Influence of Water Quality on Copper Toxicity to the Marsh Ramshorn Snail (*Planorbella trivolvis*)

by

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Abstract

Catfish growing in aquaculture ponds can be affected by infectious diseases and environmental conditions that cause fish mortality. One problem is trematode infections. The marsh ramshorn snail, *Planorbella trivolvis* is the primary host of several trematodes, such as *Bolbophorus damnificus*, which poses a risk to channel catfish (*Ictalurus punctatus*). To reduce the risks and harm these trematodes cause in aquaculture, active research is sought to disrupt their life cycle by first reducing the primary host, *P. trivolvis*. Copper (Cu) is an effective molluscicide to treat these snails. Studies have shown that water quality, including hardness, pH, and dissolved organic carbon (DOC) greatly influences Cu toxicity. However, the influence of water quality on Cu toxicity to *P. trivolvis* is unknown. This study sought to fill the scientific gap to understand the influence of water quality on Cu toxicity to *P. trivolvis*. Specifically, this thesis research investigated the influence of pH, DOC, water hardness, and organism age on Cu toxicity to *P. trivolvis*. Chapter 2's research focused on the influence of water quality on Cu toxicity to *P. trivolvis,* which allowed the development of multiple linear regression models describing Cu toxicity as a function of pH, DOC, and water hardness. The models can be used to calculate appropriate Cu doses for snail treatment, thereby increasing treatment efficiency, reducing treatment costs, and reducing Cu load in aquaculture pond systems. Research in Chapter 3 examined the Cu sensitivity of the snail at various life stages, which is also helpful for determining appropriate Cu doses for treating snails at specific life stages. Overall, the research has implications for snail and trematode management in catfish aquaculture.

Artificial Intelligence (AI) Use Disclosure Statement

In the preparation of this thesis, no Artificial Intelligence (AI) tools were used.

Digital Accessibility Use Disclosure Statement

In the preparation of this thesis, the following digital accessibility tools were used to ensure this document complies with federal requirements: [Word Accessibility Checker]. The author acknowledges full responsibility for the intellectual content of this work and has made a good faith effort to comply with digital accessibility requirements in publishing, wherein the nature of the content does not significantly change in order to do so. Furthermore, all content has been reviewed and revised to meet these requirements prior to final publication.

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List of Abbreviations

DOC	Dissolved organic carbon
DOM	Dissolved organic matter
USDA NASS	United States Department of Agriculture National Agriculture Statistics Service
US EPA	United States Environmental Protection Agency
MLR	Multiple linear regression
BLM	Biotic ligand model

Chapter 1

Literature review: Introduction and research goals

1.0. Introduction

1.1. *Bolbophorus damnificus* infestation in catfish farms and economic consequences

In the United States of America, channel catfish, *Ictalurus punctatus,* and hybrid catfish (*I. punctatus* ♀ x *I. furcatus* ♂) are farmed prominently in the Southeast, especially in Arkansas, Mississippi, and Alabama (Hegde et al., 2022). According to the United States Department of Agriculture National Agriculture Statistics Service (USDA NASS) (2024), catfish is one of the top 10 seafoods that humans consume annually. The most recent census of aquaculture reported that the US catfish industry produced about 152.8 million kilograms of catfish in 2024. Mississippi leads production, with Alabama being the second-largest producer of US farmed-raised catfish. Alabama produced roughly 30 percent of the annual 152.8 million kilograms of catfish, which is equivalent to approximately 45.3 million kilograms (USDA NASS, 2024). Catfish farming in 2023 accounted for $2 billion in profits in the USA and supported job creation in local communities (Hegde et al., 2022; USDA NASS, 2024). Despite the benefits, the catfish industry faces challenges, such as diseases affecting catfish. One of the most common and important fish health issues is the infection by trematodes (Overstreet et al., 2002; Yost et al., 2008, 2009). The trematode infections can hurt farmers financially, with farmers reporting a net return with a reduction anywhere from 61% to >100%, where the net return did not cover the costs of production, depending on the severity of the infections (Wise et al., 2008).

There are many different types of trematodes which can infect catfish. *Bolbophorus damnificus* is one of the more prevalent trematode species in commercial catfish farms (Overstreet and Curran, 2004). The life cycle of *B. damnificus* goes through various stages. (Overstreet and Curran, 2004; Yost et al., 2008).

Free-swimming newly hatched miracidium trematodes in catfish ponds are first contracted by planorbid snails (Overstreet & Curran, 2004). The miracidium feeds off the snail until it develops into a cercaria, which is shed from the snail (Yost et al., 2008). The free-swimming cercaria penetrates the catfish and produces negative effects on the fish primarily in the fall and spring seasons due to the life cycle of *P. trivolvis* (Tucker & Robinson, 1990). The American White Pelican, *Pelecanus erythrorhynchos*, or the Double-Crested Cormorant, *Nannopterum auritum*, feeds on the catfish and then releases the trematode eggs into the waters through feces. The eggs will hatch into larval trematodes and repeat the life cycle (Overstreet & Curran, 2004). The American White Pelican and Double-Crested Cormorant can also transport parasites and infect parasite-free ponds (Overstreet & Curran, 2004).

Wise et al. (2008) reported that trematodes have different effects on channel catfish. Trematodes cause anemia leading to reduced appetite in catfish, which may result in reduced growth over time (Labrie et al., 2004; Wise et al., 2008). As a result, catfish infected with trematodes are usually smaller than those that are not affected by trematodes. As a result, trematode infection can increase harvest and production time, thereby increasing catfish costs. In extreme cases, the trematode can cause severe illness in the catfish (Wise et al., 2008; Griffin et al., 2014). In addition, trematodes can make catfish more susceptible to bacterial infections, such as *Edwardsiella ictaluri,* because trematodes affect the fish's immune system, which decreases their capacity to respond to infection and decreases catfish feed intake (Labrie et al., 2004; Gunn

et al., 2023; Wise et al., 2021). Therefore, controlling trematodes in catfish aquaculture ponds to promote fish growth and overall fish health is an important priority for commercial catfish producers.

1.2. Trematode management and treatment strategies

The life cycle of trematodes involves different stages and host organisms. Therefore, the treatment of trematodes needs to consider their life cycle. Treating their first host organism, the planorbid snail, to break the trematode life cycle has been suggested, and at one point, it was the only host organism appropriate to target (Mischke et al., 2005). The marsh ramshorn snail (*Planorbella trivolvis*) is considered the most common first host organism of trematodes (Mischke et al., 2005). Different chemicals, such as lime, copper sulfate, and organic pesticides, have been used to control these snails (Mischke et al., 2005). Some organic chemicals can control snails more effectively, but potential effects on catfish and other aquatic organisms in aquaculture pond systems are of concern (Mischke et al., 2005). In addition, organic chemicals are usually expensive and are not a preferred option for farmers. As a result, copper sulfate pentahydrate (hereafter called copper (Cu)) has been widely used for treating marsh ramshorn snails in commercial catfish ponds as well as other planorbid snails and non-targeted aquatic species (Mitchell & Hobbs, 2003; Wise et al., 2006). Currently, farms use 0.64 to 1.27 mg/L for whole pond exposures (Wise et al., 2006), 150 g of Cu in a 2-m swath every 10m (Mitchell, 2002; Mitchell & Hobbs, 2003), or four weekly whole pond exposure doses of <0.38 mg/L Cu (Mischke et al., 2021) However, the drawback of using Cu is that it can accumulate in the system over time, particularly in the sediments of static catfish ponds, because it Cu is not degraded in the environment like organic chemicals. Therefore, determining optimal Cu doses for treating snails to reduce treatment costs and Cu load in catfish ponds is important for farmers. To

accomplish this goal, understanding the mechanisms of Cu toxicity and factors influencing Cu bioavailability and toxicity to marsh ramshorn snails is necessary.

1.3. Copper bioavailability and toxicity to aquatic organisms and the influence of water quality parameters

Copper toxicity in aquatic organisms has been studied extensively. In the aquatic environment, Cu exists in various forms (also called species), such as Cu^{2+}, $Cu(OH)_2$, $CuCO_3$, $CuHCO_3^+$, $CuSO_4$, etc. Among these forms, Cu^{2+} is believed to be the most bioavailable and able to enter the organism to cause a toxic effect (Allen et al., 1980; Winner, 1985; Paquin et al., 2002). Copper has been reported to cause toxic effects to aquatic organisms by disrupting the gills' ability to regulate ions and oxygen, which can lead to a variety of physiological and biochemical effects, such as imbalanced osmotic pressure due to a reduction in ion uptake, swimming and feeding patterns, and eventual death (Wood, 2001). Additionally, Cu can cause a reduction in hemolymph osmolality of snails (Brix et al. 2011), which is attributed to the competition of Cu^{2+} with other osmotic ions (i.e., sodium) at the cell membrane, such as the gill membrane of fish (Wood, 2001). Brix et al. (2011) reported that Cu acts similarly in snail gills, disrupting their ability to conduct ion flow and other processes. This competition between Cu^{2+} and osmotic ions impairs the sodium-potassium pump, causes cellular disruptions, and eventually leads to cell death (Wood, 2001; Grosell et al., 2002; Brix et al., 2011). Other research found that calcium ion (Ca^{2+}) uptake was inhibited by Cu^{2+} in snails, resulting in reduced growth and shell formation (Brix et al. 2011; Gao et al. 2017). Additionally, Brix et al. (2011) reported that Cu inhibited proteins in the apical membrane of snails that formed ion channels. As a result, the carbonic anhydrase system contributed to this phenomenon. This component of the snail's mantle hydrates carbon dioxide (CO_2) to form bicarbonate (HCO_3^-), which is used for shell

growth and strength (Brix et al., 2011). Because calcium uptake was inhibited, the eggs' shells were not properly formed, resulting in shorter shell length (Gao et al., 2017). Gao et al. (2017) further showed that Cu can affect embryonic development in snails, including the formation of lesions, hemorrhaging in larvae, abnormal eyes, and even polynuclear egg capsules.

These effects of Cu to aquatic organisms are influenced by water quality. It has been reported that Cu is more toxic when water has lower hardness, pH, and natural organic matter (Taylor et al., 2004; Klinck et al., 2007; Bourret et al., 2008; Gauthier et al., 2009; Pierron et al., 2009). Salinity can also affect the bioavailability and toxicity of Cu (Grosell et al., 2007). Water with higher pH and higher levels of natural organic matter will promote more Cu complexation with anions and organic matter. As a result, less Cu will remain in the free ionic form (Cu^{2+}). Therefore, increasing water pH and the concentration of natural organic matter will decrease Cu bioavailability and toxicity. As described above, Cu^{2+} has been shown to compete with ions at ion channels in fish gills, including Na^{+}, Ca^{2+}, Mg^{2+}. Therefore, in water with high hardness levels, greater competition between Ca^{2+}, Mg^{2+} with Cu^{2+} will occur, leading to less Cu accumulation and reduced toxicity. Considering the influence of water quality on Cu toxicity, Paquin et al. (2002) developed the Biotic Ligand Model (BLM) to explain the relationship between water quality and Cu toxicity to aquatic organisms.

The BLM includes two components. The chemistry component describes chemical reactions of Cu with anions and natural organic matter in water to form different Cu forms (species). The physiology component describes the interaction of Cu and organisms and how they respond to Cu exposure.

The BLM was calibrated and validated with metal toxicity data for fish and other aquatic organisms, such as Cu and *Ceriodaphnia dubia* and *Pimephales promelas* (Di Toro et al., 2001;

Santore, 2001), Ni and *Ceriodaphnia dubia* (Santore et al., 2021), Zn and *Oncorhynchus mykiss* (Santore et al., 2002). For the chemistry component, as pH increases, more hydroxide ions are available in the water to interact with metals and form metal hydroxides such as MOH^-, $M(OH)_2$, etc. (Boyd 2020). Therefore, at a lower pH, more Cu^{2+} will form, which is expected to cause more toxic effects to organisms than in a higher pH environment. Recent studies with the Florida apple snail (*Pomacea, paludosa*) found a similar influence of pH on Cu and Zn toxicity to the Florida apple snail (Rogevich et al., 2008; Hoang and Tong 2015). Similarly, DOC, which is abundant and exists as fulvic and humic acids in the natural environment, can complex with Cu, reducing Cu bioavailability and toxicity to aquatic organisms. The effect of hardness on metal toxicity to snails is not consistent. For example, Hoang and Tong (2015) found a strong influence of hardness on Zn toxicity to Florida apple snails, but Rogevich et al. (2008) did not find such an effect for Cu and the same snail species. These findings suggest that the influence of hardness on metal toxicity to snails is metal-and species-specific.

When Cu inhibits ion transport across the cell membrane, organisms can lose important ions, such as Na^+, Ca^{2+}, and Mg^{2+}. As a result, aquatic organisms, especially fish, have been reported to lose osmotic pressure, which can cause acute toxicity (Grosell et al., 2002; Grosell et al., 2007). In addition, Cu in aquatic organisms has been reported to interfere with biological processes and cause molecular effects, including alterations in enzyme activities (i.e., Na/K ATPase), oxidative stress, and mitochondrial function (Brix et al., 2022). These physiological effects of metals represent the physiological component of the BLM. Overall, the BLM describes the toxicity of metals to aquatic organisms, accounting for water quality. Mathematically, the toxicity of metals can be described as a function of water quality parameters. Hoang et al. (2004) described the influence of water quality on nickel toxicity to the fathead minnow through a

multiple linear regression model that involved alkalinity, hardness, DOC, and organism age. The model parameters are presented in Figure 1.1. The model was used to predict nickel toxicity to fathead minnows. The prediction was comparable to the toxicity prediction by the BLM (Figure 1.1).

