EXPOSURE ROUTES OF COPPER AND THEIR EFFECTS ON THE GREAT POND SNAIL (*Lymnaea stagnalis*)

by

Stephanie Nicole Tubbs Aselage

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Thesis Committee:
Professor Allen Burton, Chair
Professor Mike Wiley

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CHAPTER 1 – Literature Review

INTRODUCTION

Elevated levels of copper (Cu) are becoming more dominant in aquatic ecosystems, but their consequences are not fully understood. It is an essential element for all of the organisms in the aquatic environment to survive. Natural sources of Cu include the earth's crust and weathering of rocks (Flemming &Trevors 1989) yet there are many anthropogenic sources of Cu, including industrial processes, electrical wiring (Girard 2010, pp. 15), smelters, power stations, fertilizers, fungicides (WHO 1998), combustion sources (WHO 1998; Rice et. al. 2002), motor vehicle break and tire wear (Rice et. al. 2002). These and other sources of Cu allow excess amounts of Cu to enter the environment, which has been shown to have negative effects on many organisms.

Natural and anthropogenic sources of Cu can enter aquatic ecosystems through both direct and indirect paths. Cu is intentionally applied to surface waters in the form of copper sulfate to kill algae (WHO 1998; Huggett 1999) and surface runoff from roads and agriculture fields can contain elevated levels of Cu from combustion sources, brakes, or fertilizers (Flemming & Trevors 1989; WHO 1998; Rice et. al. 2002). When Cu enters an aquatic environment, it can ultimately partition to either water, sediment or periphyton. All three mediums can become exposure routes of Cu to organisms in aquatic ecosystems. Importantly, it is not known to what degree Cu is bioavailable to organisms through all of these routes and at which Cu concentrations would toxicity be observed for each exposure route. A better understanding of the chemistry of Cu within these exposure compartments and their effects to various organisms is needed to know if there is any risk for the organism and if it will expand to higher trophic levels.

Within aquatic ecosystems, snails are grazers on periphyton communities (Brönmark 1989) and major food sources for many birds, fish, reptiles and mammals (Hoang et. al. 2008). Therefore, either loss of snail biomass or contamination of snail tissues could potentially cause adverse effects on the functioning and structure of ecosystems. Anthropogenic disturbance in aquatic ecosystems, such as channelization and impoundment of rivers, have already caused almost 50 percent of freshwater snails in the Southeastern United States to be endangered or extinct (US EPA 2010) and a better understanding of Cu and its potential toxicity to snails is important for protecting the diminishing snail diversity.

CHEMISTRY OF COPPER

Cu is classified as a heavy metal and has many physical properties that makes it useful for various industrial applications. Its high electrical and thermal conductivity as well as its resistance to corrosion makes it an important element in the use of combustion sources (i.e. municipal incinerators and combustion of coal, gasoline, diesel and lubricating oils), tires and brakes of vehicles (WHO 1998; Rice et. al. 2002). However, once it enters aquatic environments, it is only slightly soluble in freshwater, saline waters or mildly acidic solutions, but carbonate, which can be found in copious amounts in freshwater, can more readily dissolve Cu (WHO 1998).

In aqueous solutions, Cu (II) is the most common oxidation state (Nriagu 1979; Flemming & Trevors 1989). This form of Cu can be dissolved in water, precipitate out of water and settle in sediments or accumulate in periphyton where it can become a dietary exposure route for organisms (Nriagu 1979; Flemming & Trevors 1989). As point and non-point sources with elevated levels of Cu enters the surrounding aquatic environments, the amount of Cu, the duration of exposure, and the frequency it enters the system can influence the toxicity of Cu to organisms. Knowing the physical and chemical properties of Cu is critical in order to understand how different speciations of Cu interact with the environment and its effects within various medias, such as water, sediment and periphyton.

The amount of Cu actually interacting with an organism (bioavailable fraction) is less than the total amount of Cu due to chemical complexation. The bioavailability of Cu in water is dependent upon multiple water quality parameters, such as pH, hardness, alkalinity, and temperature (Nriagu 1979; Flemming & Trevors 1989; Rogevich et. al. 2008). Typical background levels of Cu in water are 1 to 30 µg Cu/L (Flemming & Trevors 1989; WHO 1998). However, if the water quality parameters are optimal for Cu to precipitate out, Cu may no longer be available for organisms to take up and cause toxicity through water exposure (Stiff 1971; Flemming &Trevors 1989). The biotic ligand model (BLM) is a good model that estimates dissolved metal toxicity, including Cu, based on natural occurring ions in the environment (Cruz & Delos 2010). The BLM was first derived to look at the effects of metal toxicity to fish gills, but has recently been extended to other aquatic organisms, such as algae and crustaceans (Cruz & Delos 2010, Vijver et. al. 2004). Using pH, temperature, dissolved organic carbon, major cations, major anions, alkalinity and sulfide along with the knowledge of aqueous chemistry of Cu, a more accurate estimate of Cu toxicity from water exposure to organisms can be made.

Sediment in aquatic environments can act as a sink for Cu collecting precipitated or depositional Cu, often at high concentrations (Flemming & Trevors 1989; WHO 1998). Although background levels of Cu in freshwater sediment vary from 16 to 5000 mg Cu/kg of dry weight, the median concentration in uncontaminated sediment is reported at 30 mg/kg (range 2-250 mg Cu/kg) (Flemming & Trevors 1989; WHO 1998). Cu can be deposited in sediments from absorption by organic matter, hydrous iron, manganese oxides and clay that are in the sediment and water column, and by settling or precipitating out of the water column (Flemming & Trevors 1989; WHO 1998). Within sediment complexation by organic matter, adsorption to metal oxide surfaces and strong sulfide binding in anoxic sediments can decrease the amount of bioavailable Cu (WHO 1998). Also, free copper in oxic sediments with low pH values were tenfold higher than anoxic sediments with identical pH values (Calmano et. al. 1993). Since there is such a wide range of Cu bioavailability found in sediments, the chemistry of Cu as well as other bind agents needs to be understood to determine Cu exposure and accumulation to aquatic organisms.

Periphyton (i.e., the attached algal and bacterial community) is a major food source for many species in aquatic ecosystems. Periphyton takes up Cu through adsorption and absorption, and has been found to take in Cu more efficiently than other metals, such as zinc, manganese and cadmium. The ability of periphyton to efficiently take up Cu can create high internal

concentrations, which can surpass the concentrations of Cu in its surrounding surface water or sediment (Knauer et. al., 1997; Serra et. al., 2009). These high internal concentrations can be toxic to algae causing biomass declines and community assemblage shifts (Serra et. al., 2009). Acute exposure to elevated Cu can cause inhibition of photosynthesis and metabolic processes associated with growth, while chronic exposure causes algal community shifts and adaptations over time (Serra et. al., 2009). Cu bioavailability in periphyton is not thoroughly understood due to periphyton complexity, but it has been determined that the amount of time after exposure to elevated levels of Cu, the amount of cell densities and Cu speciation all have effects on Cu bioavailability (Franklin et. al. 2002; Meylan et. al. 2004; Serra et. al. 2009). Franklin et. al. (2002) has found that higher cell densities and the release of algal exudates will reduce Cu bioavailability. Understanding the toxicity of Cu to periphyton is a challenge in itself, and trying to understand the potential dietary exposure route to higher trophic levels is another challenge.

EFFECTS ON SNAILS

Freshwater snails are an important species in aquatic ecosystems because they have rapid grazing rates and are a food source for various species (Brönmark 1989; Hoang et. al. 2008), which make them important components of the ecosystems in which they live. Snails belong to the class gastropods, which are the largest class within the Phylum Mollusca (Barnes 1987). Snails can be found in a variety of habitats such as lakes, streams and ponds, and when found in shallow benthic communities in abundant numbers they can have big impacts on the local environment due to their grazing abilities (O'Gorman et. al. 2010). Studies have shown a strong correlation between snails and periphyton grazing with positive effects on the macrophyte growth and survival (Brönmark 1985; O'Gorman et. al. 2010). The general importance of freshwater snails within the food web is understood, but the need for more research that focus on the values and services of freshwater snails within an aquatic ecosystem and their ability to accumulate metals is needed to know if there is a risk to higher trophic levels (Covich 2010; O'Gorman et. al. 2010).

Lymnaea stagnalis, also known as the "great pond snail," is a freshwater pulmonate snail species in which the mantle cavity acts like a lung and allows gas exchange to occur by diffusion (Barnes 1987). Major predators to *L. stagnalis* are fish and crayfish while *L. stagnalis* are very efficient grazers of periphyton and macrophytes (Gomot 1998). *L. stagnalis* has a distribution that is Holarctic, they have an important position in the food web, and they are easily cultured in the laboratory, which makes them a good species to use in toxicity tests (Ducrot et. al. 2006). Although this species feeds at the sediment-water interface, it must surface for air and gas exchange at least every 60 minutes (Barnes 1987). Because of their vertical mobility across the entire aquatic ecosystem, they may be exposed to all compartments that may contain Cu.

General effects of Cu toxicity observed in aquatic taxa include overwhelmed homeostatic control mechanisms, and adverse reproductive, biochemical, physiological and behavioral effects (WHO 1998). Mussels, which share the same phylum as snails, are more sensitive to Cu than most other aquatic taxa (US EPA 2008). Since snails share some of the same physiological and biological characteristics as mussels, the implications for Cu toxicity suggests potential increased stress on endangered snail species. The type of metal, the unionoid species and size are factors that

determine the rate and location of metal accumulation in mussels (US EPA 2008). Although it is know that freshwater mussels bioaccumulate metals, it is important to understand the chemistry of Cu and the biological and physiological characteristics of snails to determine if Cu bioaccumulation occurs through any of the three exposure routes. There are studies that focus on the water and dietary routes of exposure on *L. stagnalis*, but there are not many that look at the effects from Cu in sediments on *L. stagnalis* (Croteau and Luoma 2008; Besser et. al. 2009; Croteau and Luoma 2009; Brix et. al. 2011).

For freshwater snails exposed to Cu dissolved in the water column, only the BLM can be used to determine if there is sufficient Cu available for uptake by snails and cause toxicity (Cruz & Delos 2010). Studies have shown that water columns with high DOC, pH and hardness levels will decrease Cu toxicity to snails (Cruz & Delos 2010, Flemming & Trevors 1989, Nriagu 1979; Hoang et. al. 2008; Rogevich et. al. 2008). Besser et. al. (2009) found the LC50 for *L. stagnalis* less than seven days old over a 28-day exposure period was 21.7 and 36.2 µg/L (95% CI 20-30 and 30-44, respectively). Brix et. al. (2011) found for a 96 hour exposure period that the LC50 for *L. stagnalis* less than seven days old was 30.7 µg/L (28.9-32.7 95% CI). Continuing to research the effects of Cu on snails and understanding the importance of water quality parameters on Cu toxicity in water can help protect endangered freshwater snail species.

In sediment, complexation, adsorption or precipitation of Cu can occur (Flemming & Trevors 1989). When complexation with Cu (II) occurs in anoxic sediments, it can bind to acid volatile sulfides (AVS), iron oxides, manganese oxides or organic ligands, and can reduce the amount of Cu available for snail to uptake (Costello et. al. 2011). However, if anoxic sediments contain very low or none of those compounds, Cu can become available for uptake through pore water. Huggett et. al. (1999) showed that sediments with Cu concentrations as high as 2,010 mg Cu/kg, Cu was not bioavailable to aquatic organisms due to high organic carbon content and oxyhydroxides. Heng et. al. (2004) showed a strong positive correlation with concentrations observed in snails and sediments. These studies demonstrate the importance of sediment chemistry by showing that higher concentrations of Cu (<2,000 mg Cu/kg) in sediment were not bioavailable to organisms while the sediment with concentrations well below 100 mg Cu/kg showed similar amounts of Cu accumulated in snails.

For Cu in snail food sources, like periphyton, it is also important to understand how much Cu is bioavailable to snails through the dietary route. There are multiple studies that show Cu toxicity effects on periphyton (e.g., Knauer et. al. 1997; Franklin et. al. 2002; Meylan et. al. 2004; Serra et. al. 2009), but there is little published research on Cu bioavailability from contaminated periphyton to other species. Hoang et. al. (2008) believed that the dietary exposure route had a greater potential for toxicity on Florida apple snails than the soil exposure route due to the high bioaccumulation factors (BAFs) calculated in the foot and viscera. This also shows the potential trophic transfer of Cu to higher trophic levels through the consumption of contaminated snails (Hoang et. al. 2008). However, if Cu levels in food are too concentrated, Croteau and Luoma (2009) have shown that snail will not feed as much, and therefore, decrease the amount of Cu the

snail uptakes. It needs to be determined how much Cu is taken up, where in the snail it is taken up and how much Cu is excreted to determine bioaccumulation and toxicity effects on snails.

