Comparison of Acute Testing Methods of *Utterbackia imbecillis* (Say) with Variation on Age, Diet, and *In vitro* Transformation Method

Margaret L. Barfield, Sarah A. Clem, and Jerry L. Farris
Arkansas State University, Aquatic Ecotoxicology Facility, Jonesboro

Abstract. The development to date of *in vitro* culture techniques has expanded the use of freshwater mussels in toxicity testing. Large numbers of juvenile mussels can be made available for determining effect response levels for mussels to various toxicants. Although acute test methods for juvenile mussels have not been standardized, the two test methods employed in this study have been widely used. The first acute method is a 96-hour exposure of juveniles to a waterborne toxicant without a food source or sediment available. This method was used to determine the effects of organism age, diet deprivation in culture, and *in vitro* transformation method on acute toxicity response to Cu and KCl. The second acute test method exposes juveniles to toxicants with both water and sediment available for 9 days. This method was used to determine the effects of organism age and *in vitro* transformation method on acute toxicity response to Cu. Comparison of results reveals a decrease in sensitivity of juveniles with an increase in test organism age with both 96-hour (Cu and KCl) and 9-day acute test methods. Results of test on juveniles deprived a diet in culture prior to testing showed an increase in sensitivity of juveniles with age of the test organism. Comparisons of acute response with *in vitro* transformation method initially show lower LC_{50} values for rabbit serum transformed juveniles responding in 96-hour and 9-day exposures to Cu in comparison to juveniles transformed in fish plasma. Response comparisons of *in vitro* method to KCl showed little response variation between the two *in vitro* media.

Introduction

North American freshwater mussels have experienced great declines in number and range. Some factors affecting this decline include habitat loss and alteration, overharvesting, introduction of exotic species, and pollution from anthropogenic sources. The use of freshwater mussels in toxicity testing is to gain insight into the latter of these factors. Toxicity testing is relevant to the protection of remaining mussel populations as well as populations with regards to specific effects of source contamination and habitat degradation. Acute test methods were developed with juveniles for several reasons. Large numbers of juveniles can be harvested by culture techniques, thereby preventing the sacrifice of comparable numbers of adults to determine mortality estimates. In addition, adults are prone to include an avoidance response to toxicants which confounds resulting time and concentration estimates. Representing an early life stage, juveniles are likely more sensitive than adults to toxicants (Jacobsen 1993). While there is currently no standardized method for acute toxicity testing with mussel juveniles, two distinct test methods are being employed to date. The first method, similar to established U.S. Environmental Protection Agency (EPA) acute testing methods for freshwater and marine organisms (Weber 1993), exposes juveniles to a dissolved toxicant for a period of 24-96 hours (Keller and Zam 1991; Warren and Klaine 1994; Jacobsen 1989). The second test method, developed at the Tennessee Valley Authority's Toxicity Testing Laboratory and similar to EPA's proposed methods for sediment organisms, exposes juveniles to toxicants with both water and sediment available for 9 days (Wade et al. 1989). The accumulation and comparison of mortality estimates and effective concentrations are reflective of variation in test methods. This study utilizes both test procedures for comparisons of response within each method to factors of age, diet, and *in vitro* culture technique.

Materials and Methods

Culture and Maintenance

Juveniles utilized in the study were cultured *in vitro* from gravid adult *Utterbackia imbecillis* using a technique developed by Isom and Hudson (1982). Fish plasma and rabbit serum were employed in culture media (Table 1) for comparison of acute

response of juveniles from different media. Prior to testing, juveniles were maintained in flow-through or static 48-hour renewed cultures of dechlorinated tap water with high organic, nontoxic sediment and algal concentrate available. Algal concentrate was administered daily and cultures were maintained at 25°C. A variation to these culture parameters was utilized with those organisms isolated following transformation and deprived of the diet source of algal concentrate and organic sediment.