	Coefficients[a]				
	Unstandardized		Standardized		
Model	B	SE	β	*t*	*p*
Intercept	−0.642	0.296		−2.171	0.035
Fish age	0.270	0.012	0.859	23.035	0.000
Alkalinity	0.005	0.001	0.168	3.589	0.001
Hardness	0.018	0.005	0.166	3.576	0.001
DOC	0.138	0.047	0.110	2.927	0.005

[a] The dependent variable was the 96-h median lethal concentration.

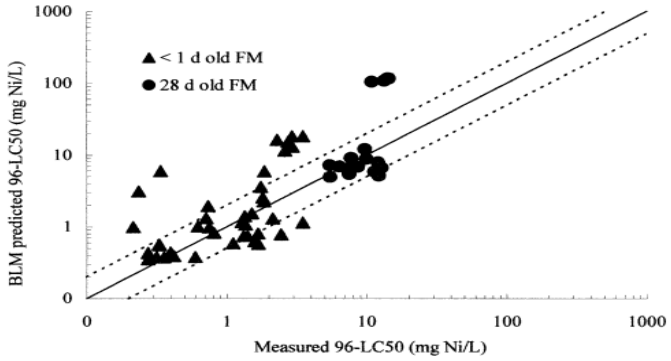

Figure 1.1: Multilinear regression model using the Biotic Ligand Model (BLM) to estimate nickel toxicity to the fathead minnow.

With the Florida apple snail, a multiple linear regression model was also developed to predict Cu toxicity to the Florida apple snail based on pH, DOC, and organism age, but not hardness (LC50 = 0.540 + 0.008(age) + 0.024(DOC) + 0.120(pH)) (Rogevich et al., 2008). Other research by Hoang and Tong (2015) found that hardness, pH, and DOC strongly influenced zinc

toxicity to the Florida apple snail. These results indicate that although water quality influences metal toxicity to aquatic organisms, this influence is metal-and organism-specific.

1.4. Treatment of marsh ramshorn snails using copper and the need for taking water quality into consideration

Concerning *P. trivolvis*, only a few studies on Cu toxicity to have been conducted. Results highlighted the influence of temperature on Cu toxicity to *P. trivolvis* (Mischke et al., 2005; Mischke et al., 2021). While the influence of water quality on metal toxicity to snails is metal-and organism-specific, research on the influence of water quality on Cu toxicity to *P. trivolvis* has not been conducted. Commercial aquaculture systems can have pH values of 5 to 6 in the morning and up to 9 in the afternoon due to the photosynthesis and respiration reactions of phytoplankton (Boyd 2020). A wide range of hardness (86 to 284 mg/L as $CaCO_3$) and DOC concentrations (1.8 to 31.8 mg/L) have been reported in aquaculture ponds (Mitchell et al., 2002; Mitchell et al., 2007; Mischke et al., 2021; Gladfelter et al., 2024). These variations in water quality in aquaculture pond systems will greatly affect Cu bioavailability and toxicity to aquatic organisms in the systems. The lack of understanding of the influence of water quality on Cu toxicity to *P. trivolvis* prevents commercial catfish farmers from determining appropriate Cu dosages to treat the snail. This can lead to overdosing, which will result in increased treatment costs and Cu load into the environment. Underdosing will result in less treatment efficacy. In addition, due to Cu reactions with abiotic and biotic components of aquatic systems, Cu availability can vary depending on the water composition. An aquatic system with a higher abundance of phytoplankton and solid particles would absorb more Cu, resulting in shorter residence time of Cu in the water column than other systems that have a lower abundance of water components. Therefore, it is important to conduct research to understand how exposure

time and water quality influence Cu toxicity to *P. trivolvis* and to apply these findings to calculate appropriate Cu doses for snail treatment.

1.5. Research aims and objectives

The main goal of this research is to help commercial catfish producers determine appropriate Cu doses to treat *P. trivolvis* in their pond systems based on the pond's water quality to increase treatment efficiency and reduce treatment costs and Cu load into their commercial ponds. To achieve this goal, this research focused on two important objectives. The first objective was to determine the influences of exposure time and water quality parameters, including hardness, pH, and DOC on Cu toxicity to *P trivolvis*. The toxicity is anticipated to be described as a function of hardness, pH, DOC, and exposure time. The second objective was to understand the relative sensitivity of snails to Cu at different life stages and the potential Cu tolerance of snails due to repeated Cu applications in aquaculture systems over time by comparing the toxicity of Cu to snails at different ages, as well as field-collected and laboratory-grown snails.

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Chapter 2

Copper toxicity to the marsh ramshorn snail as a function of water quality and exposure time

2.1. Abstract

The marsh ramshorn snail, *Planorbella trivolvis*, is an intermediate host of trematodes such as *Bolbophorus damnificus*, which is harmful to channel catfish (*Ictalurus punctatus*). To reduce harmful effect of trematodes during catfish aquaculture operations, methods are being sought to interrupt the *B. damnificus* life cycle by reducing marsh ramshorn snail populations in aquaculture ponds. Copper (Cu) is an effective molluscicide used to treat snails. While Cu toxicity is influenced by water quality parameters, such as hardness, pH, alkalinity, and dissolved organic matter, our understanding of how these parameters affect Cu toxicity to *P. trivolvis* is unknown. This study aims to investigate the effects of hardness, pH, and dissolved organic carbon (DOC) on Cu toxicity to *P. trivolvis*, and to develop multiple linear regression models to predict Cu toxicity to *P. trivolvis* based on water quality parameters and exposure time. The standard 96-h toxicity test was used to assess the toxicity of Cu to the snail. The results showed that hardness, pH, and DOC strongly influenced Cu toxicity. The 96-h LC50 increased from 12 µg/L Cu to 41 µg/L Cu when hardness was increased from 40 to 165 mg/L as $CaCO_3$. When increasing pH from 6.2 to 8.1, the 96-h LC50 increased from 4 µg/L to 14 µg/L Cu. The 96-h LC50 increased from 23 to 105 µg/L Cu when DOC was increased from 0 to 20 mg/L. Overall, the 96-h LC50 was a function of pH, hardness, DOC, and exposure time. The model predictions explained 70-94% of the experimental data, depending on exposure time. The models can be used to calculate appropriate Cu doses for treating *P. trivolvis* to minimize Cu load into aquaculture pond systems and reduce treatment costs for commercial catfish producers.

2.2. Introduction

The marsh ramshorn snail (*Planorbella trivolvis*), a planorbid snail, is the most understood primary host of the trematode parasite which is transmitted to the channel catfish (*Ictalurus punctatus*) (Mischke et al., 2005). These snails transmit trematodes such as *Bolbophorus damnificus, Bursacetabulus pelecanus, Austrodiplostomum compactum, and Hysteromorpha cf. triloba* to the channel catfish and are of concern to catfish farmers (Overstreet and Curran 2004; Mischke et al., 2021). These trematodes cause economic harm to catfish producers by increasing production time, increasing the cost of fish management, and killing catfish. Therefore, the primary focus has been on eliminating the primary host of the parasites, the planorbid snails (Overstreet et al., 2002; Mischke et al., 2005; Yost et al., 2008, 2009). Although the focus is on planorbid snails, our understanding of the sensitivity of *P. trivolvis* to chemicals and the factors influencing its sensitivity to chemicals is limited.

Copper has been reported to be a sensitive chemical to snails and has been used to treat invasive snails (Mischke et al., 2005; Wise et al., 2006). Water quality has been shown to influence Cu bioavailability and toxicity to snails, with this effect being species-specific (Mischke et al., 2005; Rogevich et al, 2008; Mischke et al., 2021). Therefore, using Cu to treat snails without accounting for water quality effects on Cu toxicity would result in either overdosing, which will increase treatment costs and Cu load into the system, or underdosing which lowers treatment efficacy. According to Mischke et al. (2005), increasing temperature increased Cu toxicity to *P. trivolvis*. Additional research conducted by Straus et al. (2026), did not identify a relationship between increasing alkalinity/hardness/pH and Cu toxicity to *P. trivolvis*. However, there is no other information on how these separate water quality parameters may influence Cu toxicity to *P. trivolvis*. The goal of this research was to determine the influence

of water quality parameters, including pH, hardness, and dissolved organic carbon (DOC) on Cu bioavailability and toxicity to *P. trivolvis*, and based on the results, to develop a multiple linear regression model to predict Cu toxicity to *P. trivolvis* based on water quality. The model can be used to calculate appropriate Cu doses for treating *P. trivolvis* under specific water quality parameters, minimize harm to non-target organisms in the system, and minimize treatment costs.

2.3. Materials and methods

2.3.1. Collection and culturing of *P. trivolvis*

Adult organisms were collected from commercial catfish farms in Alabama. Collected snails were transported to Auburn University in sealed watertight containers supplied with aerated water from the same ponds where snails were collected. Aeration was supplied via portable aerator and an air stone. These snails were used to lay eggs for the experiments. Organisms were maintained in moderately hard water using a water recycling system equipped with a UV light and a sediment filtration system in the Ecotoxicology and Risk Assessment Laboratory of Auburn University. Moderately hard water was prepared by dissolving equivalent amounts of salts ($CaSO_4 \cdot 2H_2O$, $MgSO_4$, $NaHCO_3$) in reverse osmosis (RO) water using the US EPA Method for Acute Toxicity Test Method with aquatic organisms (US EPA 2002). The water quality parameters for the culture were within the target values of moderately hard water with an average pH of 8.02 ± 0.19, conductivity of 290.36 ± 43.61 µS/cm, hardness of 95.42 ± 28.79 mg/L as $CaCO_3$, and alkalinity of 62.89 ± 11.64 mg/L as $CaCO_3$. The average temperature and dissolved oxygen concentration were 25.5 ± 1.1 ºC and 7.45 ± 0.40 mg/L, respectively.

The adult snails laid eggs on cut PVC pipes and the walls of 8L glass aquaria. Eggs were collected from containers and PVC pipes and transferred to a beaker filled with moderately hard

water with aeration. The eggs' ages were documented, as were their ages at hatching and during growth. These hatched snails were used for experiments, which were set to be one-week-old snails. Because previous research showed that age was an important factor in Cu toxicity to snails, controlling age is an important factor to consider (Rogevich et al., 2008). The snails grew in moderately hard water with the conditions above. Adult snails and hatched larval snails were fed frozen lettuce/spinach regularly.

2.3.2. Experimental design and acute toxicity testing

To understand the influence of water quality on Cu toxicity to *P. trivolvis*, the US EPA 96-h acute static renewal acute toxicity test method (US EPA 2002) was used to measure acute toxicity of Cu to *P. trivolvis* under different water quality parameters. The major measurement endpoint was mortality, and the data were used to calculate lethal concentrations (LCs).

To determine the influence of hardness on Cu toxicity to the snail, five acute toxicity tests were conducted at hardness of 40 mg/L as $CaCO_3$ (soft water), 93 mg/L as $CaCO_3$ (moderately hard water), 165 mg/L as $CaCO_3$ (hard water), 241 mg/L as $CaCO_3$ (harder water), and 321 mg/L as $CaCO_3$ (very hard water). Other water quality parameters, such as pH and DOC for the hardness series tests were kept constant at 8.0 and ~1 mg/L (background DOC in moderately hard water), respectively (Figure 2.1). To assess the pH effect, four acute toxicity tests were conducted at pH 6, 7, 8, and 9. Hardness and DOC of the pH effect test series were kept constant at 100 mg/L as CaCO3 and 1 mg/L, respectively (Figure 2.1). For DOC effect, four acute toxicity tests were conducted with DOC concentrations of 1 mg/L (based on DOC in moderately hard water), 5, 10, and 20 mg/L. Hardness and pH of the DOC effect tests were kept constant at 100 mg/L as $CaCO_3$ and 8.0, respectively (Figure 2.1). Other tests including soft water with a DOC of 5 mg/L, moderately hard water with a DOC of 10 mg/L and pH of 6, 7, and 8, and hard

water with a DOC of 0, 5, and 10 mg/L were conducted for model validation purposes. The water quality chosen for these experiments corresponds to that of aquaculture ponds in the Southeastern USA (Silapajarn et al., 2004; Boyd 2020; Gladfelter et al., 2024; McDonald et al., 2025).

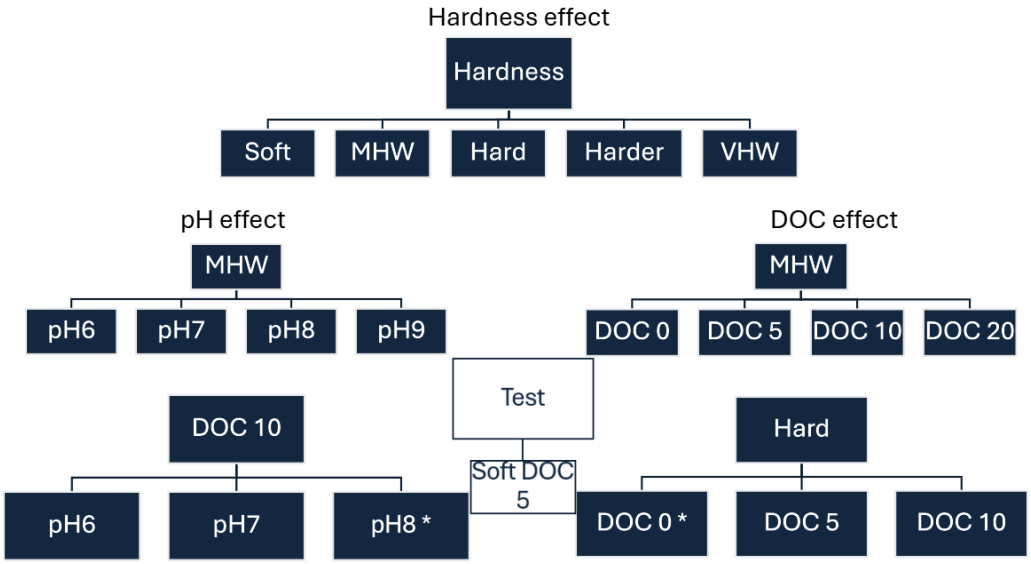

Figure 2.1: Experimental design to study the influence of water quality on copper toxicity to *P. trivolvis* (The base water for all experiments was moderately hard, unless it was specified to be hard water or soft water. * denotes that the experiment was from another effect, for example, the DOC 10 pH 8 experiment result was already calculated in the DOC effect under the MHW, DOC10).