When looking at the overall effects of toxicity on snails, it is important to look at the effects of bioaccumulation and the effects that lead to snail mortality. There are fewer consequences on higher trophic levels if Cu toxicity results in snail death, because there is less likely of a chance that the elevated Cu levels in the snail will be transferred to higher trophic levels. However, once bioaccumulation occurs in snails with no mortality, the snail has the potential to become tolerant to elevated Cu levels where soft tissue and the shell continue to accumulate more Cu. Also, if Cu levels become too high, feeding habits will change and this could have an effect on the first level of the food chain due to decreased grazing rates on periphyton and macrophyte communities. Studies have shown that that higher concentrations of Cu in water and food (lettuce and diatoms) will decrease the amount of food snails will consume (Peña and Pocsidio 2007; Croteau and Luoma 2009).

CONCLUSION

The potential for snail mortality, reduced feeding, and bioaccumulation from Cu exposure needs to be studied further to determine if there is any potential for snail populations to continue to diminish in the ecosystems. A better understanding of the chemistry in Cu, and where and how it accumulates in snails needs further investigation to determine if there are any potential threats to higher trophic levels. There are multiple studies that agree that water, sediment and dietary routes are the three major routes of exposure to snails, but there is no agreement between which route of exposure can cause greater toxicity (Heng et. al. 2004; Vijver et. al. 2004; Hoang et. al. 2008; Hoang and Rand 2009). More recently, the dietary exposure route appears to be the more potentially dangerous route due to high bioaccumulation rates and calculated BAFs of Cu (Heng et. al. 2004; Hoang and Rand 2009). *There is a deficiency in the knowledge of the mechanisms of Cu that causes toxicity in snails and Cu speciation, and Cu behaves differently from other metals which makes it difficult to find data that supports the dietary route of exposure being most dangerous for snails.* Also, determining which water quality parameters (hardness, DOC or pH) are more influential on Cu bioavailability and toxicity in snails needs further research (Rogevich et. al. 2008).

As more knowledge is gained in this field, improvements to our models and water quality standards for Cu toxicity to organisms, specifically snails, can be made. In 2007, freshwater quality criteria for Cu was updated to incorporate additional water quality parameters within the BLM to make a better prediction of Cu toxicity on fish gills, crustaceans, algae and potential future species (Vijver et. al. 2004; Cruz & Delos 2010). These updates and improvements can stimulate more research about freshwater snails and help us recognize the importance of snails and Cu in aquatic ecosystems.

CHAPTER 2 - Manuscript

ABSTRACT

Populations and diversity of freshwater snails are declining in the United States. The current study looks at the different exposure routes (water, sediment and dietary) of copper (Cu) for the great pond snail (*Lymneae stagnalis*) to determine which route of exposure has the greatest potential for inducing toxic effects and bioaccumulation in snails. *L. stagnalis* were exposed to environmentally relevant concentrations of Cu through each of the three exposure routes for 28 days and survival, growth (length and wet weight), feeding rates (weekly) and whole body Cu concentrations were measured to estimate potential toxic effects. Overlying water Cu was significantly correlated with snail survival and whole body Cu concentrations. The sediment exposure route had the least toxic effects and lowest snail mortality. The dietary exposure route showed the highest Cu concentrations in snails yet no measurable toxic effects. Regardless of exposure route, feeding rates were not affected by Cu. These findings suggest that elevated levels of Cu in overlying water and food sources may have negative effects on snail population size or lead to elevated Cu body burden.

INTRODUCTION

Elevated levels of copper (Cu) are becoming an ecological issue on aquatic environments due to its widespread use, but their consequence are not fully understood. Although Cu is an essential element for many organisms, excess Cu can stress organisms and even cause mortality. Excess amounts of Cu can enter the environment from weathering of rocks (Flemming &Trevors 1989), industrial processes, electrical wiring (Girard 2010), smelters, power stations, fertilizers, fungicides (WHO 1998), combustion sources (WHO 1998; Rice et. al. 2002), and motor vehicle break and tire wear (Rice et. al. 2002). Cu can enter aquatic ecosystems directly through intentional application of copper sulfate to water to control algal blooms (WHO 1998; Huggett 1999) or indirectly through poorly treated wastewater or surface runoff from roads and agriculture fields (Flemming & Trevors 1989; WHO 1998; Rice et. al. 2002). Once in aquatic ecosystems, Cu may remain dissolved in the water column, or precipitate out and accumulate in sediment or periphyton (Nriagu 1979; Flemming & Trevors 1989). Additionally, chemical complexation of Cu in all of the compartments within aquatic ecosystems can affect the amount of Cu that is bioavailable to organisms (Stiff 1971; Nriagu 1979; Flemming &Trevors 1989; WHO 1998).

As elevated levels of Cu continue to enter the environment, it can become a threat to benthic organisms in the aquatic ecosystem, including snails. Anthropogenic disturbance in aquatic ecosystems, such as channelization and impoundment of rivers, has caused almost 50 percent of freshwater snails in the Southeastern United States to be endangered or extinct (US EPA 2010). Within aquatic ecosystems, snails are grazers on periphyton communities and a major food source for a variety of predators making them an important link in food webs (Brönmark 1989; Hoang et. al. 2008). It is important to identify if they are accumulating a surplus of Cu and if there is a potential for it to be bioavailable for higher trophic species. Because snails live on the benthos, they are potentially exposed to Cu through three major routes of exposure; water, sediment and

diet. Although past research has focused on each exposure route independently (Huggett et. al. 1999; Real et. al. 2003; Heng et. al. 2004; Croteau and Luoma 2008; Hoang et. al. 2008; Peña and Pocsidio 2008; Besser et. al. 2009; Croteau and Luoma 2009; Hoang and Rand 2009), limited studies have attempted to compare the different routes of exposure to determine which is potentially most important for snail toxicity and bioaccumulation (Hoang et. al. 2008). Notten et. al. (2005) has also shown that on the terrestrial landscape, the transfer of metals from leaves to snails is more important than the transfer of metals from soil, and therefore, it needs to be better understood which route of exposure is important in the aquatic landscape.

With freshwater snail populations declining in the United States (US EPA 2010), it is important to determine whether sediment and water quality guidelines are protective of all potential Cu exposure routes. This study compared the three major exposure routes of Cu for *Lymnaea stagnalis*, the great pond snail, which is a commonly used snail model organism. I hypothesize that snails exposed to Cu through dietary routes will be most susceptible while the sediment exposure route will display the least effects to *L. stagnlais*. The endpoints measured in this study are survival, growth (length and weight) and feeding rates of the great pond snail.

METHODS AND MATERIALS

Test Organism

Lymnaea stagnalis were obtained from existing laboratory cultures. Snails in culture were fed spinach on a daily basis and kept on a 16:8 hour light:dark cycle. Egg cases were isolated on a weekly basis and snails used in the experiments were 14 to 21 days post-hatch. Preliminary tests and previous literature (Rogevich et. al. 2008) indicated that snails at this life stage were highly sensitive to contaminants with good survival of organisms held under control conditions.

Experimental Design

Three separate experiments were conducted in which Cu was added to one of three exposure routes: water, sediment or periphyton (Table 1). Each experiment involved 28-day static exposures of 10 *L. stagnalis* in 300-mL beakers at ambient temperature and light. Water and dietary exposure route experiments used 200 ml of culture water (i.e., Ann Arbor city water passed through a carbon filter) and the sediment exposure route experiment used 100 ml of sediment and 100 ml of culture water. Hardness and alkalinity (Clesceri et. al. 1996) were measured at the beginning and end of each 28 day experiment. *L. stagnalis* were fed spinach *ad libitum* in between feeding rate tests. Water exchanges occurred three times weekly either by overflow (Zumwalt et al. 1994) for the contaminated sediment and food exposure or by manual replacement of 90% of the water for the contaminated water exposure. Water quality measurements (temperature, pH, and dissolved oxygen) were also taken at each water exchange.

For the water exposure, $CuCl_2$ was dissolved in deionized water (18MΩ cm^{-1}, Millipore) and added directly to culture water to create four Cu concentrations and a control (Table 1). For the sediment exposure, sediment from the Saline Fisheries Research Station (Saline, MI) was removed and amended with $CuCl_2$ to create three Cu concentrations and a control (Table 1). For dietary

exposure, periphyton grown on nylon mesh (400 µm) for a minimum of three weeks at the Saline Fisheries Research Station was soaked in water at the same Cu concentrations used in the water exposure for 24 hours (Table 1) and then placed in 200 ml of non-spiked culture water. Each experimental treatment was replicated three times.

Table 1. Nominal Cu concentrations for the three exposure scenarios

Treatment	Water	Dietary[a]	Sediment
1	0 µg Cu/L	0 µg Cu/L	0 mg Cu/kg
2	5 µg Cu/L	5 µg Cu/L	100 mg Cu/kg
3	10 µg Cu/L	10 µg Cu/L	200 mg Cu/kg
4	20 µg Cu/L	20 µg Cu/L	400 mg Cu/kg
5	30 µg Cu/L	30 µg Cu/L	

[a]*Concentrations are for the soaking water and not the actual concentrations within the periphyton disks*

Toxicity Tests

During the 28-day exposure period feeding rates were measured and at the end of the exposure period snails were enumerated, rinsed with deionized water, and stored in 95% ethanol. Growth was measured as shell width between the aperture and the apex and wet weight (±1 mg) after removing excess water with a Kimwipe. Feeding rates were measured weekly by allowing the snails to feed on disks of periphyton for 4-24 hours. An equal number of controls (periphyton and exposure chambers with no snails) were used to estimate variability in initial periphyton biomass (Peterson and Renaud 1989). For the water exposure, circles (diameter = 3.8 cm) were cut from periphyton covered mesh whereas for sediment and dietary exposure periphyton was homogenized with either a stir bar or blender and collected on to 25 mm glass fiber discs (Pall Cooperation, Ann Arbor, MI).

Analytical Procedure

For all exposure scenarios, overlying water from each beaker was collected three times weekly and stored with 2% nitric acid for Cu and calcium (Ca) analysis. Sediment samples were collected before and after the 28-day exposure, stored in a freezer and analyzed for total Cu, acid volatile sulfide (AVS),simultaneously extracted metals (SEM) (US EPA 1991), and total organic carbon (TOC). To estimate feeding rates, ash free dry mass (AFDM) and chlorophyll *a* were measured by combustion at 450°C and ethanol extraction, respectively (Biggs & Kilroy 2000). Periphyton and sediment samples were acid digested with a combination of nitric and hydrochloric acids in a MARS 5 Microwave Accelerated Reaction System (CEM Cooperation, Matthews, NC). Snails were acid digested with a combination of nitric acid and hydrogen peroxide for 7 days in ambient air temperature (Croteau and Luoma 2007). All total Cu and Ca concentrations in water, sediment, periphyton and snails were measured with inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 4300 DV, PerkinElmer Instruments, Norwalk, CT).

Statistical Analysis

Results from the snail survival, growth and Cu concentrations were analyzed using one-way ANOVAs with Cu concentrations as factors. The LC_{50} (lethal concentration for 50% of the organisms) from significant one-way ANOVA results were calculated using the EPA Toxicity Relationship Analysis Program (TRAP) using a three parameter threshold sigmoid nonlinear regression. Results from the feeding rate tests were analyzed using two-way ANOVA with snails/no snail and Cu concentration as factors. For all statistical tests, a *p* value <0.05 was considered significant and statistical differences between treatments from significant ANOVA results were determined using Tukey post hoc test (Appendix A). Assumptions were tested using Shapiro-Wilk and Kolmogorov-Smirnov tests and data not meeting normality assumptions were arcsine square root or natural log transformed as appropriate (Appendix A). All statistical analyses were completed using PASW Statistics 18 (WinWrap Basic, Nikiski, AK).

RESULTS

Measures of water quality did not differ greatly between treatments or experiments with the exception of elevated hardness and alkalinity in the sediment exposure (Appendix A). Total Cu concentrations in water and sediment were similar to nominal concentrations for each of the experiments (Appendix A). For the dietary and sediment experiments, surface water Cu concentrations did not exceed 30 µg Cu/L with the majority of the measurements reported lower than 15 µg Cu/L (Appendix A). Dissolved Ca in the overlying water was highest in the sediment exposure route and the lowest in the dietary route (Appendix A). The growth rates (length and weight) were not significantly different throughout any of the exposure scenarios (Appendix A).

Water Experiment

For exposure to Cu in water, snail survival was significantly different between Cu treatments ($p = 0.013$), with significant reductions at the 30 µg Cu/L treatment relative to treatments at 0 and 5 µg Cu/L. The LC_{50} for survival was 25.1 µg Cu/L (95% confidence interval = 19.5-27.7). Whole body Cu concentrations in the snails ranged from 66.37 to 1887.85 µg Cu/g DW (Figure 2), with snails in the 30 µg Cu/L treatment having significantly greater Cu body burden ($p < 0.001$) as well as all other treatments relative to the control. Snail length ranged from 10.63 to 24.82 mm/day and snail weight varied from 2.87 to 10.05 mg/day across all five treatments with no significant results (Fig. 3a and 4a). For feeding rates, neither chlorophyll *a* nor AFDM indicated any significant differences among Cu concentrations at any of the time periods ($p > 0.05$, Table 3).

