitreum vitreum; namely, complete encapsulation within 6 hours post-infection at 20-22 C.

The encysted stage in the life cycle of the freshwater mussel constitutes its chief means of dispersal (Stein 1971). The parasitized fish can move upstream or downstream and disperse the excysting juvenile mussels to a new location. Prior to this metamorphosis however, the fish could enter waters containing contaminants. It was therefore of interest to determine the possible sensitivity of encysted glochidia to toxicants.

There are no data currently available on the susceptibility of encysted glochidia to toxicants. Moles (1980) conducted exposures of coho salmon, Oncorhychus kisutch, encysted with the glochidia of Anodonta oregonensis, to various organic pollutants and demonstrated a positive correlation between the number of glochidia attached to host fish and their sensitivity to a toxicant. Levels of infestation of 35 or more glochidia significantly increased the sensitivity of the host fish. He did not, however,

examine the effect of various toxicants on the encysted glochidia.

Mortality of host fish, due to over-encystment, has been reported to occur in the absence of any other negative factors. Lefevre and Curtis (1912) observed that over-infection of the gills and fins was easily accomplished and fatal, when inducing encystments of fish. This was particularly true for unhooked glochidia attaching to the gills. They found levels of approximately 2,500 glochidia lethal to 10-12 cm largemouth bass, Micropterus salmoides. Glochidia attaching to the gills interfere with oxygen uptake by physically reducing the surface area available for gas exchange.

These levels of infestation are primarily from artificial encystments with captive animals. Table 19 shows levels of encystment for various combinations of mussels and their hosts in the wild. They suggest that lethal encystments are unlikely to occur in natural populations.

Neves and Widlak (1988) investigated the levels of glochidial encystment on fish in the upper North Fork Holston River, near McCrady, Virginia, and found that most fish were infested with 1-10 glochidia. This is well below levels found to cause mortality, even in very small fish. However, Moles (1980) did see an effect upon host fish sensitivity to toxicants at levels of 20-35 glochidia. Thus, the possibility

Table 19. Levels of glochidial infestations in wild populations of fish.

Mussel	Host Fish	# Encysted Glochidia	Reference
Amblemine glochidia	Notropis galacturus	140	Neves & Widlak, 1988
Anodonta cataracta	Gasterosteus aculeatus	7.5 +/- 7.5 (1-40)	Threlfull, 1986
Anodonta cygnea	Gasterosteus aculeatus	< 20	Dartnell & Walkey 1979
Anodonta grandis	Lepomis macrochirus	1 - 20	
	Ambloplites rupestris	1 - 20	Lefevre & Curtis, 1910
	Pomoxis annularis	1 - 20	
A. grandis corpulenta	Pomolobus chrysochloris	24	Surber, 1913
Anodonta oregonensis	Oncorhynchus kisutch	3 - 35	Moles,1980
Lampsiline glochidia	Ambloplites rupestris	230	Neves & Widlak, 1988
Lampsilis radiata	Perca flavescens	60 - 100	Telda & Fernando, 1969
L. siliquoidea radiata	Perca flavescens	1 - 58	
	Micropterus salmoides	8 - 20	Trdan, 1981
	Lepomis macrochirus	3 - 18	
Lampsilis teres	Pomoxis annularis	16	Surber, 1913
	Scaphirhynchus platorynchus	56 - 125	
Potamilus ohioensis	Aplodinotus grunniens	112 - 850	Surber, 1913

exists that encysted glochidia could be affecting the degree of host fish mortality in polluted streams.

There are no data on the sensitivity of encysted glochidia. Given that encapsulation occurs within 2-6 hours, the glochidium is rapidly isolated from the surrounding water after attachment. This would presumably prevent any direct exposure to a water-borne toxicant. I therefore assumed that encysted glochidia would be relatively insensitive to toxic metals such as copper.

The objective of this phase of the study was to determine the sensitivity of encysted glochidia to copper. This would determine what protection, if any, the encapsulation provided the encysted glochidium.

Materials and Methods

Glochidia of Villosa nebulosa, Actinonaias pectorosa, and Anodonta grandis were used. Gravid V. nebulosa were collected at Copper Creek Mile (CCM) 50, Scott County, Virginia. Actinonaias pectorosa were collected from Clinch River Mile (CRM) 270.5, Russel County, Virginia, and Anodonta grandis were taken from Claytor Lake, Pulaski County, Virginia. After collection, gravid mussels were transported to the laboratory in chilled, water-filled coolers. Glochidia were removed by excising the marsupia and teasing them apart in a water-filled

petri dish. They were then tested for viability by inspection of three subsamples of approximately 50 glochidia. Viability was determined by their closing in response to a drop of saturated NaCl solution. Glochidia were not used if viability was below 90%.

Largemouth bass, 5 cm in length, were used as the host fish in exposures and were obtained from Kurtz's Fish Hatchery in Elverson, Pennsylvania. Fish were held in the laboratory in dechlorinated tap water for five to seven days prior to testing.

Fish were encysted in mass by placing them in a rectangular 120 L polyethylene tank filled with approximately 40-60 L of dechlorinated tap water. Glochidia of a species were then added to the tank. One marsupium of A. grandis and A. pectorosa was used, whereas both marsupia were taken for encystments with V. nebulosa, because of its smaller size. The tank was aerated with two airstones, insuring a distribution of glochidia throughout the water column. This yielded a relatively homogenous level of encystment on all fish.

Encystment was initiated for the bass by introducing them to the tank for 5 minutes. They were then removed together with a large net, constructed to fit within the walls of the tank. The freshly encysted fish were then transferred to an adjacent tank and added at random to test chambers consisting

of 17 L polycarbonate carboys. These were held at room temperature (19-21 C) and maintained on a 16:8 light-dark photoperiod.

The toxicant solutions were made by adding an appropriate volume of a 1 g/L $CuSO_4$-$7H_2O$ stock solution to 10 L of dechlorinated tap water. Immediately after encystment, 5 or 10 of the encysted bass were added to the test chambers, each aerated with a single airstone. Each of the four concentrations, ranging from 0 to 400 ug Cu/L, were replicated three times.

The test solution was renewed every two to four days by draining off 7 L and replacing it with fresh solution. The 7 L of solution were drained through a 100 micron sieve to collect any excysted juvenile mussels. Contents of the sieve were then rinsed into a petri dish and examined under a stereomicroscope. The number of juvenile mussels was recorded for each concentration and replicate. Exposure of the encysted fish was continued until no additional excysted juveniles were recovered.

Results

Encystment of the hooked glochidiam of A. grandis, attaching primarily to the fins of the bass, was relatively easy to control. Exposure of bass to the contents of one

marsupium for 5 minutes yielded a mean encystment level of 27 glochidia per fish (S.E.=1.9,n=14). The copper exposure was continued for 12 days, and juvenile mussels were recovered in all copper concentrations and controls (Table 20). No significant difference, based upon juvenile recovery, was observed between any of the concentrations and the control.

In contrast, degree of encystment of the unhooked glochidia of A. pectorosa and V. nebulosa, which attached to the gills of the fish, was difficult to control. In both cases, over-encystment occurred, resulting in bass mortality. The glochidia of A. grandis and V. nebulosa were both encapsulated within 2-3 hours on the fins and gills, respectively, of the largemouth bass at 20-22 C. Figures 3b and 3d show these glochidia in their encysted state, 3 hours after attachment.

Exposure time for bass encysted with the glochidia of A. pectorosa was 16 days. During this time period, 73% mortality of bass was observed in the 100 ug Cu/L treatment. Similarly, 80% mortality was recorded in the 200 ug Cu/L, and 60% mortality in the 400 ug Cu/L treatments. In contrast, no mortality was observed in control fish. On day 16 of the exposure, juveniles were recovered from all four treatments. Dead fish were examined and found to have swollen and discolored gills; approximately 200-300 glochidia were present on each fish.

Table 20. Mean number of juvenile Anodonta grandis recovered during exposure of encysted largemouth bass, Micropterus salmoides, to copper at 20-22 C for 12 days (hardness = 50-55 mg/L).

Cu (ug/L)	Mean*	Standard Error (n=3)
0	22[a]	4.6
25	17[a]	1.2
100	13[a]	2.9
200	16[a]	2.3

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD (Koopman's 1987, p.252).