Tests consisted of one control group and five (minimum requirement of the EPA Test Method) or six treatment groups, depending on organism availability, in triplicate, with ten one-week-old snails in each replicate, with the first treatment group receiving the lowest Cu concentration and the last treatment receiving the highest. The Cu concentration range varied across tests, depending on water quality. More details of Cu concentrations for each test are

presented in Table 1 of the Appendix. Copper concentrations were prepared by diluting an aliquot of Cu stock with test water. Copper stock concentration was 1000 mg/L and prepared by dissolving $CuSO_4 \cdot 5H_2O$ in MilliQ water and acidifying to pH 2. Tests for hardness and DOC effects were conducted in 40 mL polypropylene beakers containing 30 mL test water, while the ph effect series was conducted in 600 mL beakers containing 500 mL test water. A higher water volume was used in the pH effect series tests to help maintain pH during the experiment. The pH of the water was controlled using 0.4 M H_2SO_4 solution or 0.1 N NaOH solution. Suwanee NOM II RO Isolation (2R101N, Suwanee, Georgia) was used to adjust the DOC concentration in the test water. One-week-old snails had a diameter and height of approximately 600 µm. The measurement was conducted on an S18 Brightfield Stage Micrometer (Electron Microscopy Sciences, Hatfield, PA). Test containers were randomly positioned in a testing room at 25 ± 1 ºC under a photoperiod of 16:8 h light: dark. Snails were analyzed daily under a Nikon DS-Fi3 Dissecting Microscope (Tokyo, Japan) to determine survival during experiments. Mortality was determined by assessing a loss of their hemolymph color and a lack of internal and external movement under the Nikon Ds-Fi3 Dissecting Microscope.

Water samples were collected from test chambers for analysis of total and dissolved Cu, DOC, and cations and anions. For dissolved Cu and anions (sulfate, nitrate, fluoride, chloride, and bromide), samples were filtered through a 0.45 µm filter into 15 mL polypropylene centrifuge tubes. Samples for DOC were filtered into 30 mL glass amber bottles. For total Cu, samples were collected directly from the test chambers into 15 mL polypropylene centrifuge tubes. Water samples for total and dissolved Cu analysis were acidified to a pH of 2 by adding 1 drop of concentrated nitric acid.

2.3.3. Water chemistry analysis

Water quality was measured daily, including pH, salinity, conductivity, temperature, and dissolved oxygen (DO). The pH was measured using a Mettler Toledo pH Probe (Seven2Go pH/mV Meter S2-Light kit, Schwerzenbach, Switzerland), and the other variables were measured using a YSI Pro 2030 (Yellow Springs, Ohio, USA). Hardness was measured by titration with 0.01M EDTA as the titrant (Eaton & Franson, 2005). Alkalinity was measured by titrating with 0.02N H_2SO_4 (Eaton & Franson, 2005). A NexION 2000 Perkin Elmer Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) (Shelton, CT, USA) was used to analyze total and dissolved Cu and mineral elements in the test water following standard methods (Eaton & Franson 2005). The multi-N/C 3300 series TOC/TNb Analyzer (Jena, Germany) was used to analyze DOC in the samples. Anions were analyzed using a 930 Compact IC Flex with a 919 IC Autosampler plus (IC) (Herisau, Switzerland).

2.3.4. Analysis of effect data

Survival data and measured dissolved Cu concentrations were used to calculate LC values (LC50, LC25, LC10) using the CETIS software (Tidepool Scientific Software,2023. CETIS. V2.1.5.4x64). The 24-h, 48-h, 72-h, and 96-h LC values were calculated. Multiple linear regression models describing LC50 as a function of hardness, pH, DOC, and exposure time were developed using the Data Analysis ToolPak of Excel v. 2602. Concentration of Cu^{2+} in each treatment was calculated based on concentrations of measured Cu, mineral elements, and anions using Visual MINTEQ 4.0 (Appendix, Table 1), and LC50s in µg/L Cu^{2+} were also calculated to determine the level of Cu^{2+} that causes a lethal effect on 50% of exposed snails.

2.4. Results

2.4.1. Water quality and measured copper concentrations

Results of the water quality of all toxicity tests are presented in Table 1. Overall, water quality was within 15% of the designed values. For the hardness tests, the pH ranged from 8.04 to 8.17, which is within the moderately hard water pH range (8.00-8.30). Similarly, alkalinity ranged from 61 to 94 mg/L as $CaCO_3$. The temperature ranged from 24.7 to 25.3°C, within the designed temperature of 25 ± 1°C. Dissolved oxygen concentration ranged from 7.0 to 7.9 mg/L. The conductivity range was 217- 452 µS/cm. Hardness increased from 40 to 321 mg/L as $CaCO_3$. Concentration of DOC at background levels ranged from 0.49 to 2.26 mg/L. Conductivity and alkalinity increased when the hardness increased from soft water to very hard water.

For DOC effect tests, the measured temperature and dissolved oxygen ranged from 25.1 to 25.3°C and 7.0 to 7.9 mg/L, respectively. The hardness ranged from 79 to 101 mg/L as $CaCO_3$, which is within the hardness range of moderately hard water. Conductivity ranged from 272 to 298 µS/cm, consistent with moderately hard water, and alkalinity ranged from 61 to 85 mg/L as $CaCO_3$. Humic acid decreased the pH as DOC increased from 0.11 to 22.49 mg/L, with pH values ranging from 7.82 to 8.08.

Similarly, for the pH effect tests, the base water from moderately hard water had a hardness range of 103 to 104 mg/L as $CaCO_3$, an alkalinity of 75 to 100 mg/L as $CaCO_3$, and a conductivity of 289 to 299 µS/cm. The temperature was kept lower at 23.1 to 23.4°C to help maintain a stable pH during the experiment. The DOC ranged from 1.04 to 1.68 mg/L. The pH values ranged from 6.20 to 8.96.

The model validation tests were conducted with water quality different from that in the hardness, pH, and DOC effects tests. The measured temperature was 23.8 to 25.2°C, pH was 5.93 to 8.02, DO concentrations was 7.1 to 7.8 mg/L, conductivity was 285 to 332 µS/cm, hardness at 39 to 103 mg/L as $CaCO_3$, alkalinity at 63 to 65 mg/L as $CaCO_3$, and DOC at 8.34 to 14.37 mg/L. Most measured Cu concentrations were within 80% of the nominal concentrations, but some values varied more, as observed in other studies (Limouni et al., 2026). A likely explanation is that lower concentrations are more susceptible to interferences such as the formation of polyatomic ions (Fialho et al., 2011). The detailed measured Cu concentrations for each test treatment are presented in Table 1 of the Appendix.

Table 2.1: Water quality of each experiment (Data are mean ± standard deviation)

Type of Experimental Test	Test	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	DOC (mg/L)
Hardness Effect	1	25.0 ± 0.2	8.04 ± 0.04	7.3 ± 0.1	217.2 ± 4.1	40 ± 0	61 ± 2	0.49 ±1.10
	2	25.3 ± 0.2	8.06 ± 0.03	7.9 ± 0.2	287.1 ± 3.6	79 ± 3	61 ± 2	1.85 ±1.97
	3	25.2 ± 0.1	8.08 ± 0.08	7.5 ± 0.2	298.5 ± 11.5	93 ± 2	85 ± 3	0.11 ± 0.23
	4	25.3 ± 0.1	8.17 ± 0.07	7.2 ± 0.1	373.3 ± 8.7	165 ± 6	88 ± 4	1.47 ±2.62
	5*	25.0 ± 0.2	8.07 ± 0.02	7.8 ± 0.0	422.3 ± 4.9	241 ± 2	94 ± 2	2.04 ± 1.47
	6*	25.2 ± 0.2	8.04 ± 0.03	7.0 ± 0.0	452.2 ± 4.0	321 ± 7	86 ± 3	2.26 ± 2.37
DOC Effect	2	25.3 ± 0.2	8.06 ± 0.03	7.9 ± 0.2	287.1 ± 3.6	79 ± 3	61 ± 2	1.85 ±1.97
	3	25.2 ± 0.1	8.08 ± 0.08	7.5 ± 0.2	298.5 ± 11.5	93 ± 2	85 ± 3	0.11 ± 0.23
	7	25.2 ± 0.1	7.92 ± 0.03	7.0 ± 0.1	272.1 ± 11.7	100 ± 3	74 ± 7	8.97 ± 4.18
	8	25.1 ± 0.1	7.91 ± 0.03	7.0 ± 0.1	283.5 ± 6.8	101 ± 2	78 ± 2	14.59 ± 5.51
	9	25.1 ± 0.1	7.82 ± 0.03	7.1 ± 0.1	290.9 ± 4.1	100 ± 3	80 ± 3	22.49 ± 3.44
pH Effect	10	23.1 ± 0.1	6.20 ± 0.17	7.3 ± 0.1	289.2 ± 8.7	104 ± 3	80 ± 4	1.04 ± 1.03
	11	23.5 ± 0.1	6.76 ± 0.10	7.4 ± 0.2	299.1 ± 5.7	103 ± 3	75 ± 3	1.87 ±1.10
	12	23.3 ± 0.1	8.07 ± 0.05	7.1 ± 0.1	289.3 ± 4.4	103 ± 3	77 ± 6	1.12 ±1.07
	13*	23.4 ± 0.4	8.96 ± 0.14	7.2 ± 0.1	290.6 ± 5.5	103 ± 3	100 ± 31	1.68 ± 1.54
Model Validation	14*	25.2 ± 0.1	7.76 ± 0.04	7.1 ± 0.0	321.2 ± 23.3	103 ± 2	63 ± 2	8.55 ± 3.55
	15*	25.2 ± 0.1	7.67 ± 0.03	7.2 ± 0.1	332.8 ± 28.5	103 ± 2	64 ± 0	14.37 ± 4.03
	16	25.1 ± 0.1	5.93 ± 0.11	7.8 ± 0.1	291.7 ± 14.4	100 ± 0	65 ± 2	11.68 ± 1.41
	17	23.8 ± 0.2	6.81 ± 0.04	7.8 ± 0.0	285.8 ± 12.6	97 ± 2	63 ± 2	12.56 ± 1.94
	18	24.9 ± 0.2	8.02 ± 0.04	7.7 ± 0.1	285.0 ± 5.8	39 ± 2	63 ± 2	8.34 ± 3.51

* denotes that data was insufficient for the model and did not fit into the models. Test 13 (pH 9) showed increased mortality in the control, preventing accurate implementation in the model. Tests 14 (hard water with 5 mg/L DOC) and 15 (hard water with 10 mg/L DOC) experienced miscalculations in their hardness measurements, preventing implementation for model validation.

2.4.2. Lethal copper concentrations for *P. trivolvis*

Mortality and measured dissolved and ionic Cu concentrations of all 18 toxicology tests were used to calculate LC50s. The response data allowed calculation of LC50 values for 24, 48, 72, and 96-h, with 95% lower and upper confidence levels (LCL, UCL). LC50 values are presented in Table 2.2.

In general, as hardness increased, Cu toxicity decreased, as shown by increasing LC50 values (Figure 2.1, Appendix). For example, when hardness increased from 40 (Test 1) to 165 (Test 4) mg/L as $CaCO_3$, the 24-h LC50 values increased from 46.94 to 171.42 µg/L Cu. Additionally, as exposure time increased from 24 to 96 h, the LC50 values decreased from 46.94 to 11.67 µg/L Cu (Test 1, Table 2.2). Similar results were found for 48-h, 72-h, and 96-h LC50s. The results indicated that hardness strongly influenced Cu toxicity to *P. trivolvis*. Ionic copper was also considered in this study. The 24-h LC50 for ionic copper ranged from 0.15 to 8.16 µg/L Cu^{2+} and the 96-h LC50 was 0.007 to 3.88 µg/L Cu^{2+} (Table 2.2).

Similarly, DOC also had a strong influence on Cu toxicity to the snails (Figure 2, Appendix). When the DOC concentrations increased from 0.11 (Test 3) to 22.49 (Test 9) mg/L, the 24-h LC50 values increased from 40.62 to 230.09 µg/L Cu. This effect is also time-dependent, with longer exposure time resulting in a decrease in the LC50 value. For example, at a DOC concentration of 8.97 mg/L, the LC50 decreased from 114.84 to 68.01 µg/L Cu when exposure time increased from 24 to 96 h (Test 7, Table 2.2). The 24-h LC50 for ionic copper ranged from 0.03 to 7.21 µg/L Cu^{2+}, and the 96-h LC50 was 0.007 to 3.88 µg/L Cu^{2+} (Table 2.2).