Sediment Experiment

For sediment Cu exposure, snail survival was similar across all Cu concentrations ($p = 0.922$). A LC_{50} could not be calculated, but unbounded no observable effect level (NOEC) of 400 mg Cu/kg was calculated. Out of the three exposure scenarios, whole body Cu concentrations of snails were the lowest in the sediment exposure (12.90 to 79.22 µg Cu/g DW) and were significantly different between treatments ($p = 0.001$, Fig. 2), with greater Cu body burden between treatments 400 and 200 mg Cu/kg relative to the controls. Treatments 400 and 100 mg Cu/kg were also

statistically different from each other. The endpoints of this experiment indicated that snail length ranged from 18.63 to 26.49 mm/day and weight ranged from 11.18 to 20.06 mg/day with no significant relationship to treatment exposure (Fig. 3b and 4b). Feeding rates were not significantly different on most days with the exception of day 14 (chlorophyll *a*, p = 0.010) and 28 (AFDM, p = 0.022), but these significant differences were not consistent along the metal concentration gradient or between any paired treatments most likely due to large variance differences (Table 3).

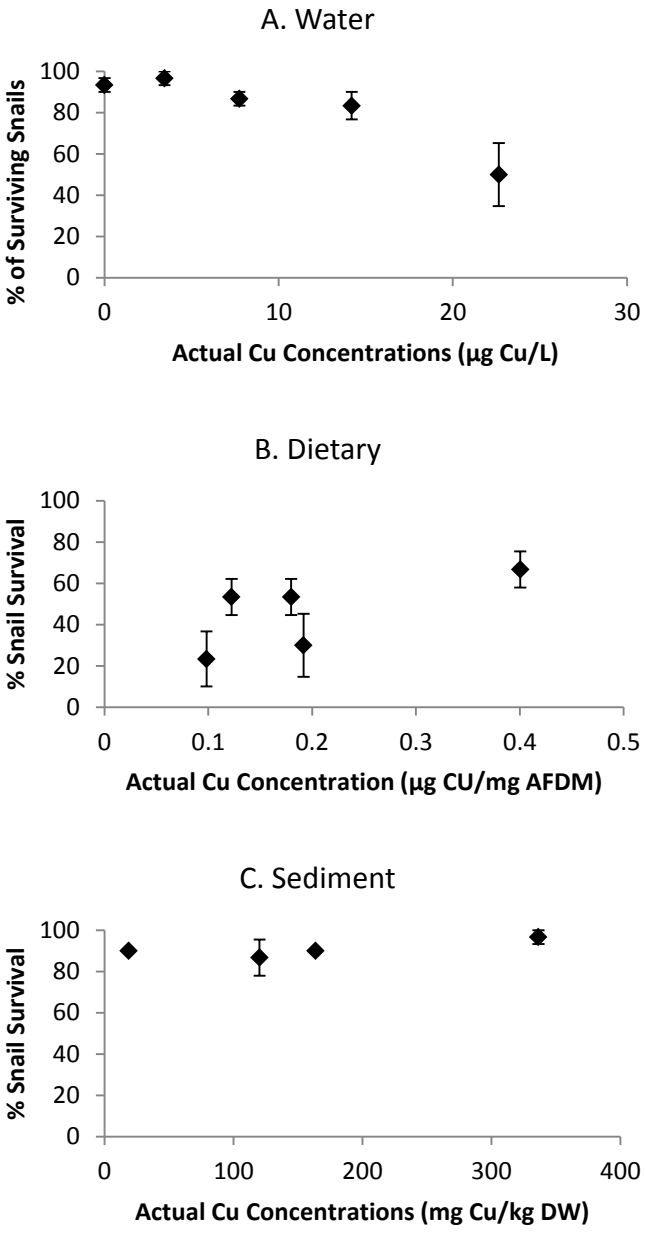


Figure 1. Average (±1 SE) snail survival after 28-day exposure to Cu through three exposure routes: water (A), dietary (B), and sediment (C).

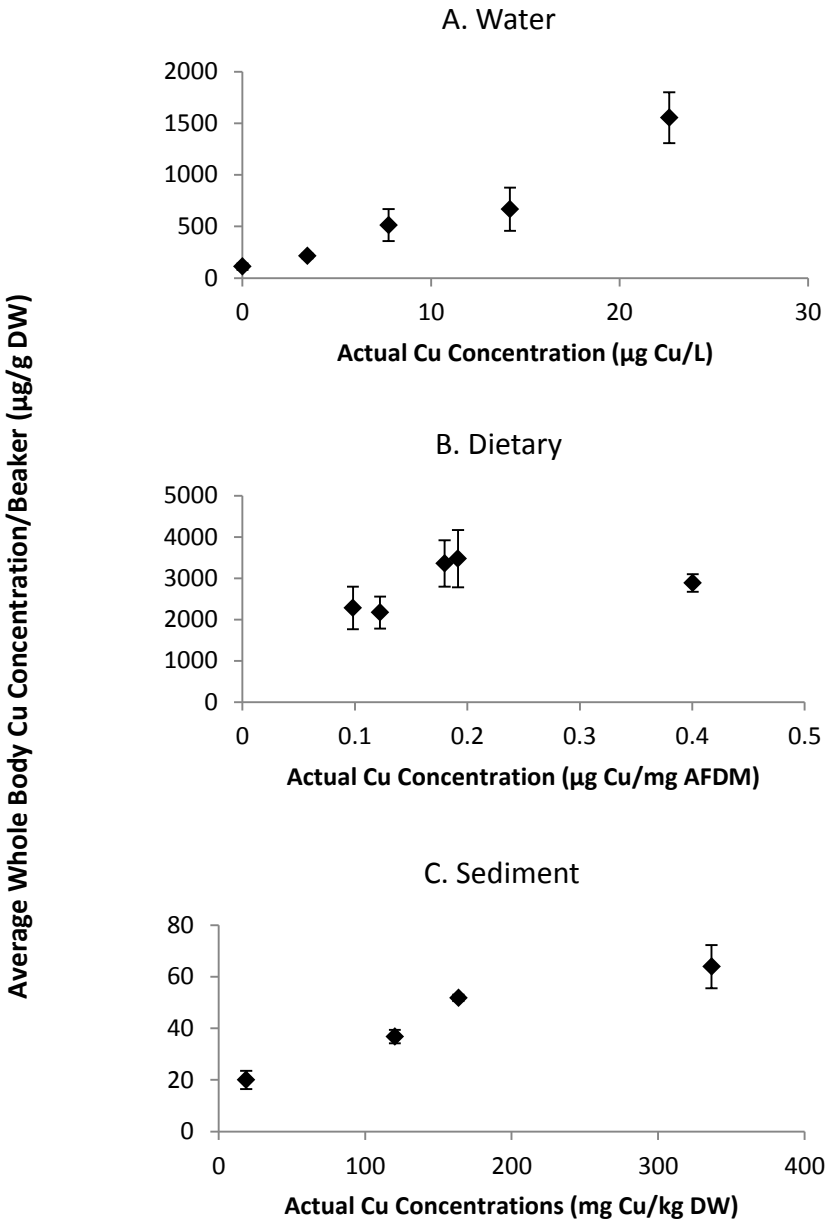
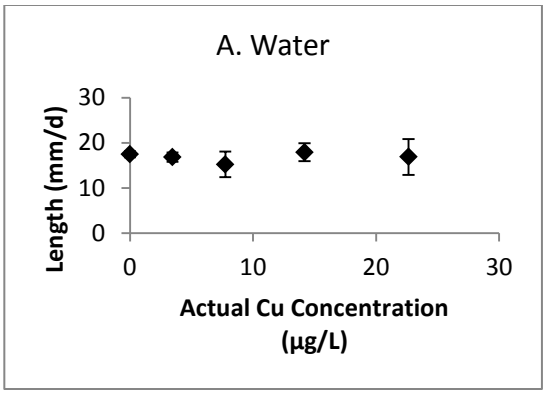
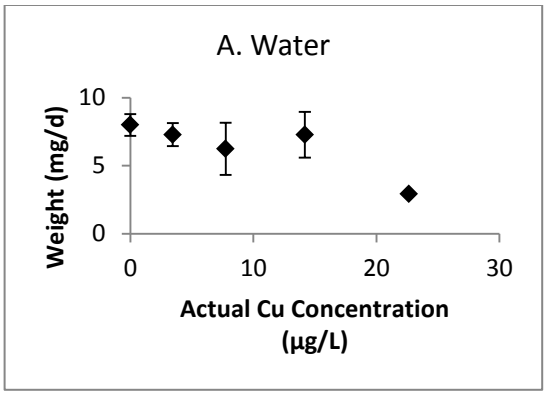
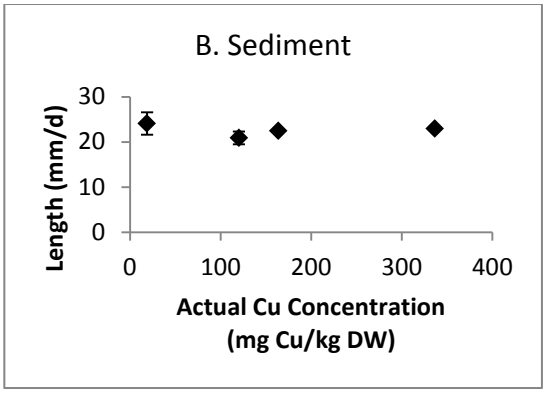
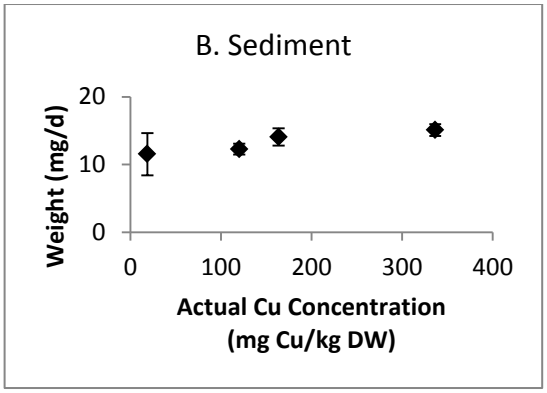


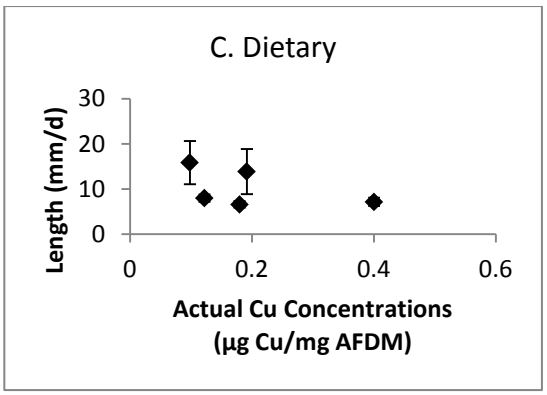
Figure 2. Average (±1 SE) whole body total Cu concentrations in snails after 28-day exposure to Cu through three exposure routes: water only (A), dietary (B), and sediment (C).











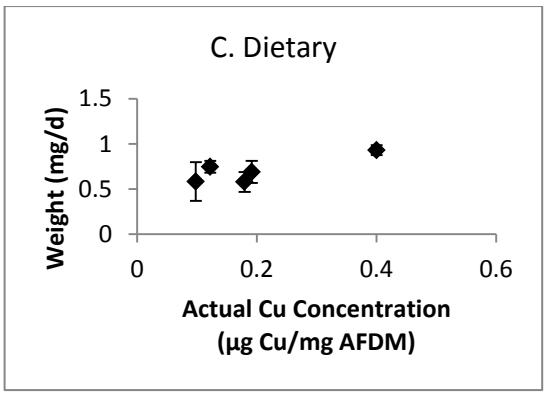


Figure 3. Average length of snail shell for each exposure route scenario: water (A), sediment (B) and dietary (C).

Figure 4. Average weight of snails for each exposure route scenario: water (A), sediment (B) and dietary (C).

Dietary Experiment

For the dietary exposure, the actual total Cu concentrations in the periphyton for each of the five treatments were 0.098, 0.122, 0.180, 0.192 and 0.400 µg Cu/mg AFDM, respectively. Survival was low and not statistically different ($p = 0.107$) among all treatments, but displayed the highest survival in the highest treatment (Fig. 1). Whole body Cu concentrations for the snails ranged from 1600.30 to 4809.09 µg Cu/g DW, but again there was no difference among treatments ($p = 0.294$, Fig. 2). The diet exposure displayed the highest whole body Cu concentrations out of all three exposure experiments. Snail weight for this experiment was low compared to the other two

Table 2. The average differences (± standard deviation of the difference of the means) for chlorophyll *a* tests between the control beakers and snail beakers (i.e., feeding rate) for each Cu treatment under each exposure scenario.