96-Hour Acute Exposures

Static, nonrenewed bioassays were conducted to determine acute toxicity of juvenile mussels to Cu and KCl. Copper in the form of $CuSO_4$ was dissolved in concentrations of 25-400 ppb at a 0.5 dilution series. Potassium chloride was dissolved in concentrations of 0.0336-2.0 ppt. The test method (outlined in Table 2) was derived from techniques given in Johnson (1990) and Warren and Klaine (1994) and detailed by Damien Simbeck (pers. comm.). Modifications of test methods included a test solution volume and chamber size of 25 ml and test temperature of ± 24°C. Several comparisons were made utilizing the 96–hour acute test method. These comparisons include test organism age, availability of diet in culture prior to testing, and *in vitro* transformation media.

Test Organism Age. All juvenile mussels exposed in this comparison were cultured from the

Table 1. Components of *in vitro* media combinations per dish prepared (final volume = 3 ml).

Component	Media Type	
	Fish Plasma (ml)	Rabbit Serum (ml)
Stock Medium	2.0	2.0
Sterile Fish Plasma	1.0	0.0
Rabbit Serum	0.0	0.66
TCH (Serum Replacement)	0.0	0.33
TCM (Serum Replacement)	0.0	0.33
Amphotercin B	0.075	0.075
Gentamicin	0.15	0.15
Rifampin	0.15	0.15
Carbenicillin	0.15	0.15

* reference (Hudson & Sherbourne, 1990)

Table 2. Test Methods

Conditions	96 Hour Acute	9 Day Acute
Test Organism	*U. imbecillis*	*U. imbecillis*
Age	1,4,7,13,15 days	6-10 days
Feeding	No food or sediment	0.75 ml Algal Concentrate 0.25 ml YCT 800 mg/l sediment 25 ml/2 l test solution
Water Temp.	24 + 1°C	24 + 1°C
Dilution Water	Mod. Hard	Mod. Hard
Toxicant	Cu, KCl	Cu
Concentration Range	Cu, 20-400 ppb	0.156-2.50 pp
KCl, 0.0336-2.00 ppt		
Replicates	4 per conc.	4 per conc.
Individuals/replicate	5	10
Renewal	none	daily
Test Container Volume	25 ml	400 ml
Test Solution Volume	15 ml	150 ml

same adult mussel. Juvenile aging begins with their placement in water after their transformation in media. Acute testing ages included 1, 4, and 15 days. Tests were conducted with juveniles cultured in both fish plasma and rabbit serum and exposed to both Cu and KCl. Juveniles were assayed for 96 hours with observations and water chemistry recorded every 24 hours.

Diet and Acute Response. Two test series were conducted with organisms deprived of diet in culture prior to testing. In the first series, organisms were deprived organic sediment and algal concentrate the first 4 days of culture. Juveniles transformed in rabbit serum and maintained in diet–deprived cultures for 4 days were then assayed with Cu at 1, 7, and 13 days. Likewise, juveniles transformed in fish plasma were assayed following maintenance for 1 and 7 days. In the second test series, only juveniles transformed in rabbit serum were deprived of diet in culture prior to testing. The diet-deprived organisms were assayed at 4 days old with both Cu and KCl. The test endpoints were then compared with those for 4 -day-old rabbit-serum-transformed organisms (cultured from the same adult) supplied a diet in culture prior to testing.

***In vitro* Transformation Media.** Test comparisons were made in conjunction with the organism age comparisons outlined above. The tests were conducted with juveniles cultured from both fish plasma and rabbit serum at ages 1, 4, and 15 days. Exposures included Cu and KCl, and the endpoints for all ages were compared for both toxicants between those test organisms transformed in both media types.

9-Day Sediment Acute Tests

Static, daily renewed sediment bioassays were conducted to determine the acute toxicity of juveniles to Cu. Nine-day sediment acute test methods are outlined in Table 2. Copper in the form of $CuSO_4$ was dissolved in moderately hard synthetic water. Organic sediment was added after Cu dilution. Minor variations within the aforementioned test method occurred between laboratories in which the assay was conducted. These variations will be discussed within each comparison as relevant. Comparisons within the 9-day sediment acute method include test organism age and *in vitro* transformation media.