The influence of pH on Cu toxicity to the snails was expected, with toxicity decreasing as pH increased (Figure 3, Appendix). For example, as the pH increased from 6.20 (Test 10) to pH

8.07 (Test 12) the 24-h LC50 value increased from 44.13 to 111.5 µg/L Cu. Additionally, toxicity decreased over time. For example, when the pH was 8.07, the LC50 decreased from 111.5 to 30.55 when the exposure time increased from 24 to 96 h (Test, 12, Table 2.2). Overall, the influence of pH showed that as the pH increased from 6.20 to 8.07, Cu toxicity decreased. The results of test 13 at pH 9 were unexpected, as they showed an increase in toxicity rather than a decrease (Appendix, Figure 2.3). This might be due to the extreme pH condition that can affect snails. The LC50 for ionic copper ranged from 0.12 to 16.91 µg/L (Table 2.2). The 24-h LC50 for ionic copper ranged from 5.95 to 16.92 µg/L Cu^{2+}, and the 96-h LC50 was 0.18 to 0.52 µg/L Cu^{2+} (Table 2.2).

Table 2.2: Lethal Cu concentrations for *P. trivolvis* (data in parentheses are 95% CI).

Type of Experiment	Exp. number	Exposure time (h)	LC50 (µg/L Cu)	LC50 (µg/L Cu^{2+})
	1	24	46.94 (41.05, 54.73)	2.51 (1.86, 3.45)
	1	48	24.9 (21.13, 28.73)	0.64 (0.46, 0.86)
	1	72	17.6 (13.65, 23.76)	0.32 (0.22, 0.95)
	1	96	11.67 (9.77, 14.01)	0.13 (0.09, 0.19)
	2	24	56.09 (47.09, 66.31)	0.15 (0.10, 0.24)
	2	48	26.18 (6.56, 39.56)	0.03 (NA, 0.072)
	2	72	16.05 (7.31, 47.72)	0.008 (0.003, 0.087)
	2	96	14.18 (5.86, 46.06)	0.007 (0.002, 0.076)
	3	24	40.62 (34.78, 47.98)	7.21 (6.10, 8.64)
	3	48	26.4 (21.33, 39.17)	4.56 (3.65, 6.87)
	3	72	20.84 (17.81, 24.38)	3.54, (2.99, 4.18)
Hardness	3	96	22.6 (21.06, 23.29)	3.88 (3.60, 4.00)
Effect	4	24	171.42 (142.71, 209.06)	8.16 (5.20, 13.21)
	4	48	100.39 (86.06, 115.73)	2.15 (1.48, 3.13)
	4	72	55.35 (48.07, 64.32)	0.52 (0.38, 0.72)
	4	96	41.37 (36.08, 47.95)	0.27 (0.20, 0.37)
	5*	24	140.76 (108.67, 178.09)	47.46 (32.69, 65.85)
	5*	48	23.6 (NA, NA)	3.74 (NA, NA)

	5*	72	22.43 (16.31, 24.88)	3.49 (2.12, 4.02)
	5*	96	17.66 (7.26, 21.56)	2.45 (0.19, 3.30)
	6*	24	109.45 (90.83, 127.37)	0.92 (0.61, 1.35)
	6*	48	65.76 (55.47, 78.18)	0.30 (0.21, 0.43)
	6*	72	55.47 (30.32, 86.91)	0.27 (NA, 0.63)
	6*	96	22.39 (15.71, 29.93)	0.0002 (7.57E-06, 0.001)
	2	24	56.09 (47.09, 66.31)	0.15 (0.10, 0.24)
	2	48	26.18 (6.56, 39.56)	0.03 (NA, 0.072)
	2	72	16.05 (7.31, 47.72)	0.008 (0.003, 0.087)
	2	96	14.18 (5.86, 46.06)	0.007 (0.002, 0.076)
	3	24	40.62 (34.78, 47.98)	7.21 (6.10, 8.64)
	3	48	26.4 (21.33, 39.17)	4.56 (3.65, 6.87)
	3	72	20.84 (17.81, 24.38)	3.54, (2.99, 4.18)
	3	96	22.6 (21.06, 23.29)	3.88 (3.60, 4.00)
	7	24	114.84 (99.32, 132.51)	0.03, (0.02, 0.04)
DOC	7	48	79.71 (66.64, 95.4)	0.014 (0.010, 0.02)
Effect	7	72	73.08 (67.31, 80.31)	0.012 (0.010, 0.014)
	7	96	68.01 (60.82, 72.89)	0.010 (0.008, 0.012)
	8	24	262 (130.06, 390.63)	0.09 (NA, 0.23)
	8	48	134.64 (116.13, 156.29)	0.014 (0.010, 0.021)
	8	72	100.01 (87.14, 114.98)	0.007 (0.005, 0.009)
	8	96	72.93 (63.54, 83.69)	0.003 (0.002, 0.004)
	9	24	230.09 (202.01, 258.04)	0.03 (0.02, 0.05)
	9	48	173.75 (75.25, 240.03)	0.02 (NA, 0.05)
	9	72	130.78 (113.40, 151.70)	0.008 (0.006, 0.011)
	9	96	105.22 (92.06, 121.57)	0.005 (0.004, 0.006)
	10	24	44.13 (37.80, 51.33)	16.92 (13.52, 20.96)
	10	48	13.85 (8.21, 19.42)	3.18 (1.52, 5.15)
	10	72	8.05 (NA, 17.96)	1.77 (NA, 4.91)
	10	96	3.93 (3.33, 4.92)	0.54 (0.43, 0.71)
	11	24	98.85 (80.26, 126.35)	12.98 (8.42, 21.63)
	11	48	26.9 (21.39, 31.92)	0.80 (0.48, 1.18)
	11	72	16.48 (11.45, 20.74)	0.34 (0.21, 0.45)
pH Effect	11	96	14.69 (10.72, 17.75)	0.29 (0.19, 0.37)
	12	24	111.5 (64.25, 158.75)	5.95 (NA, 13.87)

	12	48	39.6 (33.82, 45.97)	0.28 (0.19, 0.40)
	12	72	27.92 (24.26, 32.43)	0.12 (0.09, 0.17)
	12	96	30.55 (19.45, 35.18)	0.18 (0.04, 0.24)
	13*	24	41.67 (33.18, 50.96)	0.002 (0.001, 0.004)
	13*	48	18.52 (13.47, 22.83)	0.006 (0.004, 0.007)
	13*	72	17.01 (16.72, 17.66)	0.0004 (0.0001, 0.0006)
	13*	96	13.15 (11.58, 14.16)	0.00026 (0.00022, 0.00029)
Model Validation	14*	24	86.2 (75.06, 97.85)	0.027 (0.018, 0.038)
	14*	48	56.68 (54.23, 59.40)	0.011 (0.010, 0.013)
	14*	72	49.97 (30.57, 56.43)	0.009 (0.001, 0.0135)
	14*	96	33.34 (33.34, 33.34)	0.002 (0.002, 0.008)
	15*	24	68.56 (59.00, 81.95)	0.007 (0.006, 0.009)
	15*	48	57.93 (53.47, 63.51)	0.005 (0.005, 0.006)
	15*	72	55.81 (55.81, 55.81)	0.005 (0.005, 0.005)
	15*	96	40.16 (31.80, 51.39)	0.003 (0.001, 0.005)
	16	24	81.88 (65.11, 133.69)	1.05 (0.72, 2.87)
	16	48	38.99 (33.16, 45.82)	0.26 (0.20, 0.35)
	16	72	30.70 (27.09, 34.79)	0.17 (0.14, 0.21)
	16	96	13.75 (11.12, 16.50)	0.05 (0.04, 0.07)
	17	24	199.89 (174.85, 228.36)	1.00 (0.71, 1.37)
	17	48	95.36 (84.76, 109.48)	0.16 (0.13, 0.20)
	17	72	77.6 (30.14, 89.47)	0.06 (0.06, 0.06)
	17	96	64.42 (22.76, 89.14)	0.0605 (0.0602, 0.0608)
	18	24	98.63 (86.88, 111.57)	0.015 (0.011, 0.210)
	18	48	65.88 (57.17, 75.44)	0.005 (0.004, 0.008)
	18	72	53.52 (15.54, 72.53)	0.004 (NA, 0.008)
	18	96	24.01 (16.37, 32.15)	0.0008 (0.0004, 0.001)

* The data were insufficient for the multiple linear regression models, and NA indicates that the data were insufficient to calculate the 95% confidence intervals.

2.4.3. Multiple linear regression models

Multiple linear regression models were developed using water quality data (pH, DOC, and hardness data from Table 2.1) and LC50 data from Table 2.2. Tests 5 and 6 were toxicity

tests with higher hardness, but higher Cu toxicity was found instead of lower Cu toxicity compared to hard water. The experiment conducted at a pH 9 (Test 13) showed higher Cu toxicity rather than lower. Results from these tests were omitted from model development because they showed increased toxicity with increasing DOC rather than decreased toxicity. Overall, Cu toxicity (LC50) was a function of pH, DOC, and hardness. The models were developed for each exposure time and shown in Table 2.3. When exposure time was considered, the model included exposure time as a variable. The models had adjusted R^2 values of 0.702 to 0.937, explaining 70.2-93.7% of the variation in the data.

Table 2.3: Multiple linear regression models describing LC50 as a function of pH, DOC, and hardness.

Exposure Time (h)	Regression Model	Adjusted R^2
24	24-h LC50 = 26.57 × (pH) + 8.12 × (DOC) + 1.01 × (hardness) - 229.90	0.702
48	48-h LC50 = 24.88 × (pH) + 6.07 × (DOC) + 0.59 × (hardness) - 218.54	0.92
72	72-h LC50 = 17.33 × (pH) + 4.96 × (DOC) + 0.29 × (hardness) - 142.30	0.937
96	96-h LC50 = 17.03 × (pH) + 3.81 × (DOC) + 0.22 × (hardness) - 135.49	0.882
24-96	24-96-h LC50 = 21.45 × (pH) + 5.74 × (DOC) + 0.53 × (hardness) - 1.11 × (exposure time) - 115.06	0.78

The models were used to predict LC50s based on water quality plotted against the measured LC50 values (Figure 2.2). Overall, when exposure time was not included in the models, the LC50s were within the 95% confidence intervals of the model prediction. Results of the model validation tests showed that the data were within the 95% CI of the model prediction (orange circles). When exposure time was included as a variable, greater variation was observed in comparison between the predicted and measured LC50s, with more data falling outside the 95% CI range (Figure 2.3).

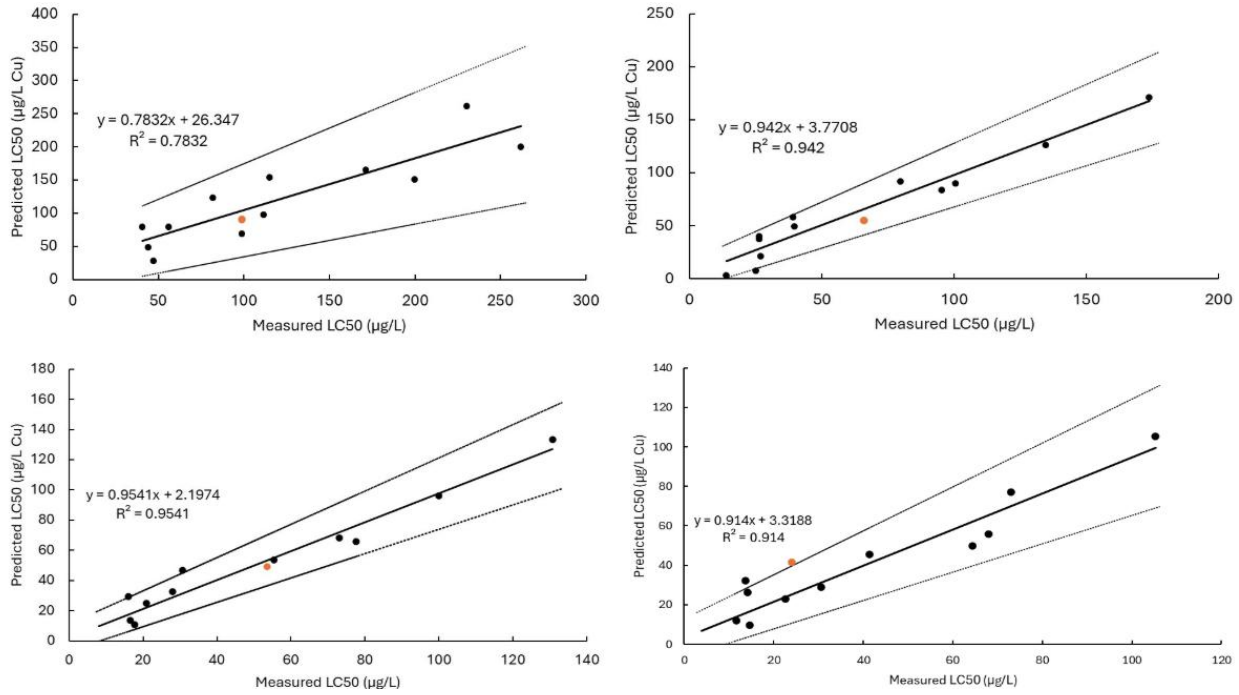

Figure 2.2: Predicted versus measured LC50s (top left: 24-h LC50 model; top right: 48-h LC50 model; bottom left: 72-h LC50 model; bottom right: 96-h LC50 model; black circles: model data; orange circles: model validation data; solid line: relationship between measured and predicted LC50s; dashed lines: 95% CI).