Chlorophyll *a*[a]		Treatment 1		Treatment 2		Treatment 3		Treatment 4		Treatment 5	
WATER		***0 µg Cu/L***		***5 µg Cu/L***		***10 µg Cu/L***		***20 µg Cu/L***		***30 µg Cu/L***	
	Day 7	0.229	(0.40)	0.097	(0.53)	0.136	(0.47)	0.214	(0.43)	-0.217	(0.35)
	Day 14	0.067	(0.38)	0.205	(0.31)	0.332	(0.33)	0.255	(0.32)	0.082	(0.20)
	Day 21	0.289	(0.38)	0.174	(0.23)	0.099	(0.15)	0.061	(0.17)	0.051	(0.14)
	Day 28	0.570	(0.23)	0.265	(0.34)	0.549	(0.30)	0.351	(0.25)	0.467	(0.34)
DIETARY		***0 µg Cu/L***		***5 µg Cu/L***		***10 µg Cu/L***		***20 µg Cu/L***		***30 µg Cu/L***	
	Day 7	-1.122	(1.23)	2.182	(0.76)	1.846	(0.73)	0.892	(1.10)	2.466	(0.92)
	Day 14[b]	-0.054	(0.62)	1.031	(0.64)	1.862	(0.49)	0.864	(0.85)	0.803	(0.51)
	Day 21	3.051	(2.70)	9.851	(2.28)	6.277	(2.73)	-1.631	(2.02)	3.504	(2.51)
	Day 28	2.980	(1.94)	7.550	(1.47)	5.803	(1.84)	5.697	(1.52)	6.637	(1.72)
SEDIMENT		***0 mg Cu/kg***		***100 mg Cu/kg***		***200 mg Cu/kg***		***400 mg Cu/kg***			
	Day 7	0.023	(0.21)	0.048	(0.25)	0.003	(0.22)	-0.003	(0.12)		
	Day 14[b]	0.537	(0.66)	-0.136	(0.55)	-0.506	(0.49)	0.840	(0.53)		
	Day 21	0.036	(1.31)	2.897	(1.56)	0.206	(1.23)	-0.280	(1.26)		
	Day 28	6.740	(1.96)	-1.096	(1.74)	4.210	(1.65)	3.460	(2.10)		

[a] µg/hour

[b] Feeding Rate Tests were significantly different between treatments ($p < 0.05$)

Table 3. The average differences (± standard deviation of the difference of the means) for AFDM tests between the control beakers and snail beakers (i.e., feeding rate) for each Cu treatment under each exposure scenario.

AFDM[a]		Treatment 1		Treatment 2		Treatment 3		Treatment 4		Treatment 5	
WATER		***0 µg Cu/L***		***5 µg Cu/L***		***10 µg Cu/L***		***20 µg Cu/L***		***30 µg Cu/L***	
	Day 7	0.013	(0.12)	0.016	(0.12)	0.021	(0.11)	0.006	(0.14)	0.003	(0.11)
	Day 14	0.000	(0.09)	0.012	(0.07)	0.009	(0.08)	0.012	(0.10)	0.005	(0.08)
	Day 21	0.023	(0.09)	0.018	(0.09)	0.013	(0.07)	0.014	(0.07)	0.001	(0.07)
	Day 28	0.029	(0.09)	0.017	(0.07)	0.019	(0.08)	0.019	(0.08)	0.028	(0.07)
DIETARY		***0 µg Cu/L***		***5 µg Cu/L***		***10 µg Cu/L***		***20 µg Cu/L***		***30 µg Cu/L***	
	Day 7[b]	0.011	(0.10)	0.011	(0.11)	0.054	(0.10)	-0.011	(0.13)	0.038	(0.12)
	Day 14	0.011	(0.09)	0.407	(0.58)	0.025	(0.08)	0.012	(0.10)	0.014	(0.14)
	Day 21	0.027	(0.14)	0.083	(0.16)	0.120	(0.21)	0.243	(0.47)	0.073	(0.17)
	Day 28	0.061	(0.22)	-0.017	(0.18)	0.000	(0.21)	0.055	(0.20)	0.002	(0.13)
SEDIMENT		***0 mg Cu/kg***		***100 mg Cu/kg***		***200 mg Cu/kg***		***400 mg Cu/kg***			
	Day 7	0.008	(0.09)	-0.002	(0.13)	0.005	(0.08)	-0.008	(0.10)		
	Day 14	0.023	(0.08)	-0.006	(0.11)	0.001	(0.10)	0.004	(0.08)		
	Day 21	-0.039	(0.23)	-0.014	(0.15)	-0.033	(0.28)	-0.017	(0.25)		
	Day 28[b]	0.175	(0.44)	0.270	(0.38)	-0.108	(0.37)	-0.325	(0.36)		

[a] mg/hour

[b] Feeding Rate Tests were significantly different between treatments ($p < 0.05$)

exposure scenarios which ranged from 0.20 to 1.03 mg/day across all five treatments and the length ranged from 5.18 to 23.75 mm/day with no significant results (Fig 3c and 4c). Again, feeding rates were not significantly different on most days with the exception of day 7 (AFDM, p = 0.019) and 14 (chlorophyll *a*, p = 0.029) with no statistical significance between paired treatments.

DISCUSSION

Our current study found that Cu delivered through the sediment exposure route was least toxic to *L. stagnalis*, which is contrary to studies that showed other freshwater snails accumulating more Cu from soil than from water (Heng et. al. 2004; Hoang et. al. 2008; Hoang and Rand 2009). When studies express sediment as being toxic to snails, it is helpful to measure and compare AVS and SEM of the sediment to better determine potential toxicity of metals even though concentrations of total Cu are high in the sediment. This study shows the molar difference within all sediments were well below one indicating high amounts of AVS available to bind to Cu and not bioavailable for snail uptake (Appendix A). The spiked concentrations of Cu in the sediment exposure experiment fell within realistic environmental concentrations, but were not near some of the most toxic sediments reported. Also, spiked Cu concentrations in the sediments fell above and below Hoang et. al. (2008) and Hoang and Rand (2009) studies. However, it could be possible that other characteristics and chemistry of the Saline sediment are not representative of the other sediments used in previous studies, such as different dissolved organic carbon (DOC) and AVS. Schuler et. al. (2008) calculated an EC_{90} to be 55.3 mg Cu/kg in South Florida, which is in the lower range of Cu concentrations used in this study. If sediment types can make that drastic of a difference on Cu toxicity, then it is important to determine which chemistry parameters promote Cu toxicity to snails. Therefore, determining the difference between the sediment used in this study versus other studies is necessary to understand toxicity effects to *L. stagnalis*.

Hoang and Rand (2009) show overlying water not toxic to freshwater snails which indicate that free Cu concentrations in overlying water were not significantly correlated with survival of snails. This indicated additional Cu speciation with $Cu(CO_3)_2^{2-}$ and $CuCO_3$ were potentially occurring, and when combining free Cu and $CuOH^+$ with $Cu(CO_3)_2^{2-}$ and $CuCO_3$, there was significant results that correlated snail survival with Cu concentrations (Hoang and Rand 2009). This shows the importance of understanding basic water chemistry in each experiment and use preliminary tests such as the biotic ligand model (BLM) to determine how ions will potentially react with Cu in the overlying water such as $(CO_3)_2^{2-}$ and CO_3. The current study indicates that any overlying water with Cu concentrations below 19.4 µg Cu/L will most likely not cause toxicity, based on the hardness equation for Cu and water quality criteria during the sediment exposure scenario (Appendix A). The measured Cu concentrations in the overlying water throughout the 28 days of the sediment scenario fell within typical background levels of Cu and never exceeded 12 µg Cu/L in any of the treatments which is consistent with the lack of Cu toxicity to snails from overlying water. Also, DOC in water was measured in previous studies while it was not measured in the current study, and based on the BLM, DOC is a large influential factor on the potential toxicity of Cu in water and not just hardness criteria (Cruz and Delos 2010). However, since there are studies that show sediment exposure being more toxic than water exposure, it needs to

furthered reviewed if the spiked Cu concentrations used in the sediment were high enough, if the Saline sediment was representative of other sediments and if there are other speciations of Cu that are potentially toxic to freshwater snails. Lastly, since snails had limited direct contact with the sediment since their food source (spinach) was floating on top of the overlying water, this could account for the observed lower Cu concentrations in snails.

There can be a variety of factors that influence exposure routes and Cu toxicity on snails such as snail behavior and persistence of chemicals. Snail behavior (i.e., habitat choice) can influence which route of exposure has more toxic effects on snails. Since freshwater snails are almost constantly in contact with water, it is a very important route of Cu exposure. Food availability is another factor than can influence exposure routes on snails. If food is predominantly available at the surface of the water body, then snails will most likely have minimal contact to sediment exposure in comparison to an environment where food sources are along the bottom of the aquatic environment and more direct exposure to sediment. As snails ingest periphyton or sediment containing high concentrations of Cu, these routes can also become an important exposure route. Observations in this study showed higher Cu concentrations in snails through diet exposure even though they may be biased due to lack of depuration. Not only is snail behavior an important factor between exposure routes, but the persistence of Cu in each exposure route is significant in determining potential toxic effects on snails. Persistence of Cu in sediments is much higher than in water due to its chemical properties. Cu can quickly precipitate out of water based on pH, alkalinity and hardness properties and reside in sediments (Flemming & Trevors 1989, WHO 1998). Finally, the exposure time of Cu from each route can regulate the toxicity effects on snails. Understanding snail species behavior and site specific conditions can prioritize which exposure route is more important.

For Cu exposure through the water scenario, *L. stagnalis* did respond to Cu, both in survival and whole body concentrations. This correlation and the EC_{50} (25.1 µg Cu/L) shows that Cu concentrations near 25-30 µg Cu/L display significant differences from the control. Similar LC_{50}s have been reported (Besser et. al. 2009; Brix et. al. 2011) confirming that the water exposure route is an important exposure route to monitor Cu levels and make sure snail populations is not compromised. This is also important to recognize because the upper concentrations of background levels in water is near 30 µg Cu/L and need to be monitored to determine if these daily environmental conditions are toxic to snails.

Regardless of treatment, exposure to dietary Cu led to the highest Cu concentrations accumulated in the snails. However, survival, growth and Cu concentrations were not statistically different among the Cu treatments and depuration was not conducted at the end of the 28 days. The data shows that as concentrations in snails increase, snail survival decreases. But there appears to be a threshold between treatments 4 and 5 where the whole body Cu concentrations in snails decrease at the highest treatment and snail survival increases from the previous treatment, implying that the snails are no longer ingesting the highest contaminated food (Figures 1 & 2). This threshold could be occurring due to potential detoxification mechanisms that are happening within

the *L. stagnalis*. Since Cu is classified with other metals that tend to bind with ligands that contain sulfur (S) or nitrogen (N) as a donor atom within organs, it is possible that Cu is binding with S or N and being excreted as residual bodies (Desouky 2006). Another reason why the dietary exposure route is displaying high Cu concentrations could be due to potential accumulation of the Cu in the digestive gland. Desouky (2006) has shown that the digestive gland in pond snails accumulate the majority of metals (Al, Cd, Zn) after 30 days of exposure. Although this study did not separate specific organs to determine distribution of Cu concentrations within the snail and the concentrations may have been elevated due to the snails not being depurated, it still shows the highest Cu concentrations in *L. stagnalis* compared to all three exposure routes, which could pose a threat to higher trophic levels. As birds, reptiles, fish and other species consume snails that contain these high Cu concentrations in their soft tissues, shell and gut, it could potentially accumulate in snail predators. However, Cu distribution and speciation within snail tissue is not completely understood and may be important due to these potential detoxification mechanisms and binding of ligands occurring within the snail potentially causing less toxicity then predicted by calculated bioaccumulation factors (BAF). Overall, future research needs to incorporate higher Cu levels in periphyton to determine if the proposed threshold is occurring and to better understand the patterns observed in the dietary exposure route.

Natural periphyton is composed of a variety of algal species, bacteria, and detritus within a polysaccharide matrix (Real et. al. 2003; Serra et. al. 2009), and this complex structure and composition could be a cause for insignificant feeding rate results. It has been shown from the guts of freshwater snails that its diet volume is 50-90% detritus followed by 25% algae (Brönmark 1989). Snail feeding affects the periphyton community by increasing species that are tightly adhered to each other by consuming the more filamentous species that are less tightly adhered to the community structure (Brönmark 1989). As Cu overloads the periphyton matrix, there are a variety of effects that can occur such as community shifts and inhibition of photosynthesis in the algae (Serra et. al. 2009). These changes can potentially occur during the feeding rate experiments and could explain why the course measures of AFDM and chlorophyll *a* showed little significance during the feeding rate experiments as well as a snail's diet which consists mainly of detritus. Lastly, snails can disrupt the community and structure of periphyton and as periphyton communities change, there can be effects on snail grazing (Brönmark 1989; Brown and Carman 1994; Feminella and Hawkins 1995) which could be synergistic or additive effects when Cu is combined with snail feeding habits.