Test Organism Age. The comparison of test organism age was conducted at Presbyterian College, Clinton, South Carolina, under the direction of Dr. Robert G. Hudson. Test method variations include sediment loading of 25 ml coarse-filtered sediment per 2 l of test solution. Concentrations of Cu as $CuSO_4$ were 0.5, 1.0, 2.0, and 4.0 ppm. Test diet consisted of concentrated algal suspension of unknown species (suspected cyanobacteria dominated). Juveniles from the same adult were transformed *in vitro* with rabbit serum. Ten-day-old juveniles were assayed with the 9-day acute sediment method. The juvenile culture was maintained as previously described until 40 days when the second test series began. The test ran for the standard 9 days at which point the LC_{50} was well below that for 10-day-old organisms. The test was then extended until a comparable endpoint was reached.

In vitro *Transformation Media.* Acute response comparisons of *in vitro* transformation media were conducted at Arkansas State University's Aquatic Ecotoxicology Facility (ASU). Two series of comparisons were conducted with individuals in each series being cultured from the same adult mussel using two transformation media types (Table 1). The 9-day sediment acute test method outlined in Table 2 included 800 mg/l (dry weight) of 100 micron filtered organic sediment per liter of test solution. Available diet consisted of 0.75 ml of algal concentrate and 0.25 ml of yeast, cereal leaves, and trout chow (YCT). Copper concentrations were 0.312-2.5 ppm in a 0.5 series dilution. Additional test results from TVA's Toxicity Testing Laboratory (TTL), also utilizing this test method, will be compiled in the results section.

Results

96-Hour Acute Test Method

Age Response. Estimated LC_{50} values for all age groups increased with organism age for any given exposure time with the exception of one LC_{50} response for 1- and 4-day-old rabbit-serum-transformed juveniles responding to Cu (Figure 2). The resolution of LC_{50} values from 24 to 96 hours for all ages of both fish-plasma- and rabbit-serum-transformed juveniles responding to Cu was greater than the resolution of LC_{50} response values to KCl (Figures 3 and 4). Differences in LC_{50} values from 24 to 96 hours in both fish-plasma- and rabbit-serum-transformed juveniles responding to KCl were indiscernible until age 15 days (Figures 3 and 4).

Diet Deprivation and Acute Response. Test series 1, representing copper LC_{50} responses of fish-plasma- and rabbit-serum-transformed juveniles deprived of sediment and algal concentrate for days 0-4 in culture and are represented in Figures 5 and 6. Rabbit-serum-transformed juveniles tested at ages 1 and 7 days exhibited similar LC_{50} responses from 24-, 48-, and 72-hour exposures. Mortality estimates for 13-day-old juveniles from the same culture were well below response values for both 1- and 7-day-

old juveniles (Figure 5). Fish-plasma-transformed juveniles from the same test series had greater mortality in 7-day-old juveniles than 1-day-old juveniles for all lengths of exposure (Figure 6). Test series 2 measured Cu and KCl exposure responses of 4-day-old rabbit-serum-transformed juveniles deprived sediment and algal concentrate, and these were compared with responses of organisms from the same transformation culture-supplied sediment and algal concentrate in culture (Figures 7 and 8). Toxicity values for the diet-deprived juveniles responding to Cu at 24-96 hours were lower than those for diet-supplied juveniles responding from 24-96 hours (Figure 7). Estimated LC_{50} responses to KCl for 4-day-old diet-deprived juveniles, however, were similar to responses for 4-day-old juveniles at 24, 48, and 96 hours and greater at 72 hours (Figure 8).

***In vitro* Transformation Media Response.** Estimated LC_{50} response ranges to Cu at 24, 48, 72, and 96 hours for all ages of rabbit-serum-transformed juveniles were 211->400 ppb, 107-341 ppb, 61-245 ppb, and 38-108 ppb, respectively (Figure 1). Somewhat lower LC_{50} response ranges to Cu at 24, 48, 72, and 96 hours for all ages of fish-plasma-transformed juveniles were 90.091-224.978 ppb, 59.527-130.1 ppb, 36.434-100.11 ppb, and 30.08-74.55 ppb, respectively (Figure 2). This trend, however, was not supported by LC_{50} response-range comparisons for juveniles from both transformation media to KCl (Figures 3 and 4). One additional comparison of transformation media response can be drawn from the LC_{50} values of juveniles in the first series of diet deprivation testing (Figures 5 and 6). Responses of 1-day-old juveniles transformed in both fish plasma and rabbit serum to Cu can be compared because they are not yet subject to the diet-deprivation variable. Estimated LC_{50} response ranges from 24 to 72 hours for 1-day-old juveniles from both transformation media are almost identical.