A similar result was observed when bass, encysted with the glochidia of V. nebulosa, were exposed. Approximately 100-200 glochidia were encysted upon each fish. Within the 20-day exposure period, 10% mortality of bass was observed in the 100 ug Cu/L treatment, and 13% mortality in the 200 and 400 ug Cu/L treatments. In contrast, control fish experienced 43% mortality. Examination of the dead fish revealed swelling and discoloration of the gills in all treatments. Dead fish in the control were distinctive however, in that the gills were covered with a growth of the fungus Saprolegnia spp.. This infection was present on dying fish and undoubtedly contributed to the observed mortality. It was not observed on the dead or dying fish in the copper treatments.

Discussion

The recovery of juveniles from the bass exposed to copper at concentrations ranging from 0 to 400 ug Cu/L indicates that encysted glochidia are relatively insensitive to external Cu concentrations. The encystment level of 27 glochidia of A. grandis per fish is similar to levels reported from nature (Table 19), whereas encystment levels with glochidia of A. pectorosa and V. nebulosa were above optimal observed levels.

Exposure of bass encysted with A. pectorosa glochidia supported the observations of Moles (1980). Although no

mortality was observed in the controls, mortality did occur in the copper treatments. Previous exposures of bass, encysted with glochidia of A. grandis, showed no mortality at copper concentrations used in tests of A. pectorosa and V. nebulosa. This suggests that observed mortality of bass in the treatments may be attributable to an increase in sensitivity due to over-encystment. This observation is of little significance to wild fish however, given that infestations of 200-300 glochidia on a 4-5 cm fish would rarely, if ever, occur.

My observations of bass over-encysted with glochidia of V. nebulosa do not fit this pattern. The high incidence of control mortality versus low mortality in copper treatments is opposite to what would be expected. However, the presence of fungus on fish gills in the control treatments does suggest an explanation. Acute exposures of isolated glochidia have shown that colonization of Saprolegnia spp. is inhibited at concentrations of 100 ug Cu/L (personal observation) and could explain the differential mortality observed. The attachment of a glochidium causes local tissue damage, leaving the site potentially susceptible to fungal infection. This infection occurred in the controls and likely contributed to the high level of observed mortality. Colonization by the fungus probably was inhibited in the copper treatments, which accounted for the lower levels of mortality observed.

It is not clear why bass, over-encysted with V. nebulosa glochidia, developed fungal infections while those over-encysted with A. pectorosa glochidia did not. The physico-chemical conditions of the two tests were similar. In addition, the level of glochidial infestation was lower on the fish encysted with V. nebulosa. It is possible that the fish used in the two tests, having been received in two separate shipments from the hatchery, may have differed. The fish used in the V. nebulosa study may have been stressed upon arrival from unusual handling prior to or during shipment which may have enhanced their susceptibility to fungal infection.

It is clear however, that encysted glochidia are resistant to copper exposures up to 400 ug Cu/L. Howard and Anson (1923) exposed encysted bass to a 1000 ug/L copper solution for 2 to 3 minutes without any effect on the encysted glochidia. This resistance to copper is undoubtedly due to the rapid encapsulation which occurs shortly after attachment of the glochidium. As previously stated, both hooked and unhooked glochidia that encyst on the gills or fins of fish are encapsulated within 2-6 hours. This encapsulation, and the barrier it creates, makes the encysted glochidia relatively insensitive. This stage in the life cycle therefore appears inconsequential in assessing the impact of copper pollution on freshwater mussels.

CHAPTER 5 - Juvenile Mussels

Introduction

The newly metamorphosed glochidium or juvenile mussel (<20 mm in shell length) is the poorest known life stage of the freshwater mussel (Lefevre and Curtis 1912; Negus 1966; Ahlstedt 1979; Neves et al. 1980). After encystment and encapsulation, glochidia pass through a period of metamorphosis to excyst as a free-living member of the benthic community. Mortality at this stage could occur from predation or dropping from the fish into an area of unsuitable habitat.

Very few studies focus on the juvenile stage, principally because of the difficulty in finding individuals. In 1911, Isely provided observations for 9 species of juvenile (sexually immature) mussels from the Kiamichi, Little, and Washita rivers in Oklahoma. He detailed the importance of gravel bars or stony situations, free from shifting sand and silt, for survival of juvenile mussels. Measurements of 10 of the 32 specimens collected averaged approximately 5 to 10 mm in size.

Neves and Widlak (1987) investigated the habitat ecology of juvenile mussels in a stream in southwestern Virginia.

They defined the juvenile stage as consisting of mussels less than 3 years of age, based upon an earlier study by Zale (1980) in the same location. A total of 92 juvenile mussels were collected, ranging from 0.8 to 30.3 mm, in substrata consisting of course gravel and boulders, similar to Isely's (1911) observations. Juveniles of less than 2 months of age were not reported however, in either study. Howard (1917) reported that the juvenile shell up to 2 months of age is small (< 1mm long), transparent, and uncalcified. This probably accounts for their absence in previous studies.

A similar lack of information exists on the sensitivity of juvenile mussels to pollution. James (1985) attributed a lack of juveniles in Lake Taupo, New Zealand, to sedimentation, pollution, and eutrophication. Recently, however, several workers have begun developing laboratory tests to measure the sensitivity of juveniles to various pollutants. Wade et al. (1989) tested 8-day old juvenile Anodonta imbecillis, produced from artificial cultures, in exposures to manganese, a paper mill effluent, and several pesticides. Keller and Crisman (1989) have also worked with juvenile Anodonta imbecillis from artificial cultures.

Toxicity testing with juvenile mussels is difficult to conduct for several reasons. First, the small size (< 1 mm) of a newly excysted juvenile makes recovery and handling very difficult. Second, a realistic appraisal of an organism's

sensitivity to a contaminant demands that it be in an unaffected condition prior to the test. In long-term tests, any effect observed should be attributed to the toxicant being tested. This assumes that the effect is not an artifact of inappropriate handling or test conditions. Proper feeding before and during a test is critical to an organism's performance. Standard methods for estimating toxicity mandate well-defined feeding regimes before and during a test (E.P.A. 1989). No such protocols for feeding juvenile freshwater mussels have been established however. In fact, as is typical with other aspects of their natural history, little is known of their diet.

Coker et al. (1921) reported the observations of A.F. Shira, who analyzed the stomach contents of 60 juveniles (4.8-21.5 mm) of 6 species, taken from Lake Pepin, an expansion of the Mississippi River between Wisconsin and Minnesota. The principal component (89-96%) was organic or vegetable matter. Less than 5% of the stomach contents consisted of either inorganic material (silt), unicellular green algae, or diatoms.

Hudson and Isom (1984), in describing the rearing of juvenile freshwater mussels in the lab, validated Shira's observations. They observed poor survival of juveniles fed only a suspension of algae, but upon the addition of silt, survival was greatly improved. They attributed this to the

organic content of the silt, representing an additional food source. Given the lack of adequate information on diet of juvenile mussels, static 24 or 48 hour tests conducted in this study were within several days of excystment of juveniles. This was done to avoid the potential inaccuracies that could be associated with an inappropriate diet.

Finally, the determination of a toxicant's impact requires an accurate assessment of the condition of the organism following exposure. This is not easy with juvenile freshwater mussels, because of their small size, which necessitates close examination. A healthy juvenile is typically active, extruding the foot and moving in the test chamber. When not actively moving, its valves are partially gaped. A dead juvenile is easily identified by its widely gaped valves and immobile and rigid foot. At intermediate concentrations, the juvenile is typically immobile with its valves closed. It is, therefore, not clear what the extent of the effect has been. The condition of the juvenile could range from near death to merely avoiding an irritant.

Given the transparent valves of many juvenile mussel species, inspection under a stereomicroscope at 30-50 times magnification reveals juvenile anatomy quite clearly. The foot, cilia, and other structures can be seen. The absence of movement of the foot or cilia, or a reduction therein, can be used to quantitfy response.

A typical test contains three replicates of eight concentrations, each containing 10 juveniles. At the end of the exposure period, individual inspection of 240 juvenile mussels is required. This can require 1 to 2 hours or longer. It was therefore desirable to have a means of rapidly assessing the condition of the juvenile at the end of an exposure. Vital staining appeared to be a suitable solution.

Vital staining is commonly defined as the staining of living cells or tissues with non-toxic dyes (Henderson and Henderson 1963). A vital stain is therefore a non-toxic dye that stains only living cells. This is a misleading over-simplification in that vital stains are known to be toxic to the target organism (Shiba and Kanno 1979). Since the stain was used to determine the juvenile's condition at the end of exposure, its toxicity was irrelevant.