Overall, snail feeding rates and growth endpoints exhibited the least sensitivity to Cu while survival endpoints and whole body Cu concentrations were more sensitive to increased Cu levels throughout all three exposure scenarios. However, growth did show similarities to Ca levels where the highest snail and weight values observed matched with the highest Ca concentrations in the sediment exposure experiment which correlates to other studies that show the success of gastropods in enriched Ca waters (Covich 2010). Sediment exposure also showed the least amount of total body Cu concentrations, the highest survival rates and largest growth rates compared to all three exposure routes which demonstrates the least potential for Cu toxicity in *L. stagnalis*. The

water and dietary exposure routes displayed concern for toxicity in current aquatic ecosystems because the experiments showed how connected these two exposure routes are and the potential for toxic effects that could be posed to higher trophic levels. Periphyton was soaked in water containing the same Cu concentrations that was used in the water exposure scenario and *L. stagnalis* had double the amount of Cu in their body from the dietary exposure versus the water exposure. Also, the water exposure was the only exposure route to display significant mortality. Therefore, not only are the background levels of Cu killing snails at higher concentrations, but they're also accumulating in *L. stagnalis* at high concentrations through their food sources that uptake Cu, such as periphyton. If background Cu levels have any possibility of increasing in the aquatic environment in the future, there are large toxicity implications through water and dietary exposure routes, including the possibility of Cu accumulating and transferring though the aquatic food chain. Finally, water quality guidelines for Cu need to address this potential for dietary toxicity from background Cu levels in water and help protect freshwater snail species from declining any further in the future.

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APPENDIX A

Table 4A. Tukey post hoc test results for snail survival from significant ANOVA tests.

				95% Confidence Interval	
WATER	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	30 to 0	0.020*	1.116	-8.00	-0.66
	30 to 5	0.013*	1.116	-8.34	-1.00
	30 to 10	0.050	1.116	-7.34	0.00
	30 to 20	0.080	1.116	-7.00	0.34
	20 to 0	0.892	1.116	-4.67	2.67
	20 to 5	0.754	1.116	-5.00	2.34
	20 to 10	0.998	1.116	-4.00	3.34
	10 to 0	0.972	1.116	-4.34	3.00
	10 to 5	0.892	1.116	-4.67	2.67
	5 to 0	0.998	1.116	-3.34	4.00

*Significant difference between individual treatments ($p < 0.05$)

Table 5A. Tukey post hoc test results for whole body Cu concentrations in snails from significant ANOVA tests.

				95% Confidence Interval	
WATER	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	30 to 0	0.000*	0.19246	3.7315	4.9984
	30 to 5	0.000*	0.19246	3.0874	4.3543
	30 to 10	0.000*	0.19246	2.7404	4.0072
	30 to 20	0.000*	0.19246	2.5467	3.8135
	20 to 0	0.001*	0.19246	0.5514	1.8182
	20 to 5	0.105	0.19246	-0.0927	1.1741
	20 to 10	0.847	0.19246	-0.4397	0.8271
	10 to 0	0.003*	0.19246	0.3577	1.6245
	10 to 5	0.422	0.19246	-0.2864	0.9804
	5 to 0	0.046	0.19246	0.0107	1.2775

*Significant difference between individual treatments ($p < 0.05$)

				95% Confidence Interval	
SEDIMENT	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	400 to 0	0.001*	6.71696	22.4349	65.4551
	400 to 100	0.016*	6.71696	5.6029	48.6231
	400 to 200	0.339	6.71696	-9.4097	33.6104
	200 to 0	0.006*	6.71696	10.3346	53.3547
	200 to 100	0.193	6.71696	-6.4974	36.5227
	100 to 0	0.133	6.71696	-4.6781	38.3421

*Significant difference between individual treatments ($p < 0.05$)

Table 6A. Tukey post hoc test results for chlorophyll *a* from significant ANOVA tests.

SEDIMENT				95% Confidence Interval	
Day 14	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	400 to 0	0.078	0.188518	-0.04435	1.03435
	400 to 100	0.281	0.188518	-0.18769	0.89102
	400 to 200	0.093	0.188518	-0.06269	1.01602
	200 to 0	1.000	0.188518	-0.52102	0.55769
	200 to 100	0.909	0.188518	-0.66435	0.41435
	100 to 0	0.871	0.188518	-0.39602	0.68269

DIETARY				95% Confidence Interval	
Day 14	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	30 to 0	0.361	0.262937	-1.27997	0.29364
	30 to 5	0.533	0.262937	-1.19914	0.37447
	30 to 10	0.172	0.262937	-1.40297	0.17064
	30 to 20	0.128	0.262937	-1.44631	0.12731
	20 to 0	0.968	0.262937	-0.62047	0.95314
	20 to 5	0.878	0.262937	-0.53964	1.03397
	20 to 10	1.000	0.262937	-0.74347	0.83014
	10 to 0	0.989	0.262937	-0.66381	0.90981
	10 to 5	0.935	0.262937	-0.58297	0.99064
	5 to 0	0.998	0.262937	-0.86764	0.70597

Table 7A. Tukey post hoc test results for AFDM from significant ANOVA tests.

SEDIMENT				95% Confidence Interval	
Day 28	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	400 to 0	0.890	0.093356	-0.20043	0.33376
	400 to 100	0.998	0.093356	-0.28376	0.25043
	400 to 200	0.984	0.093356	-0.23376	0.30043
	200 to 0	0.984	0.093356	-0.23376	0.30043
	200 to 100	0.949	0.093356	-0.31709	0.21709
	100 to 0	0.809	0.093356	-0.18376	0.35043

DIETARY				95% Confidence Interval	
Day 7	**Treatments**	**p-value**	**Standard Error**	**Lower Bound**	**Upper Bound**
	30 to 0	0.402	0.009271	-0.04441	0.01108
	30 to 5	0.197	0.009271	-0.04874	0.00674
	30 to 10	0.998	0.009271	-0.03058	0.02491
	30 to 20	0.412	0.009271	-0.04424	0.01124
	20 to 0	1.000	0.009271	-0.02791	0.02758
	20 to 5	0.988	0.009271	-0.03224	0.02324
	20 to 10	0.590	0.009271	-0.01408	0.04141
	10 to 0	0.579	0.009271	-0.04158	0.01391
	10 to 5	0.320	0.009271	-0.04591	0.00958
	5 to 0	0.989	0.009271	-0.02341	0.03208

Table 8A. One-way ANOVA test results for snail survival under each exposure scenario.

Exposure Route	df	F	p-value	Shapiro-Wilk (p-value)	Kolmogorov-Smirnov (p-value)
Water	4	5.589	0.013*	0.140	0.064
Sediment[a]	3	0.478	0.922	0.145	0.032
Dietary	4	2.526	0.107	0.061	0.138

[a] Arcsine Square Root Transformation
*Statistically significant

Table 9A. One-way ANOVA test results for the rate of growth in weight (mg/d) for snails under each exposure scenario.

Exposure Route	df	F	p-value	Shapiro-Wilk (p-value)	Kolmogorov-Smirnov (p-value)
Water	4	2.586	0.102	0.638	0.200
Sediment	3	1.749	0.244	0.741	0.200
Dietary	4	1.289	0.338	0.948	0.200

Table 10A. One-way ANOVA test results for the rate of growth in length (mm/d) for snails under each exposure scenario.

Exposure Route	df	F	p-value	Shapiro-Wilk (p-value)	Kolmogorov-Smirnov (p-value)
Water	4	0.178	0.945	0.414	0.200
Sediment	3	1.211	0.374	0.104	0.059
Dietary[a]	4	2.092	0.157	0.861	0.200

[a] Natural Log Transformation

Table 11A. One-way ANOVA test results for whole body Cu concentration in snails under each exposure scenario.

Exposure Route	df	F	p-value	Shapiro-Wilk (p-value)	Kolmogorov-Smirnov (p-value)
Water[a]	4	155.619	0.000*	0.221	0.200
Sediment	3	16.015	0.001*	0.700	0.200
Dietary	4	1.428	0.294	0.597	0.200

[a] Natural Log Transformation
*Statistically significant

Table 12A. Two-way ANOVA test results for chlorophyll *a* tests between the control beakers and snail beakers (i.e., feeding rate) for differences between Cu treatments under each exposure scenario.

		df	F	p-value	Shapiro-Wilk (p-value)	Kolmogorov-Smirnov (p-value)
Water	**Day 7**	4	1.229	0.330	0.807	0.200
	Day 14	4	1.409	0.267	0.673	0.200
	Day 21[b]	4	1.759	0.177	0.041	0.200
	Day 28[b]	4	2.078	0.122	0.200	0.200
Dietary	**Day 7**	4	2.617	0.066	0.512	0.194
	Day 14	4	3.369	0.029*	0.668	0.200
	Day 21	4	0.638	0.641	0.793	0.200
	Day 28	4	0.432	0.784	0.807	0.200
Sediment	**Day 7**	3	0.317	0.813	0.576	0.200
	Day 14	3	5.321	0.010*	0.227	0.200
	Day 21	3	0.773	0.526	0.543	0.200
	Day 28	3	1.174	0.351	0.045	0.082

[b] Natural Log Transformation

*Statistically significant

Table 13A. Two-way ANOVA test results for AFDM tests between the control beakers and snail beakers (i.e., feeding rate) for differences between Cu treatments under each exposure scenario.

		df	F	p-value	Shapiro-Wilk (p-value)	Kolmogorov-Smirnov (p-value)
Water	**Day 7**	4	0.362	0.833	0.760	0.200
	Day 14	4	0.824	0.525	0.262	0.200
	Day 21	4	1.946	0.142	0.461	0.200
	Day 28	4	1.000	0.430	0.784	0.200
Dietary	**Day 7**	4	3.766	0.019*	0.499	0.200
	Day 14	4	0.372	0.825	0.616	0.200
	Day 21	4	2.007	0.132	0.324	0.200
	Day 28	4	1.038	0.412	0.523	0.184
Sediment	**Day 7**	3	0.699	0.567	0.879	0.200
	Day 14	3	2.517	0.095	0.334	0.200
	Day 21	3	0.089	0.965	0.995	0.200
	Day 28	3	4.243	0.022*	0.371	0.200

*Statistically significant

Table 14A. Average (±1 SE) water chemistry parameters measured during each snail exposure experiment. pH, temperature and DO were measured three times weekly and hardness and alkalinity was measured at the beginning and end of each experiment.

	Water	Dietary	Sediment
pH	7.29 ± 0.03	7.45 ± 0.01	7.34 ± 0.02
Temperature (^{o}C)	23.5 ± 0.03	20.6 ± 0.02	22.1 ± 0.04
DO (mg/L)	6.52 ± 0.17	8.24 ± 0.02	4.40 ± 0.12
Hardness (mg/L $CaCO_3$)	193.0 ± 3.13	168.3 ± 3.81	246.7 ± 8.61
Alkalinity(mg/L $CaCO_3$)	68.0 ± 0.96	69.1 ± 1.07	212.0 ± 7.53

Table 15A. Nominal and actual Cu concentrations in each experiment scenario.

WATER	Treatment	Nominal Concentrations (µg Cu/L)	Average of Actual Concentrations (µg Cu/L)	Standard Deviation
	1	0	0	0.0035
	2	5	3.45	0.00257
	3	10	7.75	0.00318
	4	20	14.19	0.00594
	5	30	22.64	0.0088

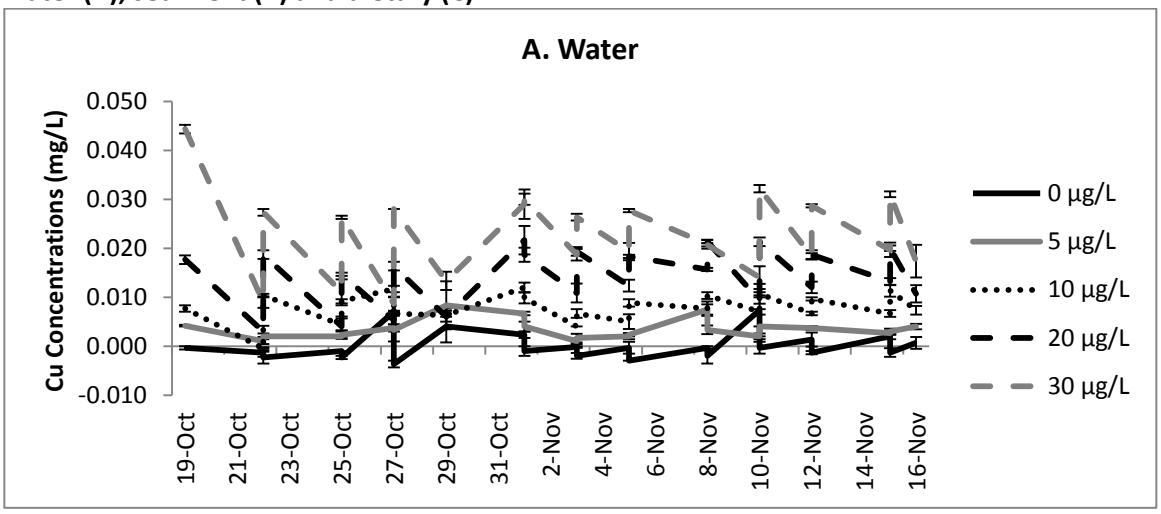
DIETARY	Treatment	Nominal Concentrations (µg Cu/L)[a]	Average of Actual Concentrations (µg Cu/mg AFDM)[b]	Standard Deviation
	1	0	0.09825	0.157043
	2	5	0.12233	0.125436
	3	10	0.17975	0.197388
	4	20	0.19167	0.215496
	5	30	0.40042	0.340874