9-Day Sediment Acute Test Method

Age Response. The LC_{50} of 10-day-old juveniles transformed in rabbit serum media was 1,756 ppb

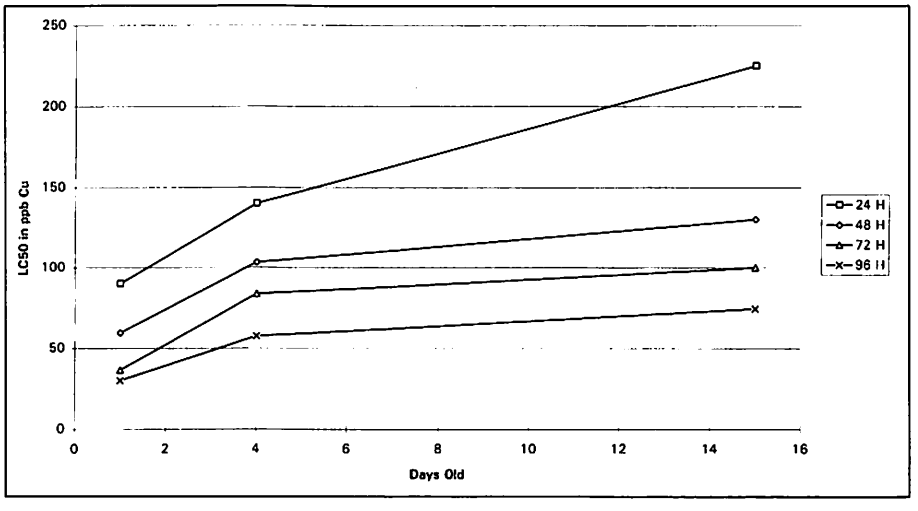

Figure 1. Age vs. acute response (96h, Cu), fish-plasma juveniles.

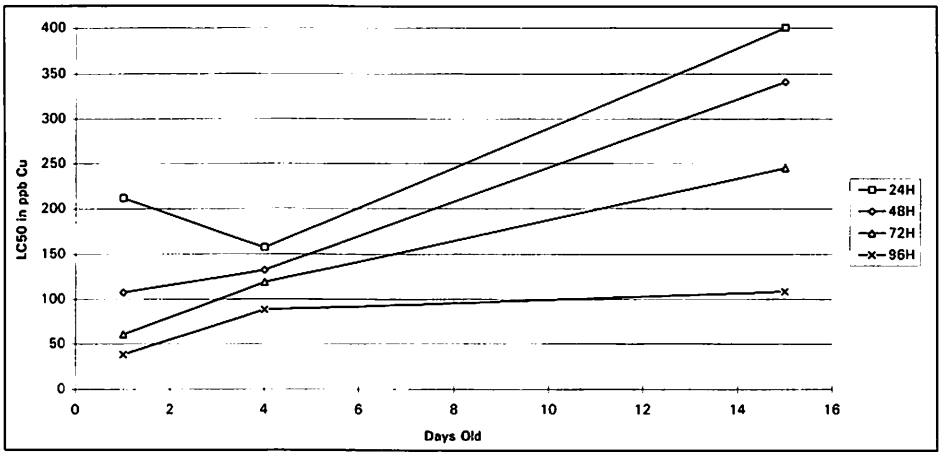

Figure 2. Age vs. acute response (96h, Cu), rabbit-serum juveniles.