Vital staining has been used in many areas of biology and medicine (Sawicki et al. 1967; Levitt 1969; Bulychev et al. 1978; Shiba and Kanno 1979; Hammond et al. 1980), and basic techniques and stains are described in several texts (Conn 1953; Lillie 1969). Dressel et al. (1972) used vital staining to sort live and dead invertebrates (copepods). Crippen and Perrier (1974) also used neutral red to determine mortality among marine plankton. Based on their results, neutral red was chosen as a potential vital stain for determining the condition of juvenile freshwater mussels.

The objectives of this phase of the study were to: (1) determine the sensitivity of juvenile mussels to copper, and (2) evaluate the suitability of vital staining with neutral red as a means of determining the post-exposure condition of juvenile mussels.

Materials and Methods

Rearing Juveniles

Gravid Villosa nebulosa were collected from Copper Creek, Scott County, Virginia (CCM 50) and gravid Anodonta grandis, from Claytor Lake, Pulaski County, Virginia. Mussels were transported to the laboratory in chilled, water-filled coolers. Glochidia were removed by excising the marsupia and teasing them apart in a water-filled petri dish. Three subsamples of approximately 50 glochidia were then tested for viability (valve closure) by adding a drop of saturated NaCl solution. Glochidia were not used if the viability was below 90%.

Largemouth bass, Micropterus salmoides, of 10-12 cm length, served as host for glochidia of V. nebulosa, and bluegill, Lepomis macrochirus, of 10-12 cm, served as host for A. grandis glochidia. The fish were obtained from Kurtz's Hatchery in Elverson, Pennsylvania. Fish were encysted five

at a time by introducing them to a 20-L bucket filled with approximately 10 L of water. Glochidia had been added previously and were kept suspended in the water by aeration with a single airstone. The contents of both marsupia were used in encystments with V. nebulosa. The fish were exposed to the glochidia for approximately 5 to 10 minutes. For encystments with A. grandis, the contents of one marsupium were used in a 10 minute encystment period. It was necessary to use caution in the encystments to avoid excessive infestations, which were often lethal. A fish was periodically anesthetized during the encystment in a bucket of 3-aminobenzoic acid ethyl ester or MS-222 (0.1 g/L) and examined to monitor the level of infestation and prevent over-encystments.

The encysted fish were transferred to 80-L flow-through aquaria with dechlorinated tap water. Encystment periods ranged from 6 to 10 days for A. grandis and 18 to 25 days for V. nebulosa at 20-22 C. The fish were fed fathead minnows, Pimephales promelas, until juveniles began to excyst. Feeding was then terminated to avoid feces accumulation and interference with juvenile recovery.

The tank bottoms were siphoned daily through a 100-micron sieve; contents of the sieve were then washed into a petri dish and examined. Juvenile mussels were removed using a flamed, wide-bore pasteur pipette, transferred to a small

beaker of water, and held at 20 C in an incubator. A photoperiod of 16:8 light-dark was maintained in the room housing the aquaria and the incubator.

Artificial Stream Exposures of Juvenile Mussels

Juveniles (1-3 days old) were held in artificial streams at the Clinch River Plant at Carbo, Virginia for a 14-day exposure. The artificial stream design and maintenance is discussed in Chapter 3 and therefore omitted here. Juvenile mussels were held in the streams by placing them in 150 ml crystallizing dishes. These dishes were then covered with a layer of 100-micron mesh, held in place by several rubber bands. This allowed an exchange of water between the container and stream. The dishes were anchored in rock-filled baskets within the streams. Each dish held 15 juvenile mussels, and three replicates per treatment were tested. Juveniles were held for the 14-day period in the control stream, receiving only Clinch River water, the 17 ug Cu/L stream, and the 10% effluent stream.

After 14 days, dishes were removed and returned to the laboratory in a chilled, water-filled cooler. Juveniles were isolated by pouring the contents of the dish through a 100-micron sieve. Contents of the sieve were then washed into a petri dish and examined.

Acute Exposures

Twenty-four hour, static exposures were conducted in 12-well polystyrene tissue culture plates. Each well, with a volume of 6.7 ml, was filled with 3.5 ml of a toxicant solution. A total of eight concentrations, ranging from 0 to 200 ug Cu/L were made by dilution of a copper stock solution. Approximately 40 mg of $CuSO_4$-$7H_2O$ were added to 1 L of filtered Clinch River water or dechlorinated tap water, to yield a stock containing approximately 10,000 ug Cu/L.

The concentration series was made by diluting varying aliquots of this stock. Hardness, alkalinity, pH, dissolved oxygen, and conductivity were then determined. A sample of each concentration was taken and acidified with 150 uL of HNO_3 for later metal analysis. Metal analysis was done at the Virginia Tech Soil Testing Laboratory using inductively-coupled, argon plasma emission spectroscopy.

Each concentration was replicated three times and randomized among and in plates by assignment using a random number table (Zar 1984). After addition of the solutions, 10 juveniles were added row by row to each well. The trays were then placed in an incubator for the duration of the 24-hour exposure period. Temperature was maintained at 20 C, and photoperiod was fixed on a 16:8 light-dark cycle. At the end of the exposure period, the trays were removed from the

incubator and examined. Juvenile condition was determined by both visual inspection and vital staining.

Visual Inspection

Each juvenile was examined at 12-50 times magnification using a Zeiss stereomicroscope. For the artificial stream exposure, two classes of condition were recorded; (1) gaped or closed and alive, or (2) gaped or closed and dead. Living juveniles were characterized by the partially open valves and the frequent extrusion of the foot or the movement of the cilia or foot inside the valves. If no movement of the foot or cilia could be seen, or the valves were widely gaped and the foot rigid and immobile, the juvenile was classed as dead. Upon completion of this visual inspection, the total number of live and dead as recorded.

Juveniles from the acute exposures were examined under the stereomicroscope only for the presence of gaped or ungaped valves. Three classes of condition were recorded; (1) gaped and alive, (2) gaped and dead, and (3) ungaped. Classes 2 and 3 were combined, representing a total number of affected juveniles, and these data were then used to calculate an EC_{50}.

Vital Staining

Juveniles from acute exposures were stained after the visual inspection for gaping using the technique described by Crippen and Perrier (1974). Juveniles were left in the wells with the toxicant solutions during the initial staining.

A stock solution of 1 g of neutral red in 1 L of water was prepared. This stock was added, 1 ml to each 100 ml of sample, to each well. Since each well contained 3.5 ml of the toxicant solution, 0.035 ml was added. The tray was then returned to the incubator and left for 1 hour. After 1 hour of staining, the trays were removed from the incubator, and the contents preserved with 0.35 ml of formalin.

The dye was then removed by draining each well with a pipette, being careful to leave the stained juvenile mussels on the bottom. The wells were then refilled with 3.5 ml of water. To each well was added 0.14 ml of an equimolar acetic acid-sodium acetate solution prepared by mixing equal parts of 1 N acetic acid and 1 N sodium acetate solution. The addition of 0.35 ml of formalin was repeated, and the trays stored overnight in a refrigerator at approximately 4 C. Juveniles were examined the next day using a stereomicroscope at 12-20 times magnification. Each juvenile was recorded in one of three categories; (1) brightly stained, (2) lightly or partially stained, and (3) unstained, which corresponded to

unaffected, affected, and dead, respectively.

Data Analysis

Results of the acute exposures were analyzed using probit analysis (Finney 1971; Buikema et al. 1982; SAS 1985) and the Spearman-Karber method (Hamilton et al. 1977; Stephan 1977). Juveniles which exhibited light or partial staining were used to calculate an EC_{50}. An EC_{50} was also calculated based on the visual observations of gaping. An LC_{50} was calculated from the number of juveniles with no detectable staining.

Results

A 14-day exposure of juvenile V. nebulosa in the 10% effluent stream resulted in 93% mortality. In contrast, no mortality was observed in the 17 ug Cu/L or the control stream (Table 21).

Juveniles of V. nebulosa, exposed to copper in 24-hour static exposures, were quite sensitive. Valve gaping, a normal behavior of healthy juveniles, was affected at test concentrations as low as 24 ug Cu/L (Table 22). An EC_{50}, calculated from the presence or absence of gaping, yielded a value of 27 ug Cu/L. Complete inhibition of gaping was observed at 59 ug Cu/L, with 100% of the juveniles having

Table 21. Results of 14-day artificial stream exposure of juvenile Villosa nebulosa. (Water chemistry given in Table 17).

Stream	# Exposed	# Dead	% Mortality
Control	45	0	0
17 ug Cu/L	45	0	0
10% Effluent	45	42	93

Table 22. Results of the 24-hour exposure of juvenile Villosa nebulosa to copper at 20 C. EC_{50} value based on presence or absence of gaping. Ungaped (closed valves) juveniles were classed as affected.