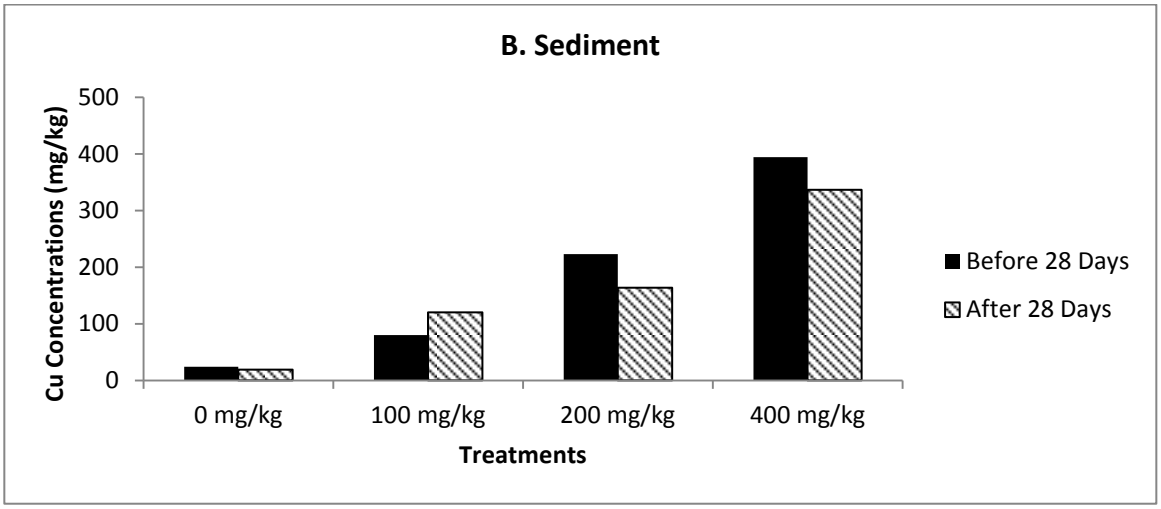
SEDIMENT	Treatment	Nominal Concentrations (mg Cu/kg DW)	Actual Concentrations (mg Cu/kg DW)
	1	0	18.76116736
	2	100	120.2587045
	3	200	163.6840024
	4	400	336.5327233

[a] Concentrations are for the soaking water and not the actual concentrations within the periphyton disks

[b] Actual concentration within periphyton disks after soaking in nominal concentrations water for 24 hours

Figure 5A. Actual Cu concentrations across 28-day exposure through three exposure routes: water (A), sediment (B) and dietary (C).





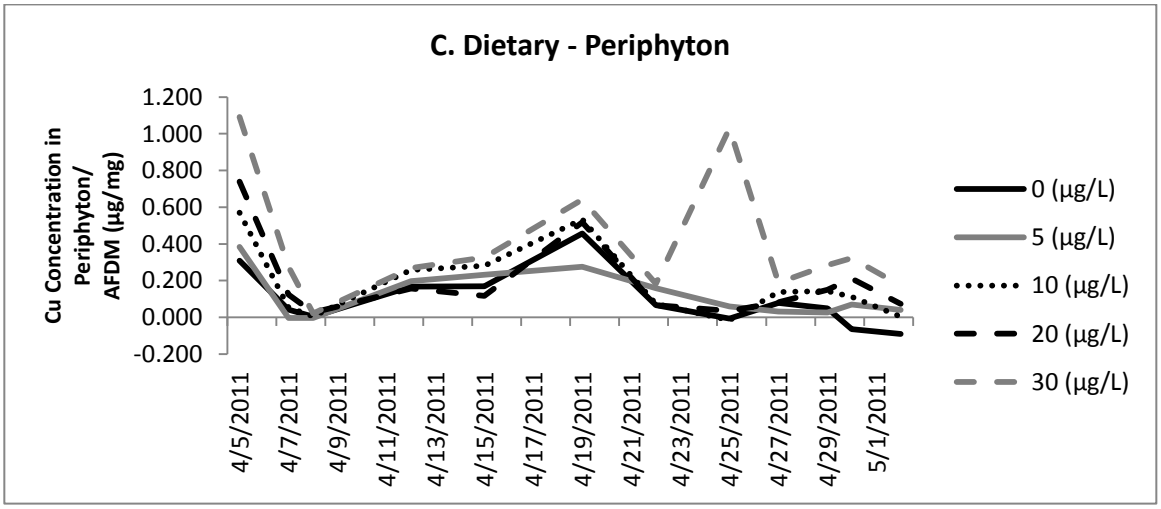


Figure 6A. Actual Cu concentrations in overlying water across 28-day exposure through three exposure routes: water (A), sediment (B) and dietary (C).

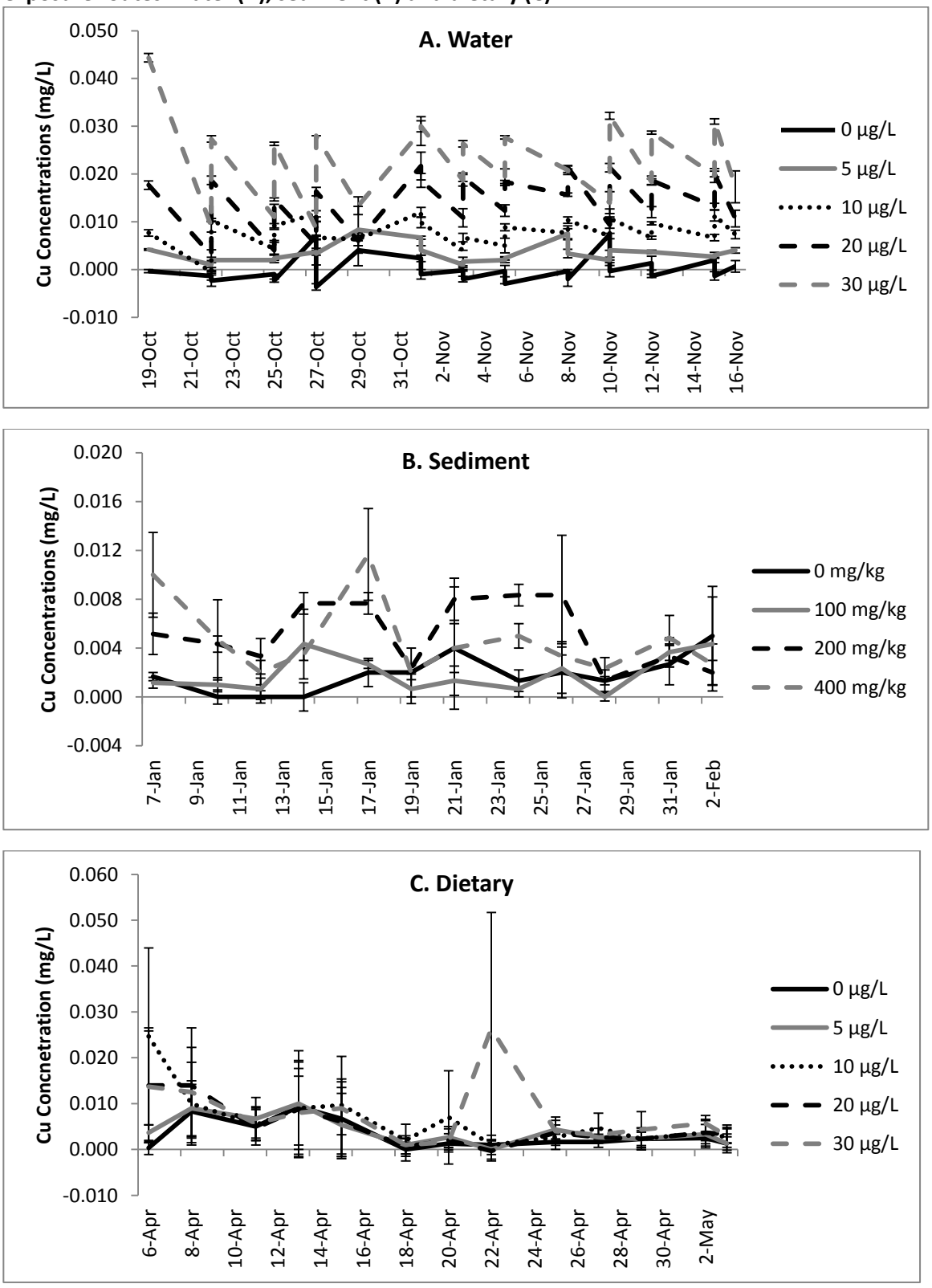


Figure 7A. Actual Ca concentrations in overlying water across 28-day exposure through three exposure routes: water (A), sediment (B) and dietary (C).

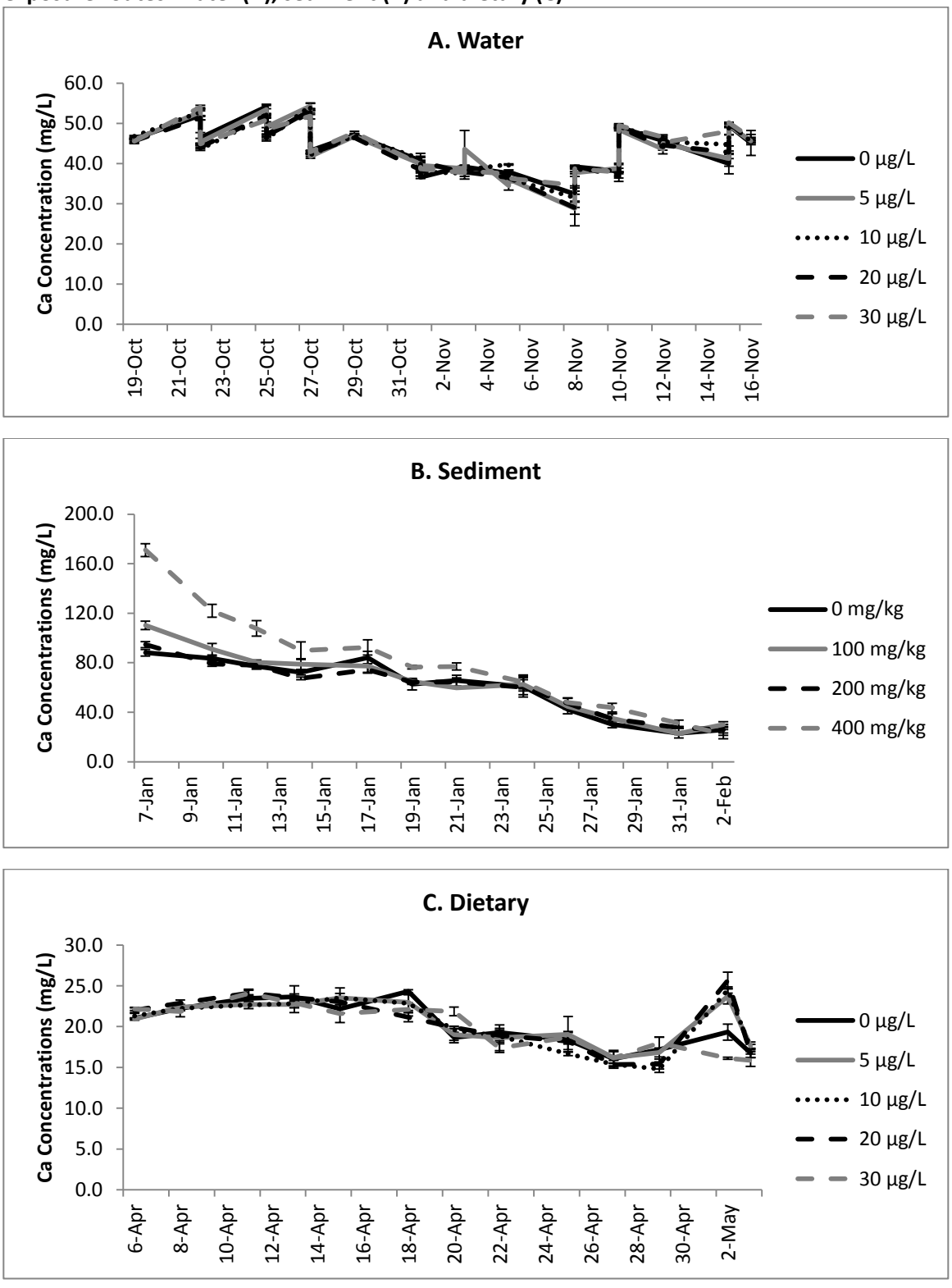


Table 16A. AVS and SEM information for each treatment in the sediment exposure scenario before and after 28-day exposure.