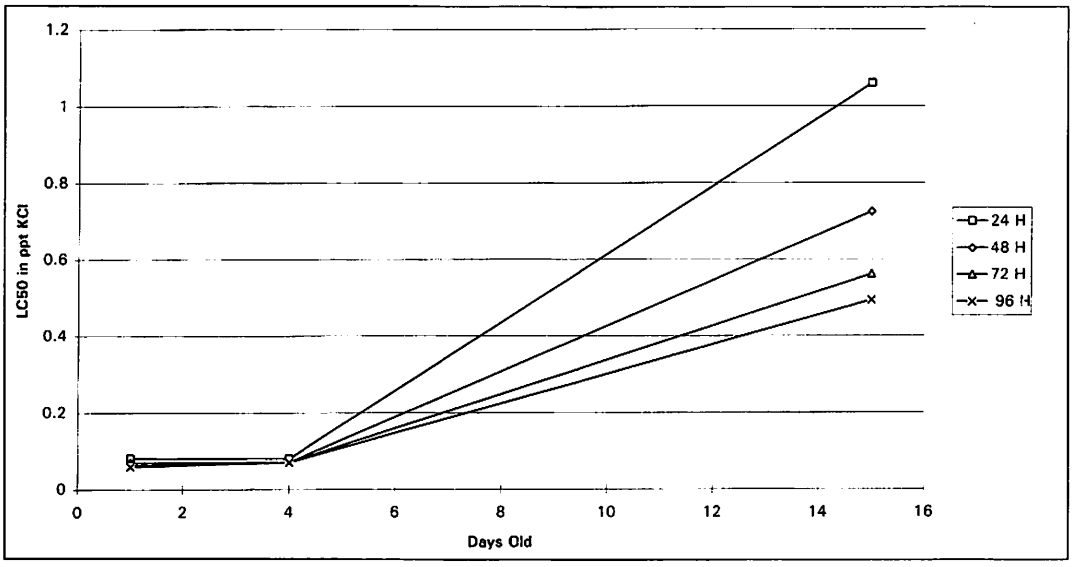

Figure 3. Age vs. acute response (96h, KCl), rabbit-serum juveniles.

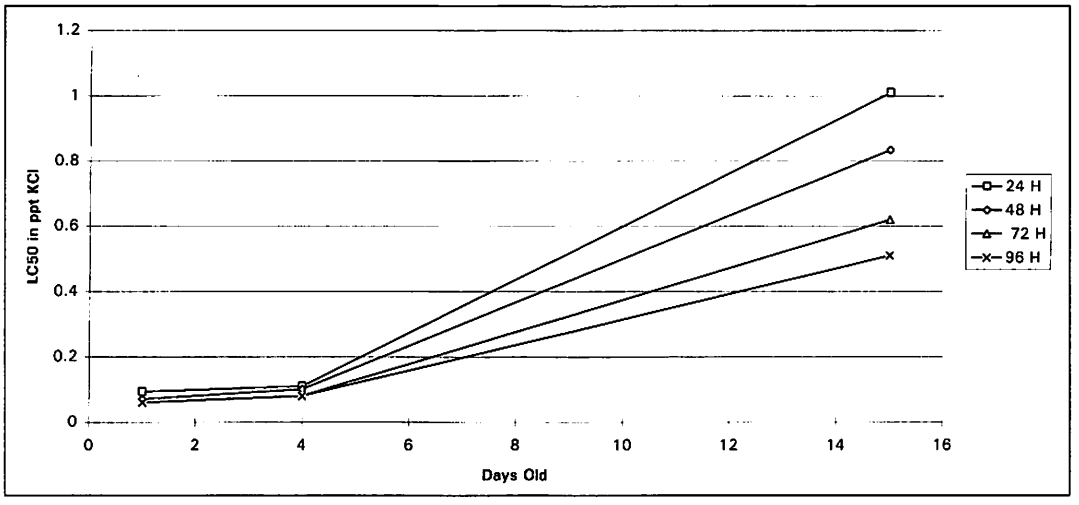

Figure 4. Age vs. acute response (96h, KCl), rabbit-serum juveniles.