Copper (ug/L)	# Exposed	# Ungaped	% Affected
5	20	0	0
24	20	10	50
37	20	15	75
42	20	17	85
59	20	20	100

	EC_{50}	95% Confidence Limits
Probit	27	23-32
Spearman-Karber	26	22-31

closed valves. In contrast, 100% of the juveniles in the controls were gaped and active.

Vital staining indicated that juveniles were affected by the copper exposure at concentrations as low as 24 ug/L. The EC_{50}, calculated by combining partially and unstained juveniles, was 29 ug Cu/L (Table 23). This value was very close to the EC_{50} calculated from the absence of gaping. Finally, an LC_{50} of 83 ug Cu/L was calculated, based on the absence of vital staining (Table 24). The water chemistry for these tests is presented in Table 25.

Juvenile A. grandis also were sensitive to copper. Exposures of 24 hours duration affected gaping at concentrations as low as 17-30 ug Cu/L (Table 26). An EC_{50} was calculated, based on the absence of gaping. This yielded a value of 33 ug Cu/L. Virtually complete inhibition occurred at 61-79 ug Cu/L, with 93-97% of the juveniles having closed valves. This is in contrast to the controls, in which 100% of the juveniles were gaped and active.

Vital staining with neutral red indicated an effect of copper at concentrations as low as 30 ug Cu/L. This was based on the absence of stain, interpreted as mortality. An LC_{50} of 44 ug Cu/L was calculated (Table 27). Vital staining was not used in the calculation of an EC_{50}. The water chemistry for the tests is summarized in Table 28.

Table 23. Results of the 24-hour exposure of juvenile Villosa nebulosa to copper at 20 C. EC_{50} calculated using vital staining. Affected animals were those which exhibited no staining or light staining relative to the controls.

Copper (ug/L)	# Exposed	# Affected	% Affected
5	20	2	10
24	20	9	45
37	20	12	60
42	20	15	75
59	20	17	85
75	20	20	100

	EC_{50}	95% Confidence Limits
Probit	29	22-35
Spearman-Karber	30	25-36

Table 24. Results of the 24-hour exposure of juvenile Villosa nebulosa to copper at 20 C. LC_{50} value based on the absence of vital stain. Unstained individuals were classed as dead.

Copper (ug/L)	# Exposed	# Unstained	% Mortality
5	20	1	5
24	20	0	0
37	20	3	15
42	20	5	25
59	20	9	45
75	20	13	65
106	20	15	75
213	20	19	95

	LC_{50}	95% Confidence Limits
Probit	83	70-100
Spearman-Karber	86	70-101

Table 25. Water chemistry for the 24-hour exposure of juvenile Villosa nebulosa (Temp. 20 C).

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
5	0	8.35	8.1	330	190	174
24	20	8.35	8.1	330		
37	30	8.35	8.1	330		
42	40	8.35	8.1	330		
59	60	8.35	8.1	330		
75	80	8.35	8.1	330		
106	100	8.35	8.1	330		
213	200	8.35	8.1	330	190	174

Table 26. Results of the 24-hour exposure of juvenile Anodonta grandis to copper at 20 C. EC_{50} value based on presence or absence of gaping. Ungaped (closed valves) juveniles were classed as affected.

Copper (ug/L)	# Exposed	# Ungaped	% Affected
2	30	0	0
17	30	4	14
30	30	12	40
38	30	25	73
61	30	28	93
79	30	29	97
99	30	30	100

	EC_{50}	95% Confidence Limits
Probit	33	22-45
Spearman-Karber	33	29-37

Table 27. Results of the 24-hour exposure of juvenile Anodonta grandis to copper at 20 C. LC_{50} value based on the absence of stain. Unstained individuals were classed as dead.

Copper (ug/L)	# Exposed	# Unstained	% Mortality
2	30	1	3
17	30	2	7
30	30	12	40
38	30	19	63
61	30	22	73
79	30	25	83
99	30	28	93

	LC_{50}	95% Confidence Limits
Probit	44	37-50
Spearman-Karber	44	38-49

Table 28. Water chemistry for the 24-hour exposure of juvenile Anodonta grandis.

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
2	0	7.97	8.0	165	70	64
17	20	7.97	8.0	165		
30	30	7.97	8.0	165		
38	40	7.97	8.0	165		
61	60	7.97	8.0	165		
79	80	7.97	8.0	165		
99	100	7.97	8.0	165		
196	200	7.97	8.0	165	75	61

Discussion

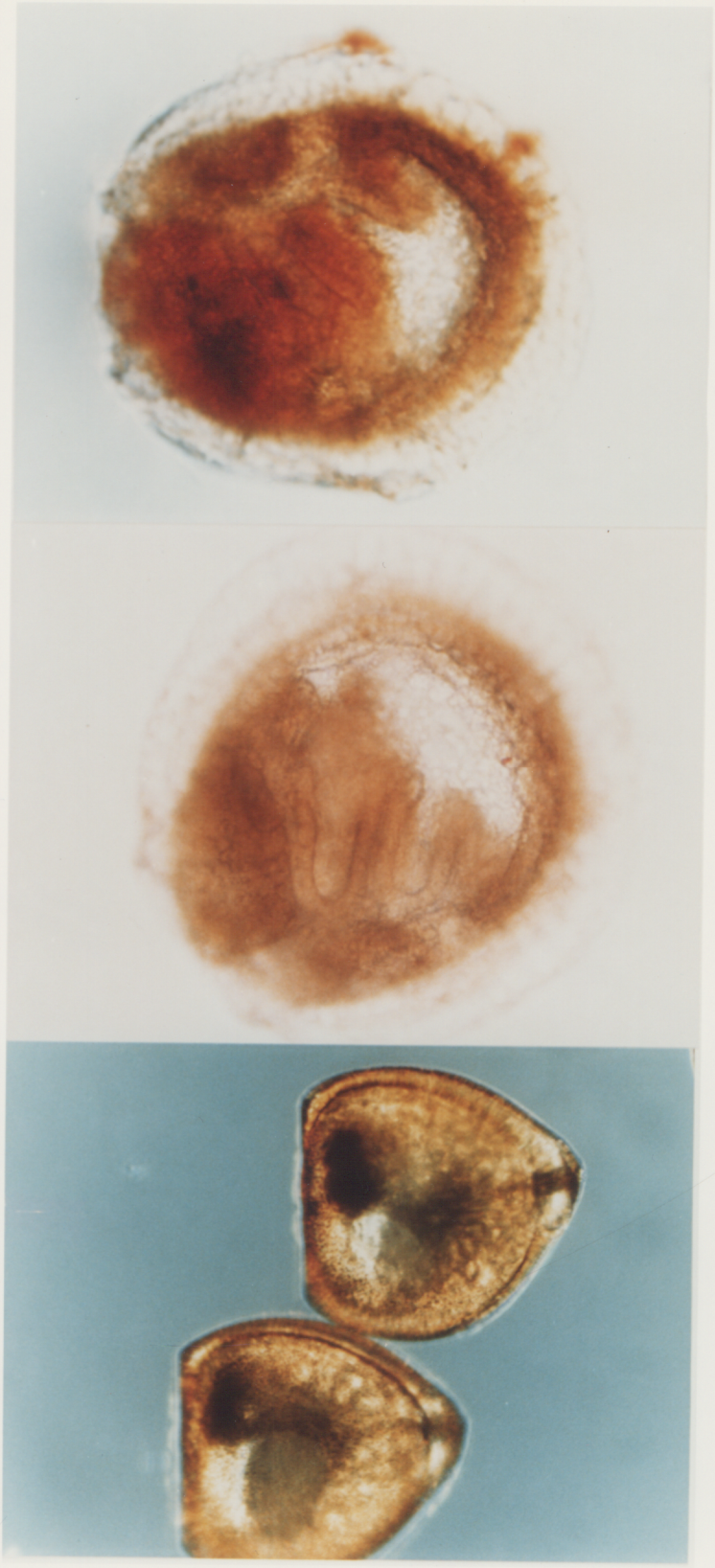
Results of the 14-day artificial stream exposure of Villosa nebulosa juveniles indicated a high level of sensitivity to the 10% effluent. The mortality observed cannot be attributed solely to the copper, however, given the elevated levels of Zn, Fe, and Al in the effluent (Van Hassel and Gaulke 1986). Also, no mortality was observed in the 17 ug Cu/L stream. Other contaminants, by themselves or in concert with the copper, were probably responsible for the observed affects.