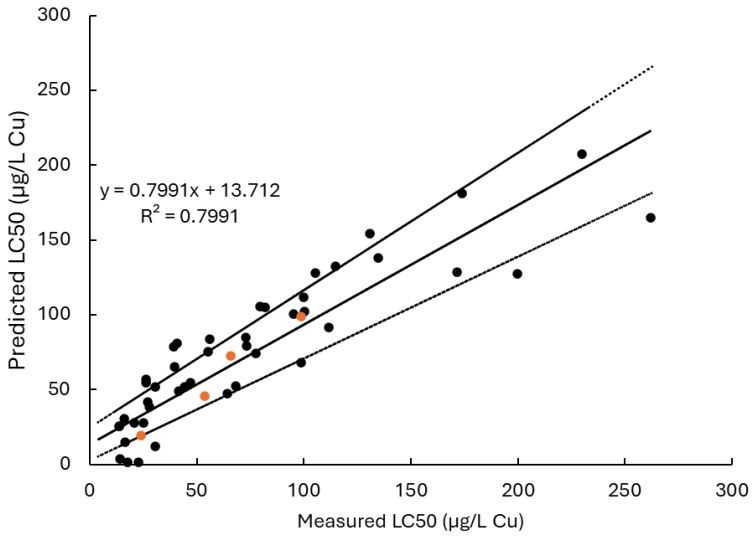

Figure 2.3: Predicted versus measured LC50 (black circles: model data; orange circles: model validation data; solid line: relationship between measured and predicted LC50s; dashed lines: 95% CI).

2.5. Discussion

2.5.1. Influence of hardness on copper toxicity

This present study showed that water hardness had a clear protective effect against Cu toxicity to *P. trivolvis*. As hardness increased, the LC50 values increased. The relationship between LC50 and hardness was strong, with R^2 values ranging from 0.7787 to 0.9959 for 24 to 96 h exposure (Figure 1, Appendix). This relationship was consistent with literature: as hardness increases, toxicity decreases, especially with fish and invertebrate species. In aquatic organisms, Cu^{2+} is known to compete with hardness cations such as Ca^{2+} and Mg^{2+} for binding sites in ion transport and osmoregulation (Winner 1985; Paquin et al., 2002). Increasing hardness increases the concentration of competing cations in the surrounding water, thereby reducing Cu^{2+} capacity to bind to the biotic ligand, making it non-bioavailable (Miller et al., 1980; Besser et al., 1996;

Yap et al., 2023). This observed decrease in toxicity with increasing hardness is consistent with previous studies on snails, which showed that hardness influences Cu toxicity (Brix et al., 2020). However, these positive effects of hardness on Cu toxicity to *P. trivolvis* appeared to be true for both hard and soft water. At higher hardness levels (Tests 5 and 6), deviation from the expected trend was observed, with increased toxicity compared to hard water (Test 4). Straus et al. (2026) observed a similar response to *P. trivolvis* when increasing their hardness values. These results suggested a threshold limitation at higher hardness levels. An explanation for these phenomena could be potentially Ca^{2+} or Mg^{2+} toxicity. At high concentrations, Ca^{2+} or Mg^{2+} has been reported to produce toxic effects to aquatic organisms (Rygało-Galewska et al., 2023; Brix et al., 2011, Brix et al. 2020). In aquaculture pond systems in the southern US, this high level of hardness rarely occurred (Silapajarn et al., 2004). However, it is important to understand the hardness range at which Cu toxicity was inhibited. Knowing the hardness range that positively affects Cu toxicity will help us determine when to apply the Cu bioavailability approach for predicting Cu toxicity based on hardness. Future research should examine how hardness ions can produce toxic effects to *P. trivolvis*.

2.5.2. Influence of DOC on copper toxicity

DOC had a clear influence on Cu toxicity to *P. trivolvis*. The relationship between LC50 and DOC was strong with R^2 values of 0.747-0.969 for 24 to 96 h of exposure (Figure 2.2, Appendix). These results were consistent with the literature, which indicates that higher DOC binds to Cu, making it less bioavailable (Rogevich et al., 2008). As DOC increased from approximately 1 to 22 mg/L, the 96-h LC50 value increased from 14.18 to 105.22 µg/L Cu, showing a seven-fold decrease in toxicity at 22 mg/L DOC. This influence was stronger than that observed for hardness, showing the importance of DOC in Cu bioavailability and toxicity.

Dissolved organic matter, typically humic substances, can bind to Cu and form organic complexes, decreasing the concentration of Cu^{2+} in solution. Because Cu^{2+} is considered the most toxic and bioavailable Cu species, a decrease in Cu^{2+} should decrease bioavailability and toxicity (Paquin et al., 2002; Boyd 2020). The observed trend of LC50 values increasing with increasing DOC is consistent with previous studies on freshwater snails and other aquatic organisms (Rogevich et al., 2008; Ng et al., 2011). Erickson et al., (1996), reported that at 5 mg/L of DOC, the 96-h LC50 increased 4-fold compared to 1 mg/L of DOC to the fathead minnow, while Rogevich et al., (2008) saw only a 2-fold increase at 15 mg/L of DOC compared to 1 mg/L of DOC to the Florida apple snail. The present study showed a 5-fold increase in the 96-h LC50 from ~1 mg/L DOC to 5 mg/L DOC. This study observed a 7-fold increase in the 24-h LC50 from 1 to 20 mg/L DOC, whereas Rogevich et al., (2008) observed a 5-fold increase at 30 mg/L DOC. These results highlight the importance of species dependency as well as time, as evident in this study. Aquaculture farms can have up to 30 mg/L of DOC in hypereutrophic ponds (Boyd, 1994, McDonald et al., 2024). Although this study considered up to 20 mg/L of DOC, our results highlight the importance of DOC in aquaculture ponds, for treating not just algae, but also trematodes by targeting *P. trivolvis*.

2.5.3. Influence of pH on copper toxicity

Water pH also significantly influenced Cu toxicity to *P. trivolvis*. The relationship between LC50 and pH was strong with R^2 values of 0.722-0.990 for 24 to 96 h of exposure (Figure 2.5, Appendix). These results, between pH 6 and 8, were consistent with the literature, which showed that increasing pH decreased Cu toxicity (Paquin et al., 2002; Rogevich et al., 2008). Increasing pH from 6.2 to approximately 8.1 resulted in higher LC50 values across all exposure durations, with the 96-h LC50 values increasing from 3.93 to 30.55 µg/L Cu. The

observed trend showed that as pH increased, the Cu toxicity decreased. This was expected due to known Cu speciation (Paquin et al., 2002). At lower pH, higher concentrations of Cu^{2+} are present due to reduced complexation with hydroxide and carbonate ions (Paquin et al., 2002; Rogevich et al., 2008; Boyd 2020). As pH increases, Cu forms carbonate complexes and DOC becomes deprotonated, thereby reducing Cu bioavailability and toxicity (Di Toro et al., 2001).

The results at pH 9 (Test 13) showed increased mortality across all treatments and controls, and the numbers closely resembled at pH 6 (Test 10). The 24-h LC50 was 44.13 µg/L Cu at pH 6 and 41.67 µg/L Cu at pH 9. This suggests that alkaline conditions might be stressful to the snails and cause mixture effects. At high pH, $CuCO_3$, $Cu(OH)_2$, and $Cu(OH)^+$ are dominant. According to Eshra (2014), Cu hydroxides can be toxic to gastropods. The present study demonstrated the importance of maintaining specific pH ranges when researching metal toxicity and considering copper speciation. Although the interpretation of Cu toxicity in the present study focused on Cu^{2+} as the most bioavailable Cu species, future work needs to look at potentially different Cu species that may contribute to Cu toxicity to snails. Understanding all bioavailable Cu species will help us better explain the influence of water quality on Cu toxicity to snails and more accurately predict Cu toxicity based on water quality. Regarding aquaculture in the southern US, it is important to consider that pH changes throughout the day (Boyd 2020). The pH is consistently between 7 and 8 between 0700 to 1800 hours and rarely below or above this range for extended periods (Silapajarn et al., 2004), although exceptions do occur. These results highlight the importance of considering pH values between 7 and 8 when treating snails, as these are the most frequent values.

2.5.4. Multiple linear regression models and implications for trematode management in catfish aquaculture

The multiple linear regression models incorporating pH, DOC, and hardness for describing the toxicity of Cu to *P. trivolvis* were developed and successfully predicted Cu toxicity to *P. trivolvis* across multiple exposure durations, explaining 70–94% of the observed variability in LC50 values. The results of the present study also indicated that exposure time was an important factor to consider in assessing the influence of Cu toxicity to *P. trivolvis* and should be included in the models. These models provide a baseline for future research on freshwater pulmonate snails and insight into the influence of Cu toxicity to *P. trivolvis*.

The models are useful for calculating relevant Cu doses based on pond system water quality to treat *P. trivolvis.* Specifically, ponds with higher hardness, elevated DOC, or higher pH should require higher Cu doses to achieve lethal concentrations appropriate for the given criteria. In contrast, soft water with low DOC and low pH should require lower Cu concentrations. It has been reported that water quality in aquaculture ponds in the Southern US varies considerably by pond. The pH values ranged from 7.5 to 10, and the hardness of soft to hard water was recorded (Table 2.4). Although DOC concentrations were not reported in the studies cited in Table 2.4, other studies found that DOC concentrations in aquaculture ponds were up to 30 mg/L (Boyd 1998; Gladfelter et al., 2024; McDonald et al., 2025). These variations in water quality will influence Cu bioavailability and toxicity. Therefore, using the models developed in the present study helps catfish producers calculate appropriate Cu doses to treat snails and avoid overdosing, which would increase treatment costs and Cu load in pond systems, or underdosing, which would result in reduced treatment efficacy.

Although the results of the present study are useful for calculating appropriate Cu doses for treating snails, protecting fish from Cu toxicity while treating snails must be taken into consideration. According to Straus et al. (2026), at a similar water quality condition, the Cu LC50s for channel catfish are higher than the Cu LC50s for *P. trivolvis*. For example, the 48-h LC50 was 26.4 µg/L Cu for *P. trivolvis* compared to 3,356 µg/L Cu (13.11 mg/L $CuSO_4 \cdot 5H_2O$) for channel catfish at a hardness of 87-93 mg/L as $CaCO_3$, a pH of 7.8-8.1, and an alkalinity of 60-85 mg/L as $CaCO_3$ (Straus et al., 2026). These results suggest that *P. trivolvis* would be more sensitive to Cu than channel catfish. However, comparisons of the relative sensitivity of *P. trivolvis* and channel catfish to Cu must be taken with caution because the influence of water quality on Cu toxicity to the organisms may not be the same. Therefore, it is important to do more Cu toxicity research with channel catfish to establish relationships between water quality parameters and Cu toxicity (e.g., LC50) that allow the prediction of Cu toxicity to channel catfish based on water quality parameters. Having such a model will allow accurate comparison of the relative sensitivity of the organisms to Cu, which helps determine appropriate Cu doses for treating snails and protecting fish.

Table 2.4: Water quality of catfish farms in the southeast region of the USA.

pH	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	DOC (mg/L)	Citation
7.8	284	220	NA	(Mischke et al., 2021)
7.6	210	180	NA	Alabama catfish farm*
NA	86-120	171-239	NA	(Mitchell, 2002)
NA	86-137	171-222	NA	(Mitchell, 2002)
8 to 9	86-103	137-257	NA	(Mitchell et al., 2007)
7.6-10	155	390	NA	(Mischke et al., 2019)

* denotes the catfish farm that was used to collect the culture snails described in the methods section.

It is important to note that after application, Cu can be adsorbed onto solid particles and precipitate in sediment, thereby decreasing Cu bioavailability. It has been reported that Cu only persists for 24-h post applications (Toth and Riemer 1968; McIntosh 1975; Button et al. 1977). This will result in 24-h exposures. Therefore, the 24-h LC50 models might be more relevant to aquaculture pond systems. If Cu persists longer in pond systems, other models, such as 48-h, 72-h, and 96-h, can be more relevant. Overall, the findings of the present study have direct implications for management of trematodes in commercial catfish aquaculture.

2.5.5. Limitations and future research

Although this study provides insight into the influence of water quality on Cu toxicity to *P. trivolvis*, this study had several limitations. Chronic and sublethal effects were not evaluated. The study was conducted with one-week-old snails, while younger snails can be more sensitive to Cu and older snails are expected to be less sensitive to Cu. More studies should be conducted to address these limitations and better understand how snails respond to Cu under different conditions. Chapter 3 examined the influence of age on Cu toxicity to *P. trivolvis*, but the data were not sufficient to include age as a model factor. Future research should investigate more life stages of the snails across different water quality conditions to help refine models that include organism age as a variable.

2.5.6. Conclusions

The present study found that Cu toxicity to *P. trivolvis* was influenced by water quality parameters (hardness, pH, and DOC) that are commonly encountered in commercial catfish

farms. Although knowledge of the influence of water quality on Cu toxicity existed, previous research showed the effects to be species-dependent, and this study corroborated the known effects to *P. trivolvis*. Each of these parameters significantly influenced Cu toxicity across the 96-h tests, with increasing hardness, pH, and DOC reducing Cu toxicity, resulting in higher LC50 values. The Cu toxicity was described as a function of pH, hardness, DOC, and exposure time. The models predicted Cu toxicity to *P. trivolvis* and can be used to calculate appropriate Cu doses for treating the snails in aquaculture ponds. Current methods have not considered water quality and used Cu based on previous studies on other ponds, which may not reflect appropriate dosages. Using the model methods will help avoid overdosing or underdosing, which can increase treatment costs and Cu load in pond systems or decrease treatment efficiency.