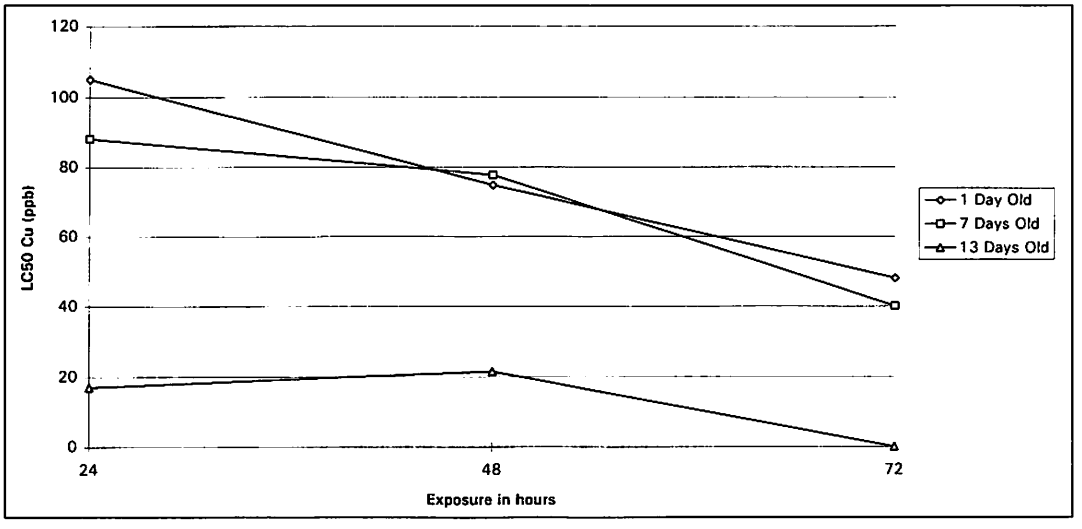

Figure 5. 72-hour response to Cu of 0-4-day diet-deprived *in vitro*-rabbit juveniles.

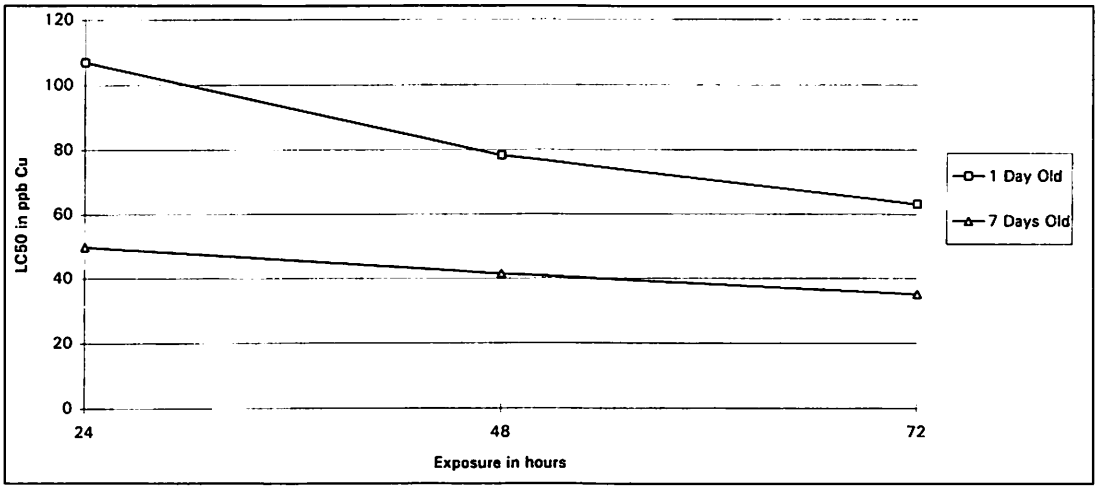

Figure 6. 72-hour response to Cu of 0-4-day diet-deprived *in vitro* fish plasma juveniles.