Sample	SEM (µmoles/g)	AVS (µmoles/g)	Ratio (AVS/SEM)	SEM-AVS	Total Organic Carbon (%)
0 mg/kg Cu[a]	0.0782	13.90	0.006	-13.82	0.0122
100 mg/kg Cu[a]	0.2351	12.88	0.018	-12.65	0.0127
200 mg/kg Cu[a]	1.2445	10.45	0.119	-9.21	0.0145
400 mg/kg Cu[a]	1.4864	5.39	0.276	-3.90	0.0147
Beaker 1[b]	0.1045	14.55	0.007	-14.44	0.0121
Beaker 5[b]	0.3029	16.36	0.019	-16.06	0.0127
Beaker 8[b]	0.1294	6.21	0.021	-6.08	0.0136
Beaker 11[b]	0.0954	12.20	0.008	-12.11	0.0144

[a] Samples of each treatment from initial spiked sediment at the beginning of 28-day exposure
[b] Samples of each treatment after 28-day exposure

Table 17A. Hardness equation based on water quality criterion for Cu for each exposure scenario.

	Hardness (mg/L of $CaCo_3$)	CMC[a] (µg/L)	CCC[b] (µg/L)	Nominal Concentration at End of 28 Days	
WATER	178.2	24.1	15.3	Day 0	
	194.3	26.2	16.5	0	(µg/L)
	193.0	26.0	16.4	5	
	199.3	26.8	16.8	10	
	200.5	27.0	16.9	20	
	178.0	24.1	15.3	30	
DIETARY	185.6	25.1	15.8	Day 0	
	158.1	21.5	13.8	0	(µg/L)
	156.8	21.4	13.7	5	
	175.9	23.8	15.1	10	
	175.9	23.8	15.1	20	
	174.6	23.7	15.0	30	
SEDIMENT	192.5	25.9	16.3	Day 0	
	264.8	35.0	21.4	0	(mg/kg)
	238.0	31.7	19.6	100	
	236.0	31.4	19.4	200	
	249.5	33.1	20.4	400	

[a] CMC – Criteria Maximum Concentration is the highest level for a 1-hour average exposure not to be exceeded more than once every three years, and is synonymous with "acute."
[b] CCC – Criteria Continuous Concentrations is the highest level for a 4-day average exposure not to be exceeded more than once every three years, and is synonymous with "chronic."
[c] NOAA SQUIRTS, Screening Quick Reference Table for Inorganics in Water, Updated November 2006.

Table 18A. Overall summary of test conditions for each exposure scenario.

	Water	Sediment	Dietary
1. Test Organisms	L. stagnalis		
2. Test Type	Toxicity test		
3. Test Duration	28 days		
4. Toxicants	Copper chloride	Copper chloride	Copper chloride
5. Dilution series	30, 20, 10, 5 and 0 µg/L	400, 200, 100, and 0 mg/kg	30, 20, 10, 5 and 0 µg/L
6. Temperature	Ambient temperature		
7. Lighting	Ambient laboratory light		
8. Aeration	None		
9. Feeding	Spinach	Spinach	Spinach
10. Test Water	Measure hardness and alkalinity at beginning and end of tests of culture water.		
11. Water addition	Change 90% of water manually with appropriate Cu concentrations on M, W, F of every week	Use ZumAlt to change water on M, W, F of every week. (2000 ml)	Use ZumAlt to change water on M, W, F of every week. (4000 ml)
12. Test Chamber	300 ml beakers (200 ml of water)	300 ml beakers (100 ml of sediment and 100 ml of water)	300 ml beakers (periphyton disks and 200 ml of water)
13. Age of test organisms	14-21 days (3 week)		
14. Organisms/ chamber	10	10	10
15. Replication	3 chambers per exposure level	3 chambers per exposure level	3 chambers per exposure level
16. Water quality	Temperature, DO, & pH before each water exchange on M, W, & F		
17. Toxicant Analysis	Dissolved Cu and Ca in water. Total Cu in periphyton and snails	Dissolved Cu and Ca in water. Total Cu in periphyton and snails	Dissolved Cu and Ca in water. Total Cu in periphyton and snails
18. Endpoints	Survival and Growth on day 28, Feeding rates on periphyton		

Figure 8A. EC_{50} curve (µg Cu/L) for snail survival in water exposure scenario and output calculated by EPA Toxicity Relationship Analysis Program (TRAP).

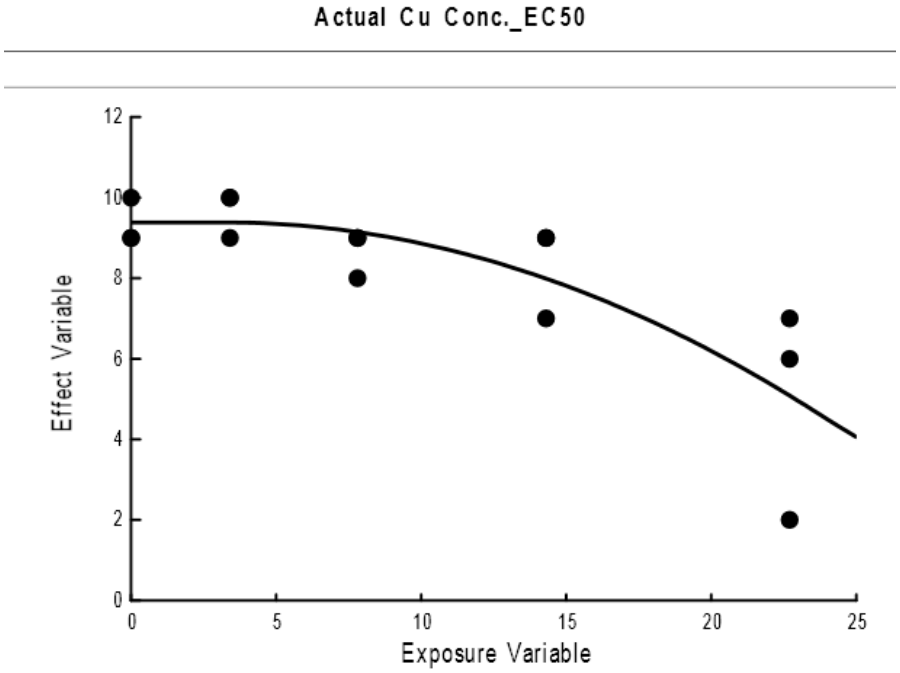


Table 19A. Parameter summary output from EPA TRAP for calculating EC_{50} (µg Cu/L).

Parameter Summary (Threshold Sigmoid Regression Analysis)

Parameter	Guess	FinalEst	StdError	95%LCL	95%UCL
X50	25.13	23.57	1.88	19.48	27.65
S	0.03999	0.04905	0.01896	0.00774	0.09035
Y0	9.500	9.388	0.521	8.253	10.523

Table 20A. Effect concentration summary output from EPA TRAP for calculating EC_{50}, EC_{20}, EC_{10} and EC_5 (µg Cu/L).

Effect Concentration Summary

%Effect	Xp Est	95%LCL	95%UCL
50.0	23.57	19.48	27.65
20.0	16.073	10.123	22.023
10.0	12.296	3.635	20.958
5.0	9.626	-1.110	20.361

12/01/2011 15:22 MED Toxicity Relationship Analysis Model, Version 1.21

Figure 9A. Average Cu concentrations in periphyton disk for each treatment in control beakers (no snails) and beakers with snails for each feeding rate test in the water exposure scenario.

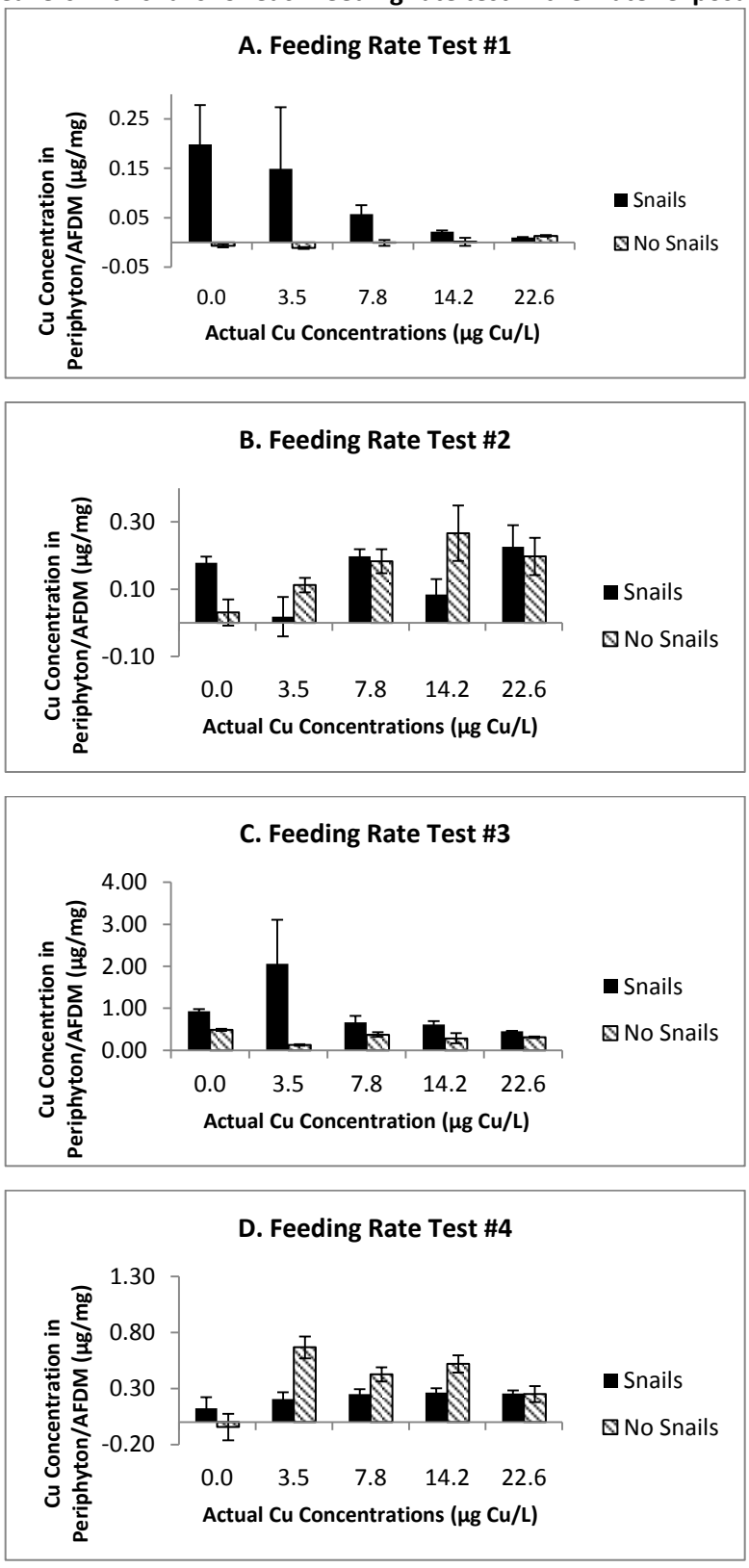


Figure 10A. Average Cu concentrations in periphyton disk for each treatment in control beakers (no snails) and beakers with snails for each feeding rate test in the sediment exposure scenario.

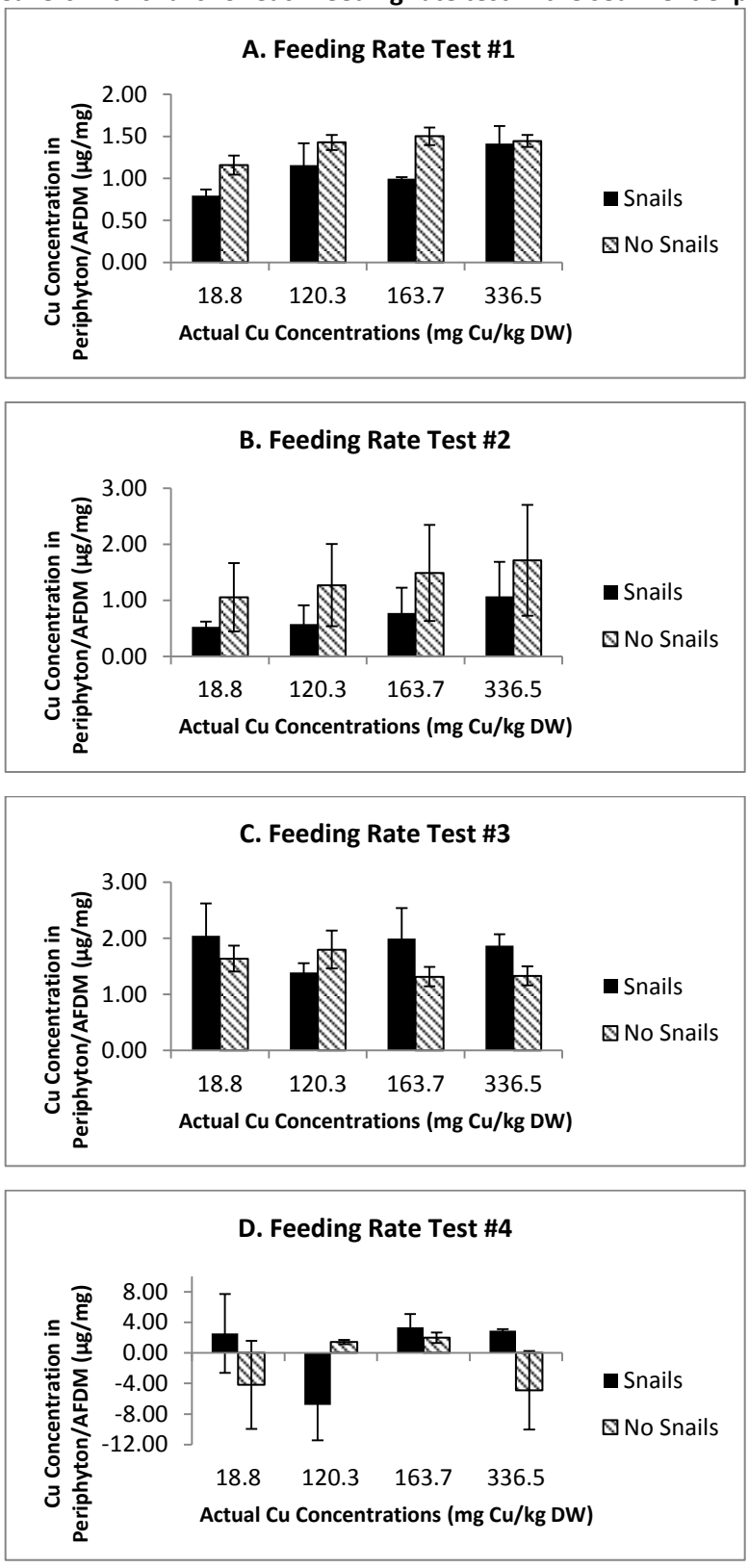


Figure 11A. Average Cu concentrations in periphyton disk for each treatment in control beakers (no snails) and beakers with snails for each feeding rate test in the dietary exposure scenario.