Results of the 24-hour exposures of V. nebulosa and A. grandis indicated a high level of sensitivity to copper. The determination of mortality in both species was greatly facilitated by the neutral red stain, which allowed the rapid separation of dead from live specimens. This could not have been accomplished as rapidly if individual inspection of the valve contents had been required. In addition, neutral red made possible the quantification of partial affects. Stain intensity ranged from bright red in the controls to non-existent in the high-concentration exposures (Figures 4a and 4b). The range of intensity over intermediate concentrations corresponded with a dose-effect response. At each successively higher toxicant concentration, the intensity of the staining decreased, grading from an intense red to a faint

Figure 4a. Live juvenile Villosa nebulosa stained with neutral red.

Figure 4b. Dead juvenile Villosa nebulosa showing absence of staining.

Figure 4c. Juvenile Anodonta grandis showing colored valves.



pink.

In tests with juveniles of V. nebulosa, EC_{50} values, based on valve gaping and partial staining, were in close agreement. In a gaped juvenile, neutral red is circulated within the valves by the flow of water created by beating cilia. It is thus in immediate contact with the soft tissues of the mussel, facilitating uptake of the stain. However, when a juvenile closes its valves in response to copper in the water, such contact would not occur and the uptake of the stain would be restricted.

Juveniles that have closed their valves in copper-containing solutions occasionally open and close their valves. This behavior may indicate that the juvenile is testing the environment, and if unsuitable, it will close. This does offer an explanation for the partial staining observed in the low to intermediate concentrations. It is also possible however, that the variation in staining could be due to the effects of copper on cellular processes, which could impair stain uptake. The range of intensity could be explained by increasing numbers of dead or damaged cells not incorporating the stain. These two explanations, physical restriction of uptake by valve closure and inhibition due to cellular damage, are not mutually exclusive. In fact, they may be operating in concert, with behavioral effects predominating at lower concentrations and cellular damage becoming increasingly

prevalent at higher concentrations.

The speculation that partial staining could be due to an inhibition of cellular processes is supported by the work of Hammond et al. (1980). They hypothesized that entry of neutral red into the cell may have been accomplished by micropinocytosis or transport/diffusion across the plasma membrane. They favored the former mechanism, based on evidence provided by inhibition studies involving azide, colchicine, and low temperatures.

Regardless of the mechanism, vital staining with neutral red was a useful technique. It allowed for rapid determinations of mortality and partial effects. However, it will not allow quantification of partial effects in species possessing colored or opaque valves. EC_{50} values, based on partial staining, could not be determined for juveniles of A. grandis. The amber coloration of the valve prevented visualization of any partial staining of the valve contents (Figure 4c).

Results of the tests on V. nebulosa and A. grandis show that newly excysted juvenile mussels are sensitive to copper, and avoid it at low concentrations (24-30 ug Cu/L). While avoidance may confer a temporary advantage in limiting exposure to a toxicant, it also prevents feeding. Given the small size of the juvenile, it does not have any substantial nutritive reserves to draw upon, as does an adult.

Therefore, prolonged valve closure and inhibition of

feeding would lead to a rapid deterioration in juvenile condition. In addition, the peak excystment of juveniles of both long-term brooders and short-term brooders is occurring in mid to late summer. This is the period during which toxicants in the water are likely to have their greatest impact. Under conditions of low flow and high temperature, newly excysted juvenile mussels will be very susceptible to copper and other pollutants.

CHAPTER 6 - SUMMARY AND CONCLUSIONS

Isolated glochidia and juvenile mussels were very sensitive to copper. Isolated glochidia were adversely affected at concentrations as low as 16 to 26 ug Cu/L, which caused 50% mortality in 24 hours to some species. Juvenile mussels reduced their activity during 24-hour exposures to copper concentrations as low as 17 to 24 ug Cu/L. Concentrations of 30 to 42 ug Cu/L caused mortality in juvenile mussels. This level of sensitivity is comparable, and in some cases, greater than that reported for Ceriodaphnia and fathead minnows, Pimephales promelas (U.S.E.P.A. 1985; Belanger et al. 1989).

In contrast, glochidia encysted on host fish were resistant to copper exposures as high as 400 ug Cu/L. No significant effect on metamorphosis to the juvenile stage was observed. Glochidia held in the marsupia of gravid adults were unaffected in 30-day exposures at a mean concentration of 19 ug Cu/L. However, high levels of organic particulates, due to the high river stage, may have reduced the concentration of biologically active copper. The relative sensitivities of the tested life stages for V. nebulosa and A. grandis are summarized in Table 29.

Table 29. Comparisons of sensitivity to copper among life stages of the freshwater mussels, Villosa nebulosa and Anodonta grandis.

Villosa nebulosa

Life Stage	Cu (ug/L)
Glochidia in marsupia	> 19[1]
Isolated glochidia	37-75[2]
Encysted glochidia	> 400[3]
Juveniles	27-29[4]
Juveniles	83[5]
Adults	> 1000[6]

[1] 30-day artificial stream exposure.
[2] LC_{50} - 24-hour static exposure.
[3] 20-day static exposure of encysted fish.
[4] EC_{50} - 24-hour static exposure.
[5] LC_{50} - 24-hour static exposure.
[6] 96-hour artificial stream exposure.

Anodonta grandis

Life Stage	Cu (ug/L)
Isolated glochidia	46[1]
Encysted glochidia	> 200[2]
Juveniles	33[3]
Juveniles	44[4]

[1] LC_{50} - 24-hour static exposure.
[2] 12-day static exposure of encysted fish.
[3] EC_{50} - 24-hour static exposure.
[4] LC_{50} - 24-hour static exposure.

As judged by my observations, juvenile mussels and glochidia within the marsupia are probably the two most sensitive stages in the life cycle of the freshwater mussel. Newly excysted juveniles are particularly susceptible to copper pollution, given their presence in mid to late summer when water temperature is high and flow is minimal. Elevated levels of copper in the river at this time could eliminate or reduce population recruitment.

Discharges from the plant at Carbo, Virginia, are having a negative impact upon the mussel community downstream. Concentrations of copper in the effluent from the plant are well above those which have been found to have serious effects on some of the life stages (Van Hassel and Gaulke 1986). The question is, how far downstream is an impact occurring?

Observations of Alhstedt (1984) and Stansbery et al. (1986) suggest that the 20 kilometer reach between CRM 256 and CRM 268 has been adversely affected by the plant's operations. The extent to which the plant's current operations are affecting the mussel fauna is unknown. The impact of copper released from the plant will vary, dependent upon the current river conditions and Plant operations.

River stage changes and the associated changes in organic and inorganic particulates will alter the effect of copper released into the river. In addition, seasonal changes in water temperature will also affect the impact of copper.

Therefore, the length of the river harmed by the releases can vary seasonally. However, the Plant is not the only anthropogenic factor responsible for declines in mussel populations.

The mussels of the Clinch River are in jeopardy throughout the watershed, not just in the 20 kilometers below the plant. Of the nearly fifty species of freshwater mussels reported from the river, eleven are on the federal endangered species list (Table 1). Along with the reported population declines have been numerous changes in the watershed. Coal mining, gas and oil exploration, municipal discharges, clear-cutting of forested lands, bridge and road construction, farming, cattle grazing, cutting of streambank vegetation, removal of gravel from the river bed, commercial and scientific collecting of mussels, and illegal dumping, among others, have all undoubtedly contributed to the observed declines in the mussel populations. Municipal outfalls are common and often located upstream of sites possessing good populations of freshwater mussels (Goudreau 1988). As was noted in Havlik and Marking (1987), many factors are affecting mussels and their habitats. In many cases, more than one of these factors contributes to the total impact but few studies have examined cumulative effects. The contaminants that all of these activities contribute to the river may act to exacerbate the impact of the Plant's releases.

In order to prevent further deterioration of the mussel fauna of the Clinch River, detrimental activities within the watershed will have to be controlled. The implementation of BMP's (Best Management Practices) within the watersheds of the two major tributaries of the Clinch River would be helpful. The Little River and Copper Creek tributaries would greatly benefit from such an implementation. This should include the restriction of livestock access to the stream, maintenance of streambank vegetation, use of low-till agricultural methods, and restricted use of agricultural pesticides and herbicides.

The future of the freshwater mussel fauna of the Clinch River relies upon the ability to identify and control these inappropriate land-use practices, as well as industrial discharges.

APPENDIX A

Appendix A. Results of the 24-hour acute exposures of glochidia from the 30-day artificial stream study. Numbers in parenthesis indicate % viability of glochidia at the start of exposure. Data presented are total number exposed, total number affected, and percent affected.