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Appendix

Table 2.1: Nominal and measured concentration of copper (µg/L)

Type of Experiment	Test Number	Nominal Copper (ug/L)	Dissolved Cu (µg/L)	Total Cu (µg/L)	Ionic Cu (µg/L)
	1	0	2.01	3.30	0.01
	1	5	4.40	5.04	0.02
	1	10	6.31	7.21	0.03
	1	20	17.60	21.06	0.32
	1	40	35.17	37.29	1.25
	1	80	57.27	61.66	4.15
	1	160	149.78	161.86	22.20
	2	0	-0.22	-0.36	0.00
	2	5	2.05	2.66	0.00

	2	25	19.82	21.66	0.01
	2	50	45.29	45.92	0.09
	2	100	87.80	89.09	0.44
	2	200	182.34	222.45	7.32
	2	400	413.28	432.79	34.72
	3	0	1.09	1.57	0.05
	3	5	3.12	4.25	0.20
	3	25	13.37	20.21	2.21
	3	50	32.48	40.69	5.66
	3	100	78.15	93.35	14.71
	3	200	163.58	183.18	28.96
	3	400	339.46	369.60	53.46
Hardness Effect	4	0	7.77	8.62	0.01
	4	25	25.17	25.44	0.09
	4	50	46.63	48.26	0.35
	4	100	101.66	100.90	1.99
	4	200	182.12	193.39	11.68
	4	400	382.88	403.39	47.35
	5	0	1.35	1.08	0.01
	5	10	7.26	7.72	0.19
	5	50	39.94	43.41	7.29
	5	125	125.18	130.55	41.95
	5	250	247.69	257.43	107.50
	5	500	519.79	530.89	275.07
	6	0	0.31	-0.22	0.00
	6	10	6.60	7.25	0.00
	6	50	43.50	47.13	0.13
	6	125	115.35	114.62	0.95
	6	250	217.65	217.75	5.78
	6	500	497.78	509.41	45.07
	2	0	-0.22	-0.36	0.00
	2	5	2.05	2.66	0.00
	2	25	19.82	21.66	0.01

	2	50	45.29	45.92	0.09
	2	100	87.80	89.09	0.44
	2	200	182.34	222.45	7.32
	2	400	413.28	432.79	34.72
	3	0	1.09	1.57	0.05
	3	5	3.12	4.25	0.20
	3	25	13.37	20.21	2.21
	3	50	32.48	40.69	5.66
	3	100	78.15	93.35	14.71
	3	200	163.58	183.18	28.96
	3	400	339.46	369.60	53.46
	7	0	-0.03	0.06	1.93E-07
	7	5	4.14	4.05	2.72E-05
	7	25	22.39	23.82	5.78E-04
DOC Effect	7	50	46.15	48.28	2.44E-03
	7	100	95.39	97.33	0.02
	7	200	189.38	191.91	0.10
	7	400	411.03	415.93	0.62
	8	0	0.19	0.21	1.37E-06
	8	5	5.19	4.99	4.42E-05
	8	30	28.88	29.11	5.07E-04
	8	60	57.98	61.06	1.87E-03
	8	120	114.80	117.25	9.04E-03
	8	240	234.26	239.63	0.06
	8	480	472.41	486.08	0.32
	9	0	0.48	0.41	5.05E-06
	9	10	9.69	9.37	1.32E-04
	9	50	47.32	48.21	1.05E-03
	9	75	72.73	73.82	2.10E-03
	9	150	148.52	150.34	9.41E-03
	9	300	300.89	314.64	0.07
	9	600	577.48	630.09	0.32
	10	0	2.51	2.66	0.28

	10	5	4.40	4.74	0.62
	10	25	23.37	23.58	6.62
	10	50	45.40	47.26	17.96
	10	100	88.41	93.17	45.07
	10	200	175.03	189.51	112.25
	11	0	1.06	1.28	5.642E-04
	11	5	3.66	4.05	7.772E-03
	11	25	21.98	22.70	0.48
	11	50	45.50	48.06	2.95
	11	100	90.67	94.24	10.96
pH Effect	11	200	186.38	197.77	44.52
	12	0	-0.76	-0.50	0.00
	12	5	2.62	2.72	2.96E-04
	12	25	20.51	22.42	6.41E-02
	12	50	45.01	47.11	0.35
	12	100	88.92	93.29	2.17
	12	200	179.20	206.73	17.27
	13	0	-0.24	1.33	4.97E-07
	13	5	4.19	2.60	2.74E-06
	13	25	20.55	22.61	4.75E-04
	13	50	45.45	43.39	2.35E-03
	13	100	95.18	92.39	1.43E-02
	13	200	181.88	187.35	0.20
	13	400	355.66	351.74	1.32
	13	800	780.48	780.48	4.00
	14	0	-0.96	-0.97	0.00E+00
	14	10	9.47	10.77	6.93E-04
	14	25	33.33	24.16	2.30E-03
	14	60	77.68	59.58	0.02
	14	125	149.71	129.20	0.12
	14	250	253.22	253.81	0.57
	14	500	545.07	512.52	3.09
	15	0	1.87	1.40	5.41E-05
	15	10	8.63	8.62	3.93E-04

Model Validation	15	30	35.68	30.89	2.09E-03
	15	65	74.94	67.06	8.19E-03
	15	150	162.05	154.38	0.07
	15	300	329.51	333.85	0.42
	15	600	604.64	625.58	1.70
	16	0	0.06	0.17	4.82E-04
	16	5	9.09	11.08	0.03
	16	25	21.66	25.19	0.10
	16	50	43.56	47.52	0.30
	16	100	92.11	96.91	1.25
	16	200	178.80	187.82	5.26
	16	400	343.88	359.32	19.16
	17	0	0.06	1.81	7.35E-04
	17	5	9.09	7.92	3.61E-03
	17	13	16.19	13.86	7.06E-03
	17	25	27.21	26.44	0.02
	17	50	54.99	55.78	0.06
	17	100	111.50	94.35	0.20
	17	200	212.46	207.37	1.25
	17	400	400.35	400.66	4.83
	18	0	0.51	0.47	3.94E-06
	18	5	5.36	5.21	6.29E-05
	18	40	41.45	40.50	1.69E-03
	18	80	83.73	82.13	0.01
	18	160	155.97	154.79	0.05
	18	320	313.19	307.39	0.26