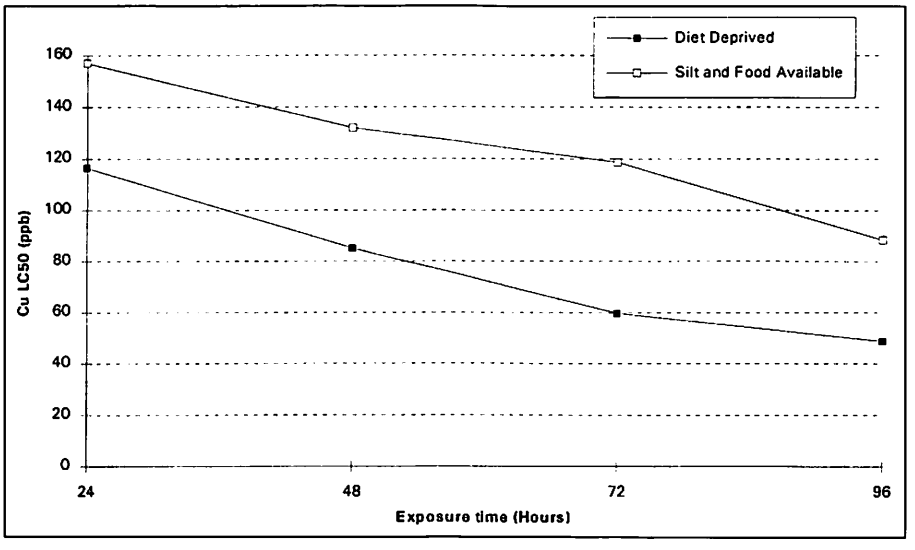

Figure 7. Cu 96-hour response of 4-day-old diet-deprived vs. diet-supplied rabbit-transformed juveniles.

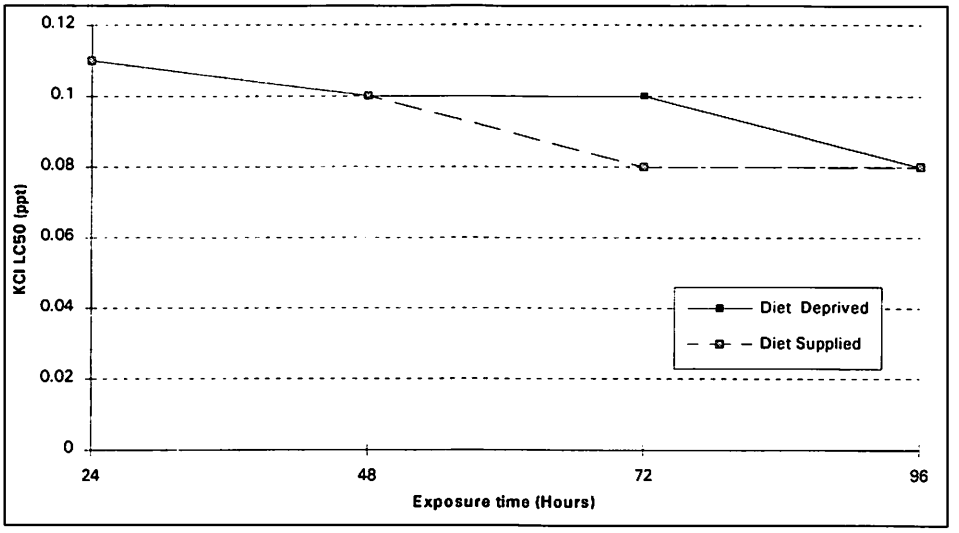

Figure 8. KCl 96-hour response comparison of 4-day-old diet-deprived and diet-supplied rabbit juveniles.

Cu. The identical test method was utilized to expose 40-day-old juveniles from the same culture; however, to elicit a comparable LC_{50} value (1833 ppb Cu), the exposure was extended to 22 days.

***In vitro* Transformation Media Response.** Two series of sediment acute test exposures to Cu utilizing individuals transformed in both fish plasma and rabbit serum media were conducted. LC_{50} values for test series 1 were 765 ppb and 990 ppb for fish plasma and rabbit serum, respectively. LC_{50} values for test series 2 were 577 ppb and 630 ppb for fish plasma and rabbit serum, respectively (Table 3). Additional responses obtained utilizing this test method in other labs were compiled in Table 3.

Discussion

Organism Age Response

Acute and chronic testing methods as outlined by the EPA (1989 and 1993) include specific consideration of test organisms ages representative of early life stages. It is therefore assumed that greater sensitivity occurs during critical life stages and differences in age of test organisms can greatly affect the observed variance in test responses. Age comparisons with copper in these tests revealed that LC_{50} estimates can support this assumption since toxicity was reduced with increasing beginning test age. This trend was consistent for all observed exposure lengths from 24 to 96 hours. The observed toxicity with increasing concentrations of KCl also suggests a beginning test age-dose dependent relationship is apparent only with juveniles tested at the 15-day-old age. Reasons for the lack of resolution in beginning age comparisons among 1- to 4-day-old individuals to acute KCl toxicity is unknown; however, implications can be drawn for the mechanisms of toxicity between metal and salt toxicants. Further test comparisons will certainly have implications for selection of suitable laboratory reference toxicant selection and even the possibility of employing multiple reference toxicants in working with juvenile mussels.