Control Stream

Cu (ug/L)	Mussel #1			Mussel #2			Mussel #3		
			(96)			(96)			(94)
5	130	14	11	217	17	8	180	15	8
22	180	21	12	115	14	8	211	9	4
34	171	41	24	221	170	77	218	90	41
58	174	133	76	201	201	100	195	190	97
82	164	164	100				201	201	100

Copper Creek

Cu (ug/L)	Mussel #1			Mussel #2			Mussel #3		
			(97)			(97)			(97)
5	161	27	17	195	22	11	121	38	31
22	164	15	9	214	11	5	100	15	15
34	172	151	88	154	31	20	163	48	29
58	166	166	100	200	142	71	160	108	68
82				191	191	100	130	120	92
93							154	143	93
136							138	138	100

17 ug Cu + 47ug Zn/L Stream

Cu (ug/L)	Mussel #1			Mussel #2			Mussel #3		
			(89)			(97)			(98)
5	188	11	6	186	8	4	185	16	9
22	165	17	10	166	24	14	136	10	7
34	167	137	82	173	169	98	117	72	43
58	180	173	96	175	175	100	187	160	86
82	175	175	100				169	169	100

10% Effluent

	Mussel #1			Mussel #2			Mussel #3		
Cu (ug/L)			(89)			(88)			(89)
5	219	32	15	218	27	12	188	22	12
22	191	36	19	223	27	12	170	13	8
34	258	226	88	182	160	88	188	38	20
58	223	223	100	240	238	99	229	183	80
82				216	216	100	240	223	93
93							203	203	100

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8 ug Cu/L Stream

	Mussel #1			Mussel #2			Mussel #3		
Cu (ug/L)			(98)			(98)			(95)
26	217	29	13	249	22	9	188	43	23
30	214	45	21	210	42	20	177	69	39
38	207	59	29	180	71	39	222	189	85
51	187	65	35	161	100	62	206	182	88
65	206	155	75	184	150	82	198	198	100
71	198	198	100	197	197	100			

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17 ug Cu/L Stream

	Mussel #1			Mussel #2			Mussel #3		
Cu (ug/L)			(98)			(98)			(98)
26	179	46	26	150	115	77	212	192	91
30	182	33	18	147	29	20	227	43	19
38	223	108	48	176	71	40	188	70	37
51	200	144	72	170	104	61	212	141	67
65	196	196	100	131	116	89	204	198	97
71				155	155	100	240	240	100

APPENDIX B

Appendix B. Results of the 24-hour acute exposures of isolated glochidia. The dates reference water chemistry data given in Appendix C.

Actinonaias pectorosa- Copper, 24 hour exposure at 10C and 25 C. 2/13/89.

10 C

Cu (ug/L)	# Affected	# Exposed	% Affected
3	3	300	1
10	3	238	1
20	2	279	1
30	3	300	1
36	2	300	1
60	12	303	4
77	27	309	9
100	70	278	25
200	320	340	94
400	277	277	100

25 C

Cu (ug/L)	# Affected	# Exposed	% Affected
3	2	279	1
10	8	308	3
20	22	312	7
30	57	524	11
36	61	351	17
60	384	384	100

Actinonaias pectorosa - Effluent, 24 hour exposure at 10 C and 25 C. 2/13/89.

10 C

% Effluent	Cu (ug/L)	# Affected	# Exposed	% Affected
0	3	0	359	0
10		0	345	0
20		0	269	0
30		0	280	0
40		0	326	0
60		0	260	0
80		0	305	0
100	346	0	300	0

25 C

% Effluent	Cu (ug/L)	# Affected	# Exposed	% Affected
0	3	3	300	1
10		21	254	8
20		13	322	4
30		4	254	2
40		10	334	3
60		35	290	12
80		154	539	29
100	346	205	312	66

Actinonaias pectorosa - Effluent, 24 hour exposure at 25 C
2/16/89

% Effluent	Cu (ug/L)	# Affected	# Exposed	% Affected
0	5	10	320	3
10		11	476	2
20		16	419	4
40		35	215	16
60		190	471	40
80		417	554	75
90		413	493	84
100	270	338	362	93

Actinonaias pectorosa - Copper, 24 hour exposure at 15C.
3/22/89.

Cu (ug/L)	# Affected	# Exposed	% Affected
5	13	239	5
23	20	267	7
41	32	325	10
87	79	409	19
108	364	445	82
217	300	300	100

Actinonaias pectorosa - Copper, 24 and 48 hour exposure at 20 C. 5/23/89.

24 hours

Cu (ug/L)	# Affected	# Exposed	% Affected
5	3	187	2
21	13	260	5
46	8	267	3
70	133	282	47
89	175	177	99
108	100	100	100

48 hours

Cu (ug/L)	# Affected	# Exposed	% Affected
5	5	201	3
21	11	153	7
46	27	188	14
70	191	195	98
89	100	100	100

Actinonaias pectorosa - Effluent, 24 and 48 hour exposure at 20 C. 5/23/89.

24 hours

% Effluent	# Affected	# Exposed	% Affected
0	4	185	2
5	0	177	0
10	7	218	3
20	15	210	7
40	2	225	1
60	4	219	2
80	20	246	8
100	55	244	23

48 hours

% Effluent	# Affected	# Exposed	% Affected
0	2	168	1
5	5	315	2
10	5	165	3
20	18	253	7
40	16	185	9
60	79	240	33
80	196	236	83
100	213	232	92

Anodonta grandis - Copper and effluent, 24 hour exposure at 10 C. 11/14/88 (no water chemistry).

Copper

Cu (ug/L)	# Affected	# Exposed	% Affected
9	0	168	0
14	0	189	0
24	0	206	0
44	0	216	0
92	3	244	12
182	16	152	11
391	108	171	63
695	212	212	100

Effluent

% Effluent	# Affected	# Exposed	% Affected
0	0	211	0
10	0	283	0
20	0	195	0
40	0	166	0
80	0	302	0
100	0	246	0

Anodonta grandis - Copper, 24 hour exposure at 10 C. 12/7/88 (no water chemistry).

Cu (ug/L)	# Affected	# Exposed	% Affected
0	1	132	0
10	1	169	0
20	0	227	0
40	0	177	0
80	5	142	4
160	33	126	26

Anodonta grandis - Copper, 24 hour exposure at 20 C. 12/14/89.

Cu (ug/L)	# Affected	# Exposed	% Affected
2	1	137	1
19	50	171	29
30	68	169	40
37	88	173	51
52	122	161	76
73	153	221	70
101	121	164	74
168	182	182	100

Lampsilis fasciola - Copper, 24 and 48 hour exposures at 20 C. 5/23/89.

24 hours

Cu (ug/L)	# Affected	# Exposed	% Affected
5	1	213	1
21	85	208	41
46	86	202	43
70	181	188	96
89	227	230	99
108	100	100	100

48 hours

Cu (ug/L)	# Affected	# Exposed	% Affected
5	5	172	3
21	24	160	6
46	178	287	62
70	196	201	98
89	100	100	100

Lampsilis fasciola - Effluent, 24 and 48 hour exposures at 20 C. 5/23/89.

24 hours

% Effluent	# Affected	# Exposed	% Affected
0	13	253	5
5	5	246	2
10	47	253	19
20	84	405	21
40	28	224	13
60	69	273	25
80	107	176	61
100	165	183	90

48 hours

% Effluent	# Affected	# Exposed	% Affected
0	14	236	6
5	8	189	4
10	26	217	12
20	16	205	8
40	41	199	21
60	157	199	79
80	217	230	94
100	100	100	100

Lampsilis fasciola - Copper, 24 hour exposure at 20 C. 5/23/89.

Cu (ug/L)	# Affected	# Exposed	% Affected
3	8	158	5
12	8	164	5
21	63	135	47
37	99	131	76
54	129	129	100

Lampsilis fasciola - Copper, (New River Water) 24 hour exposure at 20 C. 8/10/89.

Cu (ug/L)	# Affected	# Exposed	% Affected
9	10	167	6
28	9	244	4
37	47	186	25
44	86	163	53
51	124	185	67
61	167	199	84
79	173	180	96
92	214	219	98

Lampsilis fasciola - Effluent, 24 exposure at 10 C. Before and after 24 hour post-exposure period in control water. (No water chemistry).

Before

% Effluent	# Affected	# Exposed	% Affected
0	8	344	2
10	5	249	2
20	13	352	4
40	40	305	13
80	198	371	59
100	276	313	91

After

% Effluent	# Affected	# Exposed	% Affected
0	4	306	1
10	3	245	1
20	6	299	2
40	13	266	5
80	66	346	20
100	179	368	48

Medionidus conradicus - Copper, 24 and 48 hour exposures at 20 C. 5/23/89.