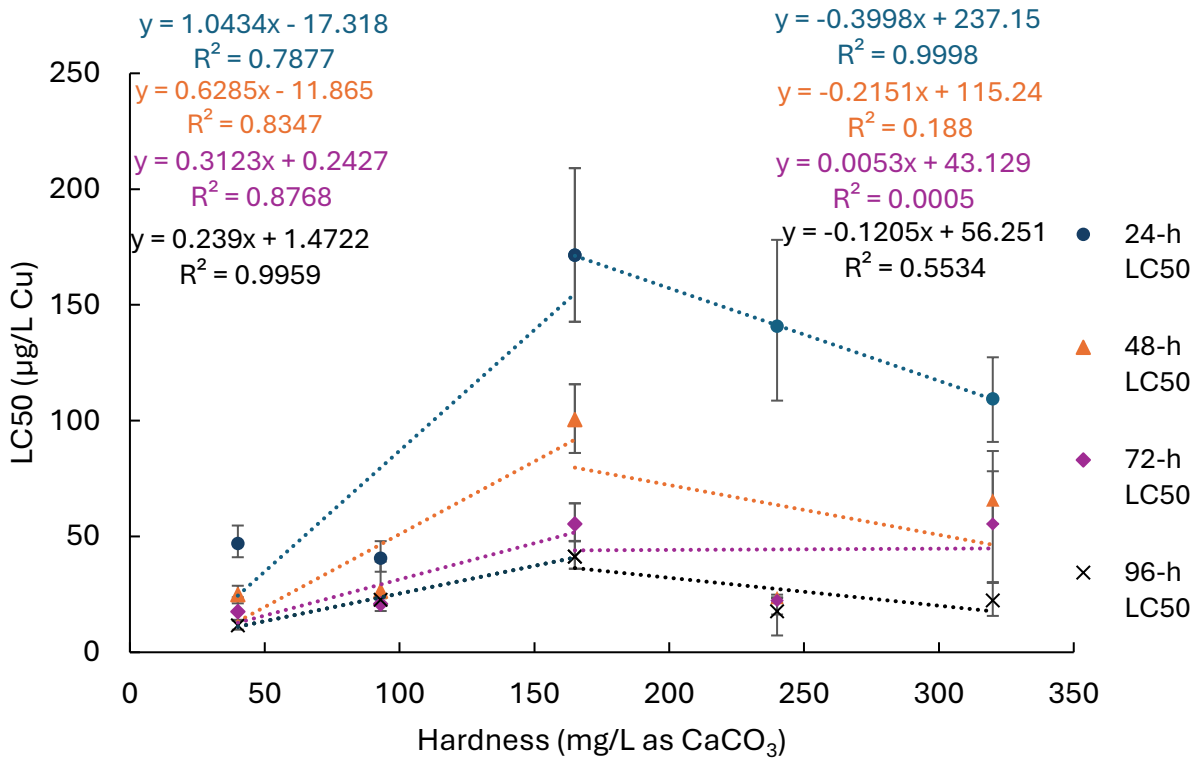

Figure 2.1: Effect of hardness on copper toxicity to the marsh ramshorn snail

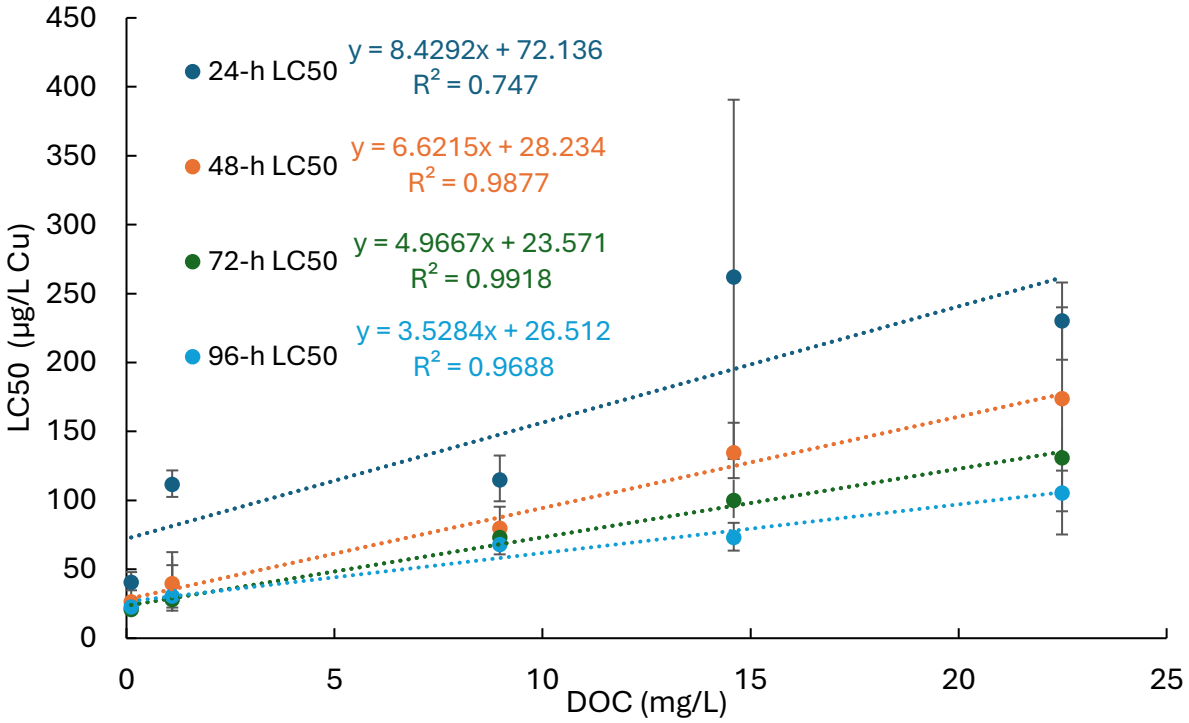

Figure 2.2: Effect of DOC on copper toxicity to the marsh ramshorn snail

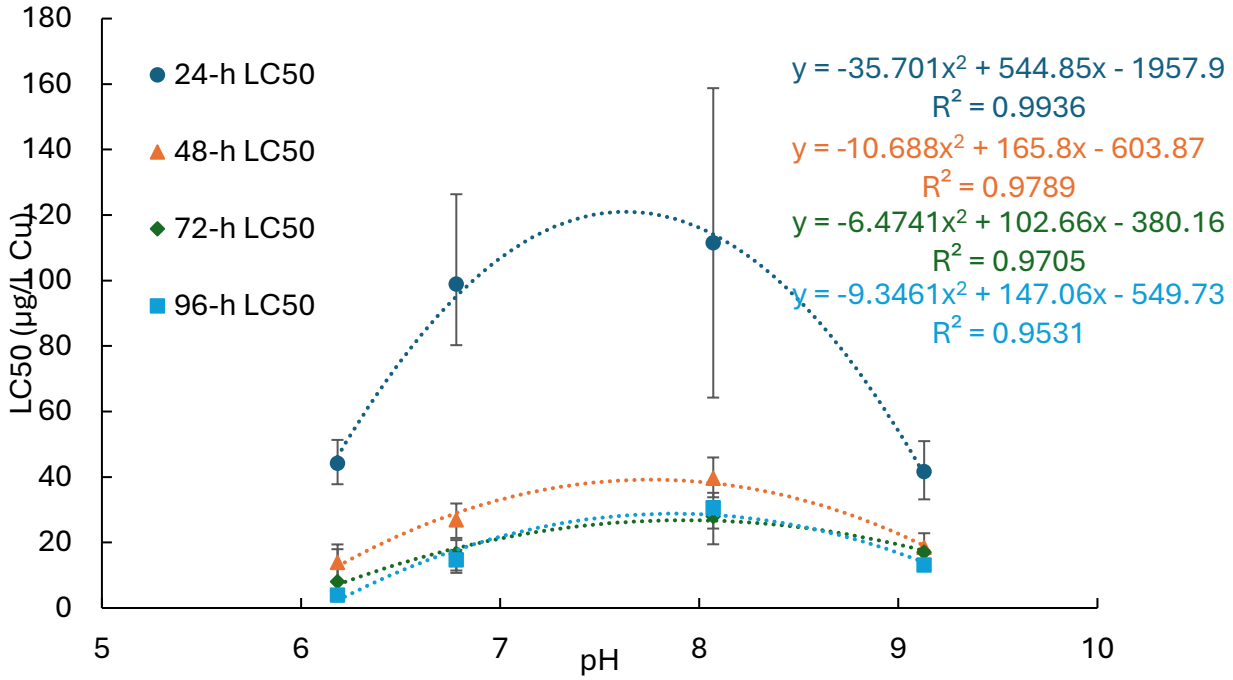

Figure 2.3: Effect of pH on copper toxicity to the marsh ramshorn snail

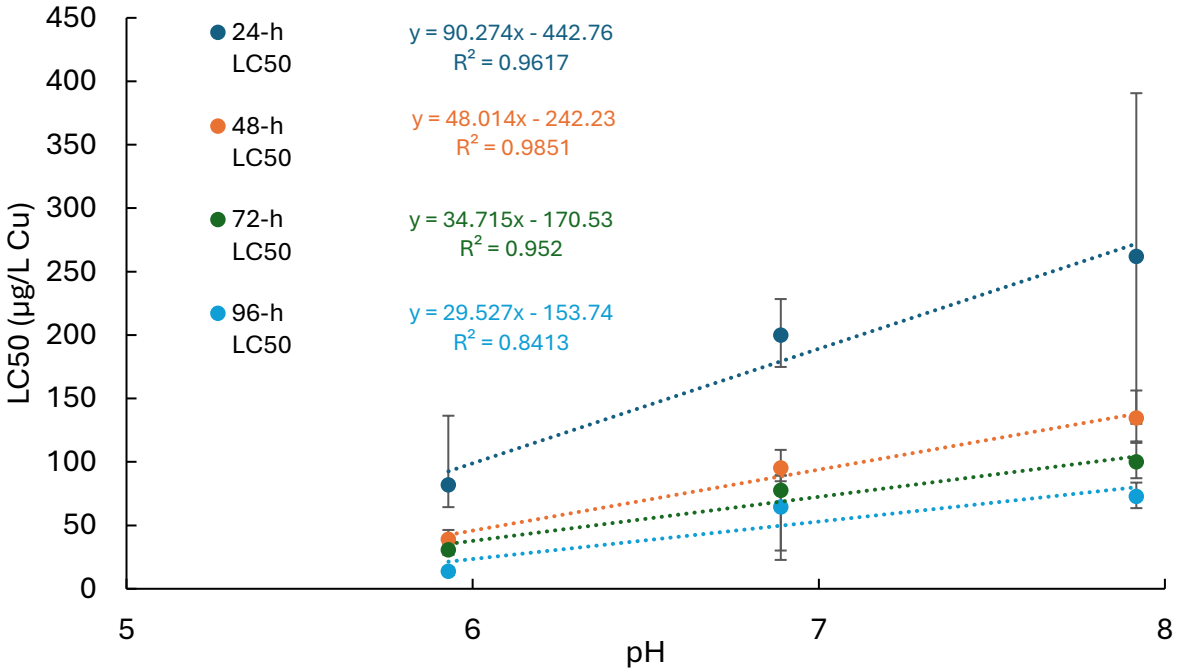

Figure 2.4: Copper toxicity to marsh ramshorn snail at DOC10 and varying pH

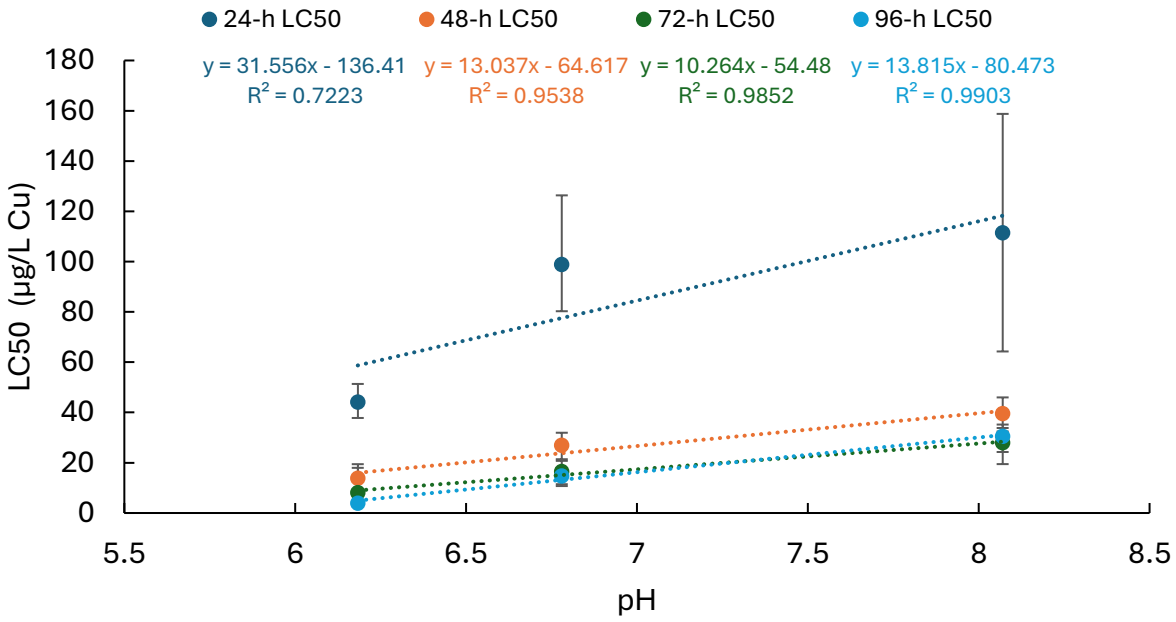

Figure 2.5: Effect of pH on Copper Toxicity to the marsh ramshorn snail (Excluding pH 9).

Chapter 3

Influence of organism age on copper toxicity to the marsh ramshorn snail

3.1. Abstract:

Copper sulfate pentahydrate (Cu) is commonly used in commercial catfish aquaculture to control the marsh ramshorn snail, *Planorbella trivolvis*, the primary host of trematode parasites that cause significant impacts on catfish. Previous work demonstrated that water quality parameters, including hardness, pH, and dissolved organic carbon, influence Cu toxicity to early life stages of *P. trivolvis*. However, the effects of age and growth environment (laboratory versus field) on Cu toxicity remain poorly understood. The present study conducted a 22-week growth assessment to examine developmental stages from the neonatal to the reproductive adult stages. The 96-h acute toxicity tests were conducted with laboratory-grown snails at one-week, one-month, and three-month-old, and on field-collected snails at one-week and three-month-old, under controlled moderately hard water conditions. The results indicated that Cu toxicity decreased with increasing organism age, with 24-h LC50 values for laboratory snails increasing from 40.62 µg/L Cu at 1-week-old to 257.07 µg/L Cu at 3-month-olds. Field-collected snails exhibited similar age-dependent trends and greater tolerance as the adult snails, with a 24-h LC50 of 499.13 µg/L Cu compared to 1-week-old snails at 56.09 µg/L Cu. Despite differences at 24-h, the 96-h LC50 values across ages and growth conditions were similar, suggesting that after a certain exposure time, Cu toxicity is independent of organism age or growth environment. These results demonstrate that early life stages are the most sensitive to Cu exposure and that the growth environment likely influences acute Cu tolerance. The findings of the present study are useful for catfish producers to determine an appropriate Cu dose for treating snails and controlling trematode infection in catfish.

3.2. Introduction

Commercial catfish ponds are designed to raise catfish. The pond systems also host other aquatic organisms, including the planorbid snail, which is the primary host of the trematode parasite that affects catfish (Overstreet et al., 2002; Overstreet & Curran, 2004; Wise et al., 2008). To combat trematodes, previous studies identified multiple chemical products as being effective against the marsh ramshorn snail (*Planorbella trivolvis*), a planorbid snail, but copper (Cu) has been identified as the most practical treatment because of its low cost and high toxicity to the snails (Mischke et al., 2005).

The toxicity of Cu to aquatic organisms depends on multiple factors, including water quality and organism age (Paquin et al., 2002; Rogevich et al., 2008). Water quality parameters to consider, but not limited to, include hardness, pH, and dissolved organic carbon (DOC), which strongly influence Cu speciation in water (Paquin et al., 2002; Rogevich et al., 2008). The research conducted in Chapter 2 of this thesis corroborated this and showed that hardness, pH, and DOC influenced Cu toxicity to *P. trivolvis*. Increasing hardness, pH, and DOC reduced Cu toxicity to *P. trivolvis*. However, Chapter 2 did not consider two major limitations that this chapter sought to investigate. The first limitation was the use of only one-week-old snails to develop the multiple linear regression models. As mentioned earlier, organism age is an important factor to consider in these types of studies, and Cu toxicity is expected to decrease with older organisms (Rogevich et al., 2008). The models from Chapter 2 worked well for one-week-old snails, but they may not accurately predict Cu toxicity in older snails. In aquaculture pond systems, snails are present at different life stages. Therefore, understanding the different Cu sensitivities of snails at various life stages would help farmers determine appropriate treatment doses to control snails. The second limitation was the use of laboratory-grown snails rather than

field snails, which may be more environmentally relevant. Previous studies on these snails have shown increased Cu sensitivity in field-collected *P. trivolvis* infested with trematodes (Richardson et al., 2025).

This study sought to address these two limitations of the previous study by examining the sensitivity of *P. trivolvis* at different life stages to Cu. First, a 22-week-growth study was conducted to examine the life stages of *P. trivolvis* from embryo to mature adult. Results of this growth study help determine the age at the mature life stage for toxicity studies. After the growth study, three age groups were selected for acute toxicity tests: one-week-old, one-month-old, and three-month-old. The one-week-old data in Chapter 2 were used for comparing with the one-month-old and three-month-old data generated in this chapter. The experiments were also conducted with one-week-old and three-month-old snails collected from the field for comparison of the sensitivity of laboratory-grown snails and field-collected snails.

3.3. Materials and methods

3.3.1. Snail collection and growth study with *P. trivolvis*

The methods for collecting and culturing larval and adult laboratory-grown *P. trivolvis* were described in Chapter 2. Briefly, adult snails were collected from a catfish aquaculture pond in Greensboro, AL and transported to the Ecotoxicology and Risk Assessment Laboratory of Auburn University. Snails were maintained in moderately hard water. Eggs were collected and hatched in a 1 L glass beaker containing 800 mL moderately hard water, and the resulting larval snails were used for a growth study. The study was conducted in an 8 L glass aquarium containing 6 L of moderately hard water and 50 larval snails for 1 month. When the snails grew larger, they were transferred to a glass fiber tank containing 50 L moderately hard water. The

tank was set up to recycle and treat water with UV light and a biofilter to prevent algal growth and ammonia generation. During the study, snails were fed with a mixture of spinach and lettuce leaves. Food fed to the snails each time was weighed and recorded (Figure 3.1, Appendix). The amount was adjusted to ensure sufficient food and to avoid excess. New food was provided when no or little old food was present. Food consumption per snail increased with time and at a rate of 0.005g/snail in week 3 to 0.1825g/snail in week 22 (Figure 3.1, Appendix). Survival of snails was measured daily. Dead snails were removed from the tank if observed.

Daily water quality measurements included temperature, dissolved oxygen (DO), conductivity, and pH. A YSI meter was used to measure temperature, DO and conductivity. The pH was measured using a Mettler Toledo pH meter. Hardness and alkalinity were measured weekly using titration methods with 0.01M EDTA and 0.02N sulfuric acid (Eaton and Franson, 2005). The average measured temperature, pH, DO, and conductivity was 25 ± 1 ºC, 8.12 ± 0.17, 7.75 ± 0.44 mg/L, and 280.49 ± 30.93 µS/cm, respectively. Hardness was 94.17 ± 29.15 mg/L as $CaCO_3$, and alkalinity was 63.42 ± 12.80 mg/L as $CaCO_3$. The diameter and height of the snails were measured every two weeks starting in week 1 for the first 11 weeks, rather than weekly, because of their fragile shell at this young life stage can be easily broken with more frequent measurements. After week 11, the diameter, height, and weight were measured weekly. The diameter and height were measured using a Nikon DS-Fi3 Dissecting Microscope (Tokyo, Japan), and the size was compared to a S18 Brightfield Stage Micrometer (Electron Microscopy Sciences, Hatfield, PA). The weight was measured using a VWR analytical balance with an accuracy of 0.1 mg. Eggs produced by adult snails were collected, counted visually, and removed from the tank. Monitoring of snail reproduction was conducted daily. Reproduction rates in the number of eggs/week and the number of eggs/adult/week were calculated using equation (1).

Cumulative reproduction was calculated by adding the reproduction rate of the previous week to the following week.

$$Reproduction\ rate\ = \frac{Total\ eggs\ produced\ in\ a\ week}{Total\ living\ snails\ in\ the\ same\ week}. \qquad (1)$$

3.3.2. Acute toxicity testing and water chemistry analysis

The 96-h renewal acute toxicity test method of the US EPA was used for experiments in this chapter. The general test procedure was described in detail in Chapter 2. The water volume used for larger snail experiments was adjusted. With one-month-old laboratory-grown snails, 400 mL of test water (500 mL beaker) was used. Experiments with three-month-old laboratory-grown snails were conducted in a 600 mL beaker containing 500 mL of test water. For three-month-old field-collected snails, experiments were conducted in 3 L beakers containing 2L test water. Aeration was provided for three-month-old field-collected snails because they were larger than three-month-old laboratory-grown snails and required more oxygen for respiration.