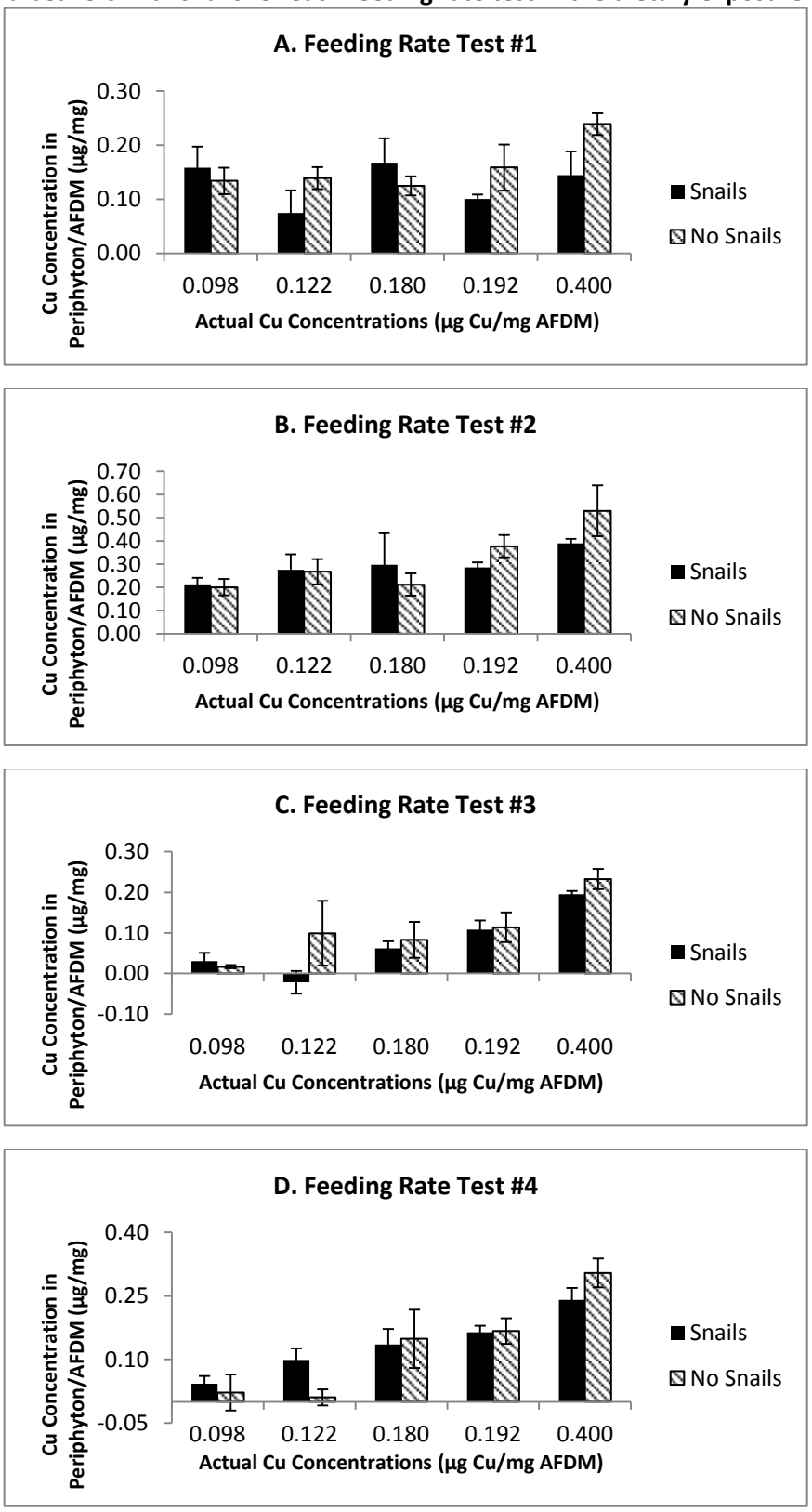


Table 21A. Descriptive statistics for 1 week old snail's shell length in mm from preliminary tests to determine appropriate age of snails to use for exposure experiments.

Treatment	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	3	11.00	2.000	1.155	6.03	15.97	9	13
10	3	6.67	5.508	3.180	-7.01	20.35	1	12
20	3	9.33	4.163	2.404	-1.01	19.68	6	14
30	3	11.33	9.018	5.207	-11.07	33.74	2	20
Total	12	9.58	5.282	1.525	6.23	12.94	1	20

Table 22A. Descriptive statistics for 3 week old snail's shell length in mm from preliminary tests to determine appropriate age of snails to use for exposure experiments.

Treatment	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	3	13.33	7.234	4.177	-4.64	31.30	5	18
10	3	10.33	6.351	3.667	-5.44	26.11	3	14
20	3	10.67	2.309	1.333	4.93	16.40	8	12
30	3	10.33	2.887	1.667	3.16	17.50	7	12
Total	12	11.17	4.589	1.325	8.25	14.08	3	18

Table 23A. One-way ANOVA test results of snail's shell length in mm from preliminary tests to determine appropriate age of snails to use for exposure experiments.

	Sum of Squares	df	Mean Square	F	Sig.
1 Week Old	40.917	3	13.639	0.410	0.750
3 Week Old	19.000	3	6.333	0.238	0.867

Figure 12A. Scatterplot of snails shell length difference for both 1 week (blue) and 3 week (green) for each treatment.

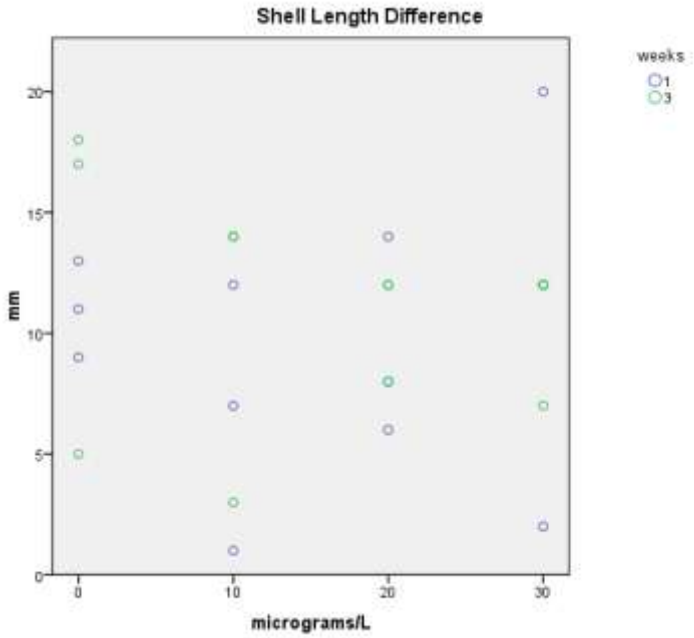


Figure 13A. Bar graph of snails shell length difference for both 1 week (blue) and 3 week (green) for each treatment.

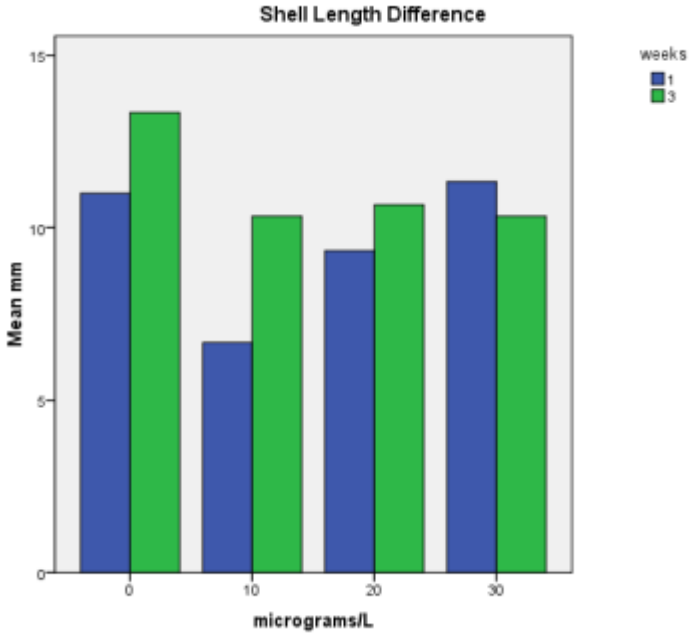


Table 24A. Descriptive statistics for 1 week old snail's weight in grams from preliminary tests to determine appropriate age of snails to use for exposure experiments.

Treatment	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	3	0.01393	0.00225	0.00130	0.00835	0.01952	0.0120	0.0164
10	3	0.01437	0.01463	0.00845	-0.02198	0.05072	-0.0008	0.0284
20	3	0.01100	0.00675	0.00390	-0.00577	0.02777	0.0061	0.0187
30	3	0.02320	0.01710	0.00987	-0.01929	0.06569	0.0036	0.0351
Total	12	0.01563	0.01114	0.00321	0.00855	0.02270	-0.0008	0.0351

Table 25A. Descriptive statistics for 3 week old snail's weight in grams from preliminary tests to determine appropriate age of snails to use for exposure experiments.

Treatment	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	3	0.04967	0.03807	0.02198	-0.04490	0.14424	0.0085	0.0836
10	3	0.03323	0.01612	0.00930	-0.00680	0.07327	0.0157	0.0474
20	3	0.01773	0.02561	0.01479	-0.04588	0.08135	-0.0101	0.0403
30	3	0.01843	0.01085	0.00627	-0.00852	0.04539	0.0062	0.0269
Total	12	0.02977	0.02524	0.00729	0.01373	0.04581	-0.0101	0.0836

Table 26A. One-way ANOVA test results of snail's weight in grams from preliminary tests to determine appropriate age of snails to use for exposure experiments.

	Sum of Squares	df	Mean Square	F	Sig.
1 Week Old	0.000	3	0.000	0.597	0.634
3 Week Old	0.002	3	0.001	1.098	0.405

Figure 14A. Scatterplot of snails weight difference for both 1 week (blue) and 3 week (green) for each treatment.

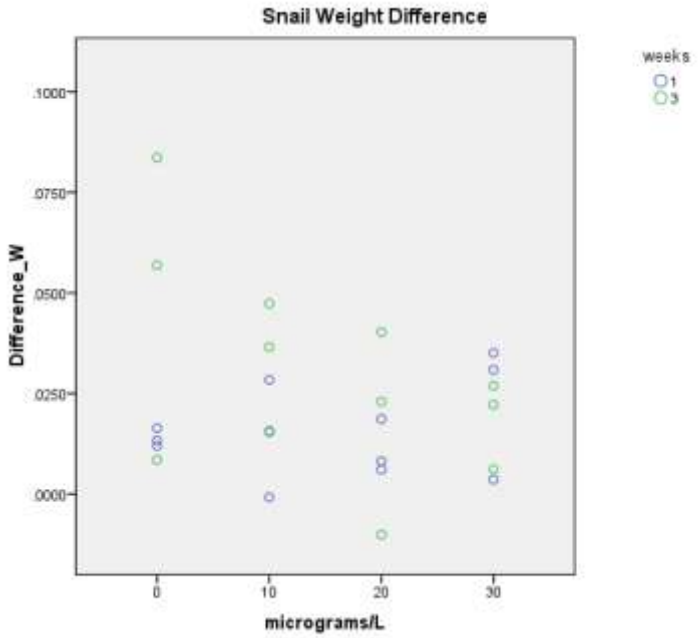


Figure 15A. Bar graph of snails weight difference for both 1 week (blue) and 3 week (green) for each treatment.