24 hours

Copper (ug/L)	# Affected	# Exposed	% Affected
5	2	125	2
21	35	117	30
46	96	150	64
70	100	150	67
89	112	140	80
108	122	143	85
161	137	162	85
217	100	100	100

48 hours

Copper (ug/L)	# Affected	# Exposed	% Affected
5	10	174	6
21	115	115	67
46	141	141	90
70	131	131	94
89	181	181	95
108	100	100	100

Medionidus conradicus - Effluent, 24 and 48 hour exposures at 20 C. 5/23/89.

24 hours

% Effluent	# Affected	# Exposed	% Affected
0	11	169	7
5	25	159	16
10	45	168	27
20	124	179	69
40	173	187	93
60	208	218	95
80	196	199	99
100	197	202	98

48 hours

% Effluent	# Affected	# Exposed	% Affected
0	19	163	12
5	17	166	10
10	153	272	56
20	152	168	91
40	159	167	95
60	226	237	96
80	189	197	96
100	177	183	97

Medionidus conradicus - Copper, 24 hour exposure at 20 C. 6/20/89.

Copper (ug/L)	# Affected	# Exposed	% Affected
3	1	133	1
12	5	127	4
21	17	140	12
37	74	135	55
54	96	130	74
70	130	141	92
86	100	100	100

Medionidus conradicus - Copper and effluent, 24 hour exposure at 20 C. 7/21/89.

Copper

Copper (ug/L)	# Affected	# Exposed	% Affected
0	17	207	8
14	19	204	9
21	8	179	4
46	20	224	9
71	75	173	43
89	131	194	68
109	133	188	71
217	126	140	90

Effluent

% Effluent	# Affected	# Exposed	% Affected
0	13	136	10
5	10	152	7
10	4	137	3
20	8	138	6
40	18	119	15
60	14	115	12
80	33	132	25
100	71	181	39

Medionidus conradicus - Copper, 24 hour exposure at 20 C. 10/5/89.

A

Copper (ug/L)	# Affected	# Exposed	% Affected
3	1	146	1
23	16	196	8
30	70	158	44
38	130	147	88
61	183	190	96
79	194	195	99
98	200	200	100

B

Copper (ug/L)	# Affected	# Exposed	% Affected
3	2	125	2
23	15	120	13
30	43	118	36
38	87	152	57
61	105	126	83
79	112	118	95
98	188	203	93
185	200	200	100

C

Copper (ug/L)	# Affected	# Exposed	% Affected
3	0	173	0
23	9	186	5
30	17	204	8
38	55	173	32
61	90	199	45
79	123	224	55
98	163	202	81
185	200	200	100

Villosa nebulosa - Effluent, 24 hour exposure at 10 C. (No water chemistry) 11/6/88.

% Effluent	# Affected	# Exposed	% Affected
0	19	191	10
10	12	165	7
20	44	213	19
40	31	194	16
80	51	186	26
100	84	228	37

Villosa nebulosa - Copper, 24 and 48 hour exposures at 20 C. 5/23/89.

24 hours

Copper (ug/L)	# Affected	# Exposed	% Affected
5	3	160	2
21	4	181	2
46	39	128	31
70	76	158	48
89	102	288	54
108	171	224	76
161	158	163	97
217	100	100	100

48 hours

Copper (ug/L)	# Affected	# Exposed	% Affected
5	2	144	1
21	0	173	0
46	38	154	25
70	92	181	51
89	112	141	79
108	156	161	97
161	100	100	100

Villosa nebulosa - Effluent, 24 and 48 hour exposures at 20 C. 5/23/89.

24 hours

% Effluent	# Affected	# Exposed	% Affected
0	4	176	2
5	0	173	0
10	0	116	0
20	4	142	3
40	46	175	26
60	69	214	32
80	45	157	29
100	57	185	31

48 hours

% Effluent	# Affected	# Exposed	% Affected
0	4	163	3
5	0	164	0
10	3	164	2
20	7	150	5
40	44	181	24
60	83	222	37
80	61	151	40
100	88	201	44

Villosa nebulosa - Copper, 24 hour exposures at 25 C. 6/20/89.

Copper (ug/L)	# Affected	# Exposed	% Affected
3	15	158	9
12	9	106	8
21	23	124	19
37	56	121	46
54	63	114	55
70	83	108	77
86	94	103	91
169	124	126	98

Villosa nebulosa - Copper, 24 hour exposures at 20 C. 6/20/89 (freshly collected and held).

Freshly collected

Copper (ug/L)	# Affected	# Exposed	% Affected
3	2	89	2
12	8	131	6
21	39	144	27
37	74	125	59
54	71	124	57
70	84	109	77
86	83	103	81
169	100	100	100

Held

Copper (ug/L)	# Affected	# Exposed	% Affected
3	11	166	7
12	6	140	4
21	14	114	12
37	72	113	64
54	147	175	84
70	128	133	96
86	100	100	100

Villosa nebulosa- Copper, 24 and 48 hour exposures at 20 C. 7/21/89.

24 hours

Copper (ug/L)	# Affected	# Exposed	% Affected
0	7	199	4
21	5	172	3
46	114	192	59
71	147	206	71
89	213	227	94
109	100	100	100

48 hours

Copper (ug/L)	# Affected	# Exposed	% Affected
0	7	191	7
21	8	195	4
46	131	230	57
71	181	201	90
89	100	100	100
109	100	100	100

Villosa nebulosa - Effluent, 24 and 48 hour exposures at 20 C. 7/21/89.

24 hours

% Effluent	# Affected	# Exposed	% Affected
0	15	150	10
5	13	140	9
10	23	174	13
20	11	144	8
40	15	148	10
60	26	150	17
80	19	150	13
100	60	159	38

48 hours

% Effluent	# Affected	# Exposed	% Affected
0	22	140	16
5	24	165	15
10	15	155	10
20	17	200	9
40	24	169	14
60	23	151	15
80	20	135	15
100	39	130	30

Villosa nebulosa- Copper and effluent, 24 hour exposure at 20 C. 7/26/89.

Copper

Copper (ug/L)	# Affected	# Exposed	% Affected
6	54	266	20
22	20	296	7
28	63	274	23
40	155	334	46
49	162	303	54
57	198	346	57
76	289	311	93
93	335	353	95

Effluent

% Effluent	# Affected	# Exposed	% Affected
0	30	212	14
5	29	209	14
10	36	237	15
20	63	241	26
40	138	257	54
60	197	240	82
80	171	234	73
100	247	273	91

Villosa nebulosa- Copper, 24 hour exposure at 20 C. 8/10/89a.

Copper (ug/L)	# Affected	# Exposed	% Affected
8	19	201	10
23	10	224	5
30	61	204	30
42	134	202	66
50	141	193	73
58	178	202	88
70	186	200	93
86	225	229	98

Villosa nebulosa- Copper, 24 hour exposure at 20 C. 8/10/89b.

Copper (ug/L)	# Affected	# Exposed	% Affected
8	12	136	9
23	11	164	7
30	52	171	30
42	139	198	70
50	138	167	83
58	164	188	87
70	170	176	97
86	174	175	100

Villosa nebulosa - Copper, 24 hour exposure at 20 C.
10/5/89.

A

Copper (ug/L)	# Affected	# Exposed	% Affected
3	0	217	0
23	5	179	3
30	5	190	3
38	0	189	0
61	103	231	45
79	251	277	91
98	181	181	100

B

Copper (ug/L)	# Affected	# Exposed	% Affected
3	1	167	1
23	6	168	4
30	27	132	21
38	62	166	37
61	65	182	36
79	119	181	66
98	162	195	83
185	100	100	100

C

Copper (ug/L)	# Affected	# Exposed	% Affected
3	3	226	1
23	34	227	15
30	78	248	32
38	82	174	30
61	135	191	71
79	233	248	94
98	245	250	98
185	100	100	100

Villosa nebulosa- Copper, 24 hour exposure at 20 C. (Clinch and Dechlorinated Water) 10/22/89.