Comparison of the 9-day acute test method to the shorter-term acute test methods also supported consideration of decreasing sensitivity with increasing test organism age. In order to induce an LC_{50} endpoint similar to that of the 10-day-old juvenile, the exposure of 40-day-old juveniles required 22 days. Although results compiled by Warren and Klaine (1994) indicate increasing sensitivity with increasing age, the authors suggested that insufficient diet may have played a role in their results. This conclusion was supported by comparisons of diet deprivation and acute responses in this study.

Diet Deprivation Response

Both test series initiated with diet-deprived organisms yielded similar results. Series 1, comparing test organisms from both transformation media responding to Cu, indicated lower LC_{50} values for older organisms. Even though the diet was reintroduced to the cultures at 5 days, responses continued to decline at 7 and 13 days. In the second series of tests, organisms transformed in rabbit serum were exposed to both Cu and KCl. Comparisons in this series were made between 4-day-old organisms withheld and supplied a diet in culture prior to testing (Figures 7 and 8). Again, the depression of LC_{50} values are correlated to withholding diet in culture. In our comparisons, only with deprivation

Table 3. 9 Day Sediment Acute Comparisons.

	LC_{50} (ppm Cu)
Age	
10-day-old *in vitro* rabbit	1.756
40-day-old *in vitro* rabbit (22 day exposure)	1.833
Culture Media Comparisons	
Fish Plasma	
Ecotoxicology Facility, Arkansas State	0.765
	0.577
TVA Toxicity Testing Lab	1.12
	0.97
	0.83
	0.67
Rabbit Serum	
Ecotoxicology Facility, Arkansas State	0.990
	0.63
Undergraduate Research, Presbyterian College	1.756

of diet were LC_{50} responses observed to decrease with age.

In vitro *Transformation Media Response*

The effects of *in vitro* transformation media type on acute response is not as clear as age or availability of diet described earlier. Initial observation of the range of test concentrations for Cu in the age comparison indicates higher sensitivity in fish media-transformed juveniles. On the other hand, comparison of the concentration range for KCl in the age comparison revealed little difference in response between fish-plasma- and rabbit-serum-transformed juveniles. Additionally, comparison of 1-day-old organisms in the first diet deprivation test series (prior to diet deprivation effects) showed almost identical LC_{50} values for both fish-plasma- and rabbit-serum-transformed juveniles responding to Cu (Figures 5 and 6). Comparison of results from 9-day sediment acute testing is first made with endpoints obtained at Arkansas State University's Ecotoxicology Facility.

Comparisons at ASU were made between juveniles transformed from the same adults on two separate occasions. Additional testing and endpoint collection should reveal the presence or absence of statistical significance between responses of juveniles from the two transformation media types. However, for the present, additional endpoints were compiled from other laboratories utilizing the 9-day sediment acute test method (Table 3). It will be of value to note minor variations in the test methods and their possible effects on endpoints. The endpoint of 1,756 ppb Cu for rabbit-serum-transformed juveniles obtained from Presbyterian College, South Carolina, is markedly higher than other endpoints. A possible reason for this difference is an increased organic sediment load of ~25 ml/2 L test solution in test solutions as compared with 800 mg/L sediment dry weight (approximately 1-2 ml sediment/L) used in other laboratories. Endpoints obtained from TVA's Toxicity Testing Lab are listed chronologically from oldest test date to most recent as they descend in Table 3. The following test method variations may have contributed to the observed increase in sensitivity over time: conversion from a Cu source as $CuSO_4$ crystals to acidified Cu reference toxicant available from EPA, and the addition of a test diet source of yeast, cereal leaves, and trout chow (Damien Simbeck, pers. comm.). Although the compilation of test endpoints does not elucidate a difference in acute response with *in vitro* transformation media, it is promising to see the potential for interlaboratory test endpoint comparisons. This will speed the collection of data for acute response of juvenile mussels to various toxicants.

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