Clinch River A

Copper (ug/L)	# Affected	# Exposed	% Affected
5	5	187	3
24	20	206	10
37	26	220	12
42	21	229	9
59	28	173	16
75	113	194	58
106	170	228	75
213	200	200	100

Dechlorinated A

Copper (ug/L)	# Affected	# Exposed	% Affected
9	6	203	3
33	5	185	3
37	33	240	14
42	117	186	63
59	175	180	97
103	200	200	100

Clinch River B

Copper (ug/L)	# Affected	# Exposed	% Affected
5	6	184	3
24	16	199	80
37	43	226	19
42	95	263	36
59	74	232	32
75	132	254	52
106	204	226	90
213	200	200	100

Clinch River C

Copper (ug/L)	# Affected	# Exposed	% Affected
5	10	235	4
24	28	261	11
37	74	265	28
42	53	196	27
59	30	258	12
75	76	220	35
106	180	244	74
213	200	200	100

Dechlorinated C

Copper (ug/L)	# Affected	# Exposed	% Affected
9	6	214	3
33	4	173	2
37	13	205	6
42	35	207	17
59	158	241	66
103	204	215	95
99	222	226	98
190	200	200	100

Villosa nebulosa- Copper, 24 hour exposure at 20 C. 12/14/89.

Copper (ug/L)	# Affected	# Exposed	% Affected
2	7	213	3
19	6	213	3
30	12	201	6
37	15	214	7
52	56	280	20
73	154	240	64
101	164	181	91
168	215	220	98

APPENDIX C: WATER CHEMISTRY

Appendix C. Water chemistry data from acute exposures referenced in Appendix B.

Water Chemistry. 11/2/88 - Effluent (Clinch River Water)

% Effluent	Cu (ug/L)	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalinity (mg/L)
0		8.41	8.5	337	85	142.5
6.25		8.34	8.5	398		
12.5		8.34	8.3	460		
25		8.27	8.4	582		
50		8.27	8.4	825		
100	265	7.89	8.3	1325	300	45.7

Water Chemistry. 2/13/89 - Copper and effluent (Clinch River Water).

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
3	0	8.32	8.4	250	140	110
10	10	8.24	8.5	260		
20	20	8.24	8.5	260		
30	30	8.24	8.5	260		
36	40	8.30	8.5	260		
60	60	8.30	8.5	265		
77	80	8.30	8.5	270		
100	100	8.30	8.5	265		
200	200	8.30	8.5	260		
400	400	8.22	8.5	265	140	110

% Effluent	Cu (ug/L)	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalinity (mg/L)
0	3	8.32	8.4	250	140	110
10		8.25	8.4	335		
20		8.20	8.4	400		
30		8.20	8.4	470		
40		8.17	8.4	550		
60		8.07	8.4	660		
80		7.94	8.4	790		
100	346	7.74	8.4	900	500	30

Water Chemistry. 2/16/89 - Effluent (Clinch River Water).

% Effluent	Cu (ug/L)	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalinity (mg/L)
0	5	8.36	9.0	235	140	108
10		8.29	9.0	290		
20		8.26	9.0	345		
40		8.17	9.0	440		
60		8.08	9.0	525		
80		7.89	9.0	625		
90		7.79	9.0	675		
100	270	7.61	9.0	700	380	20

Water Chemistry. 3/22/89 - Copper (Clinch River Water).

Cu (ug/L)		pH	D.O.	Cond.	Hard.	Alkalin.
Measured	Target		(mg/L)	(umhos)	(mg/L)	(mg/L)
<5	0	8.36	8.9	265		
22	20	8.32	8.9	280		
41	40	8.35	8.9	270		
87	80	8.34	8.9	280		
108	100	8.35	8.9	270		
217	200	8.33	8.9	280		
326	300	8.33	8.9	272		
434	400	8.27	8.9	287		

Water Chemistry. 5/23/89 - Copper and effluent (Clinch River Water).

Cu (ug/L) Measured	Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
5	0	8.41	8.3	260	135	170
21	20	8.41	8.3	265		
46	40	8.41	8.3	267		
70	60	8.41	8.3	265		
89	80	8.41	8.3	272		
108	100	8.41	8.3	280		
161	150	8.41	8.3	275		
217	200	8.41	8.3	270	135	170

% Effluent	pH	D.O. (mg/L)	Conductivity (umhos)	Hardness (mg/L)	Alkalinity (mg/L)
0	8.47	8.4	260	135	170
5	8.42	8.4	305		
10	8.39	8.4	355		
20	8.34	8.4	440		
40	8.24	8.4	610		
60	8.14	8.4	770		
80	7.99	8.4	920		
100	7.71	8.4	1050	22	620

Water Chemistry. 6/20/89 - Copper (Clinch River Water).

Cu (ug/L) Measured	Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
5	0	8.51	8.9	270	109	160
12	10	8.51	8.9	272		
21	20	8.50	8.9	275		
37	40	8.52	8.9	270		
54	60	8.52	8.9	270		
70	80	8.52	8.9	273		
86	100	8.48	8.9	275		
169	200	8.54	8.9	277	114	160

Water Chemistry. 7/21/89 - Copper and effluent (Clinch River Water).

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
<5	0	8.55	>8	300	129	150
14	10	8.58	>8	294		
21	20	8.60	>8	297		
46	40	8.58	>8	300		
71	60	8.59	>8	300		
89	80	8.55	>8	300		
109	100	8.60	>8	295		
163	150	8.54	>8	300		
217	200	8.52	>8	300	130	160

% Effluent	Cu (ug/L)	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
0		8.49	>8	300	129	150
5		8.49	>8	345		
10		8.44	>8	398		
20		8.38	>8	490		
40		8.20	>8	600		
60		8.04	>8	780		
80		7.71	>8	950		
100	269	7.58	>8	1100	24	640

Water Chemistry. 7/26/89 - Clinch River Water.

Cu (ug/L) Measured	Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
6	0	8.55	8.1	305	137	150
22	20	8.60	8.1	306		
28	30	8.62	8.1	307		
40	40	8.56	8.1	308		
49	50	8.60	8.1	308		
57	60	8.55	8.1	308		
76	80	8.60	8.1	307		
93	100	8.53	8.1	308	137	170

% Effluent	Cu (ug/L)	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
0	6	8.49	8.1	305	137	150
5		8.49	8.1	370		
10	38	8.51	8.1	430		
20		8.44	8.1	450		
40	129	8.38	8.1	660		
60		8.20	8.1	860		
80		8.04	8.1	1040		
100	289	7.71	8.1	1230	33	685

Water Chemistry. 8/10/89 - Copper (Clinch and New River Water).

Clinch River Water

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
8	0	8.56	8.3		155	136
23	20	8.56	8.3			
30	30	8.56	8.3			
42	40	8.56	8.3			
50	50	8.56	8.3			
58	60	8.56	8.3			
70	80	8.56	8.3			
86	100	8.56	8.3		155	136

New River Water

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
9	0	8.10	8.3		75	54
28	20	8.10	8.3			
37	30	8.10	8.3			
44	40	8.10	8.3			
51	50	8.10	8.3			
61	60	8.10	8.3			
79	80	8.10	8.3			
92	100	8.14	8.3		80	54

Water Chemistry. 10/5/89 - Copper (Clinch River Water).

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
3	0	8.32	8.4	325	185	170
23	20	8.32	8.4	325		
30	30	8.32	8.4	325		
38	40	8.32	8.4	325		
61	60	8.32	8.4	325		
79	80	8.32	8.4	325		
98	100	8.32	8.4	325		
185	200	8.32	8.4	325	185	170

Water Chemistry. 10/22/89 - Copper (Clinch and Dechlorinated Tap Water).

Clinch River Water

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
5	0	8.35	8.1	330	190	174
24	20	8.35	8.1	330		
37	30	8.35	8.1	330		
42	40	8.35	8.1	330		
59	60	8.35	8.1	330		
75	80	8.35	8.1	330		
106	100	8.35	8.1	330		
213	200	8.35	8.1	330	190	174

Dechlorinated Tap Water

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
9	0	7.83	8.1	125	55	39
33	20	7.83	8.1	125		
37	30	7.83	8.1	125		
42	40	7.83	8.1	125		
59	60	7.83	8.1	125		
103	80	7.83	8.1	125		
99	100	7.83	8.1	125		
190	200	7.83	8.1	130	55	39

Water Chemistry. 12/14/89 - Copper (Dechlorinated Tap Water).

Cu (ug/L) Measured	Cu (ug/L) Target	pH	D.O. (mg/L)	Cond. (umhos)	Hard. (mg/L)	Alkalin. (mg/L)
2	0	7.91	8.1	110	50	39
19	20	7.91	8.1	110		
30	30	7.91	8.1	110		
37	40	7.91	8.1	110		
52	60	7.91	8.1	110		
73	80	7.91	8.1	110		
101	100	7.91	8.1	110		
168	200	7.91	8.1	110	50	39

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Vita

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