One- and three-month-old laboratory-grown snails were produced by hatching eggs produced by field-collected adults and maintained in moderately hard water as described above. The average diameter and height of one-month-old laboratory-grown snails were 2.29 ± 0.34 mm and 2.07 ± 0.30 mm, respectively. The three-month-old laboratory-grown snails had an average diameter of 3.17 ± 0.34 mm and height of 3.01 ± 0.45 mm. The one-week-old laboratory-grown snails had an average diameter of 0.83 ± 0.06 mm and a height of 0.94 ± 0.06 mm (Chapter 2).

One-week-old field-collected snails were produced by collecting eggs on duckweed, alligator weed, and the sides of the containers from adult snails in field water. These eggs were collected and hatched in field water in laboratory conditions for one week. The diameter and height of one-week-old field snails were similar to those of one-week-old laboratory-grown

snails (0.886 ± 0.096 mm in diameter, 0.854 ± 0.069 mm in height). Three-month-old field snails were collected from a commercial catfish aquaculture pond in Newbern, AL. The snails had an average diameter of 10.05 ± 0.82 mm, and a height of 5.71 ± 0.42 mm. These sizes were larger than the three-month-old laboratory-grown snails but were similar to the size (diameter) of the three-month-old snails used by Mischke et al. (2021). Therefore, field-collected snails were considered three months old. The difference in snail size indicated that the laboratory snails did not grow as fast as field snails, likely due to the lack of diversity of food sources in laboratory conditions compared to field conditions.

Other test procedures were the same as for tests in Chapter 2. Water samples of the test water were collected for analysis of total and dissolved Cu, anions, mineral elements, and DOC. The sample collection and analysis methods described in Chapter 2 were used for research in this chapter. Water quality parameters in the test water, such as temperature, DO, pH, hardness, alkalinity, and conductivity, were measured.

3.3.3. Data analysis

Analysis of growth data, including regression and correlation analyses were conducted using the Data Analysis ToolPak in Excel. Analysis of LC values was conducted using CETIS Software, developed for toxicology data analysis. Mortality measured Cu concentration data were used for the LC50 calculations.

3.4. Results

3.4.1. Growth study

The growth study showed that snails grew in laboratory conditions. The growth was slow in the first two months but faster onward to the end of the experiment at 5.5 months. The snails'

diameter and height were 0.761 mm and 0.8 mm at the start of the experiment and increased to 7.7 mm and 6.29 mm at the end of the experiment, respectively. The weight at week 11 was 22 mg, and at the end of the experiment, it was 141 mg (Figure 3.1). Overall, the growth of the snails for 22 weeks followed a linear function (Figure 3.1).

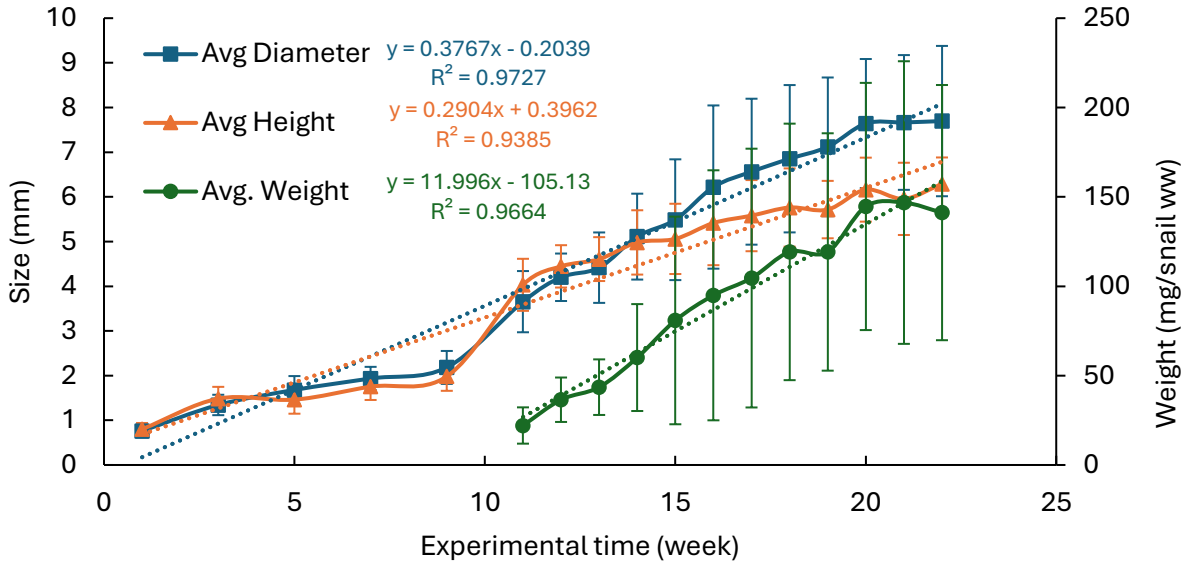

Figure 3.1: Growth of *P. trivolvis* in 22 weeks (Data are the average of 10 individual measurements; error bars represent standard deviations; Weight was not measured for < eleven-week-old snails because of their small size and fragile shell that can be easily broken while handing for measurement)

Snail survival was 100% to week 14. After this time, mortality gradually increased and continued to 60% in week 20. No further mortality was observed, and 40% survival was maintained until the conclusion of the experiment (Figure 3.2).

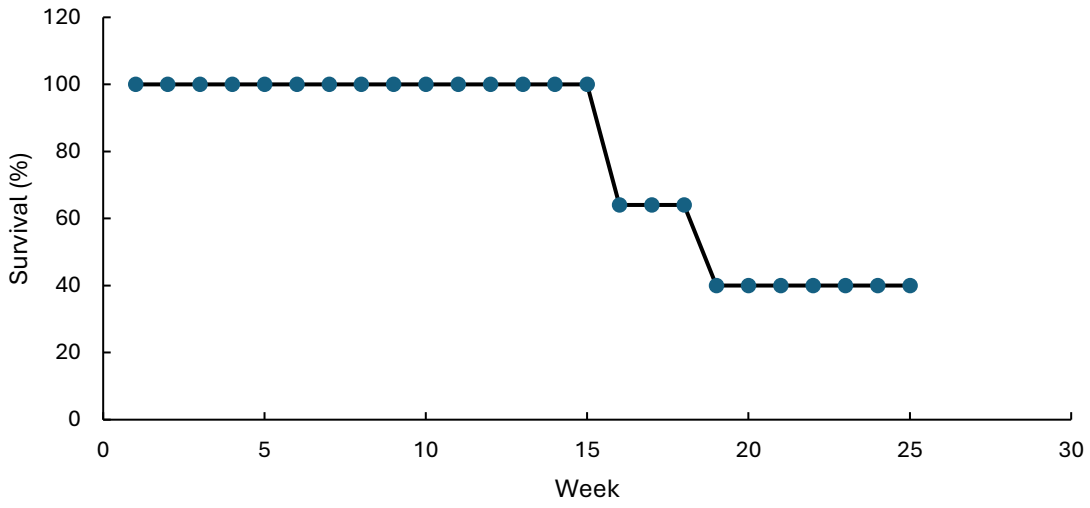

Figure 3.2: Survival of *P. trivolvis* over 25 weeks.

Eggs were first observed in week 16 and onward to week 25. Overall, reproduction rates increased with time. The reproduction rates in the first reproduction week were 23 eggs/week or 0.72 eggs/adult/week (Table 3.1). These rates increased to 162 eggs/week, or 8.10 eggs/adult week, in week 25 (Table 3.1). The reproduction rate increased with time, yielding an R^2 of 0.4028 and a slope of 0.6359, which was statistically significantly different from 0 ($p = 0.048$), Figure 3.3).

Table 3.1: Reproduction of *P. trivolvis* during the experimental period.

Week	Eggs/week	Eggs/adult/week	Cumulative eggs/adult
16	23	0.72	0.72
17	56	1.75	2.47
18	31	0.97	3.44
19	124	6.20	9.64
20	194	9.70	19.34
21	58	2.90	22.24
22	109	5.45	27.69
23	120	6.00	33.69
24	99	4.95	38.64

25	162	8.10	46.74

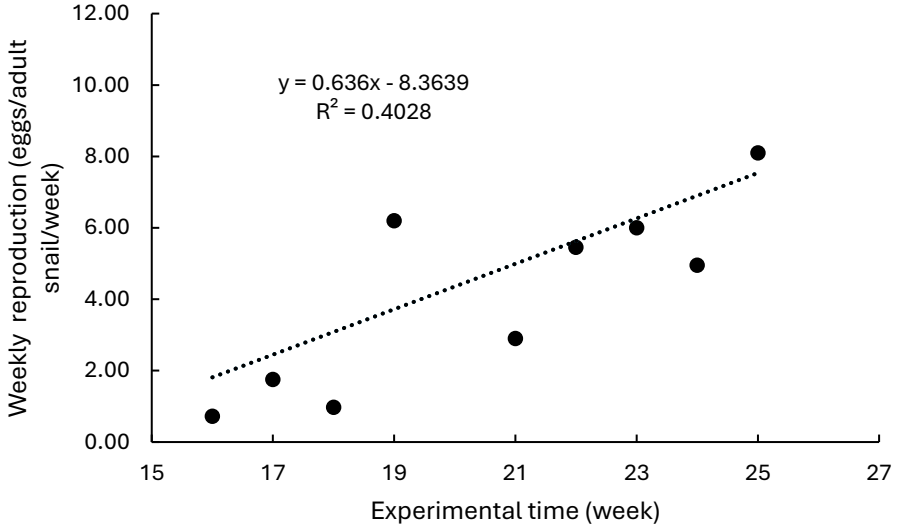

Figure 3.3: The cumulative reproductive rate of *P. trivolvis* over the course of 9 weeks.

Diameter, height, weight, cumulative reproduction, and food consumption were strongly positively correlated (Table 3.2). The results indicate that larger snails consumed more food.

Table 3.2 Correlations between size, weight, reproduction, and food consumption by *P. trivolvis.*

	Diameter	Height	Weight	Cumulative reproduction	Food consumption
Diameter	1				
Height	0.936	1			
Weight	0.927	0.921	1		
Cumulative reproduction	0.945	0.927	0.919	1	
Food consumption	0.829	0.641	0.830	0.721	1

3.4.2. Water chemistry of toxicity test water

Water quality during the toxicity tests was measured, and the results are presented in Table 3.1 of the Appendix. The pH (7.92 – 8.09), hardness (79.33- 106 mg/L as $CaCO_3$), alkalinity (60.67 – 84.80 mg/L as $CaCO_3$), and conductivity (268.18 – 298.46 µS/cm) were within the water quality of moderately hard water. The temperature ranged from 24.59 to 25.27 °C, within the designed temperature of 25 °C. Concentrations of DO were from 7.45 to 8.77 mg/L, which were above the DO requirement for toxicology test of the US EPA Method (US EPA 2002). Concentrations of DOC in most tests (0.49 to 1.85 mg/L) were approximately at the background concentration. Slightly elevated DOC concentration (3.01 mg/L) in the one-month-old laboratory-grown snail test could be due to water carryover of snail culture water when loading snails to the test chambers.

Measured total and dissolved Cu concentrations were within 80% of the nominal concentrations except for a few cases, at which the difference between the nominal and measured concentrations were more than 20%, especially at low Cu concentrations that are close to the detection limit of the method and instrument (1-2 µg/L), such as for 5 µg/L Cu treatments (Table 3.2, Appendix). Overall, dissolved Cu concentrations were lower than total Cu concentrations because of Cu complexation with DOC that was filtered by the 0.45 µm filters.

3.4.3. Lethal copper concentrations for *P. trivolvis*

The results of the experiments on the influence of organism age showed that as age increased, Cu toxicity decreased. For 24-h exposure and laboratory-grown snails, the LC50 for one-week-old, one-month-old, and three-month-old snails were 40.62 µg/L Cu, 64.62 µg/L Cu, and 257.07 µg/L Cu, respectively (Table 3.3). Overall, the LC50 increased linearly with organism age (Figure 3.4). The relationship was strongest for a 24-h exposure time, with an R^2 =

0.9711. The relationship was weaker when exposure time increased to 96 h (R^2 = 0.5362, Figure 3.4). With field-collected snails, the 24-h LC50 was 56.09 µg/L Cu for one-week-old snails and 499.13 µg/L Cu for three-month-old snails, showing an approximate ten-fold increase in the older snails compared to the younger snails. When exposure time increased, this difference decreased to approximately 1.5 times (Table 3.3).

Table 3.3: The LC50 values of Cu for the one-week-old, one-month-old, and three-month-old laboratory-grown and field-collected snails (Data in parentheses are 95% CIs).

Snail age	Exposure time (h)	LC50 (µg/L Cu)
One-week-old laboratory-grown snails	24	40.62 (34.78, 47.98)
	48	26.40 (21.33, 39.69)
	72	20.84 (17.81, 24.38)
	96	22.60 (21.06, 23.39)
One-month-old laboratory-grown snails	24	64.62 (55.79, 74.37)
	48	37.69 (30.96, 43.58)
	72	23.30 (NA, 19.53)
	96	15.08 (8.72, 31.19)
Three-month-old laboratory-grown snails	24	257.07 (236.07, 280.29)
	48	150.57 (127.23, 176.98)
	72	84.76 (68.11, 104.62)
	96	31.43 (26.64, 36.87)
One-week-old field-collected snails	24	56.09 (47.09, 66.31)
	48	26.18 (6.56, 39.56)
	72	16.05 (7.31, 47.72)
	96	14.18 (5.86, 46.06)
	24	499.13 (315.49, 726.63)
	48	71.95 (45.43, 99.79)

Three-month-old field-collected snails	72	24.41 (3.43, 34.76)
	96	21.73 (13.21, 27.89)

NA: Not available because mortality data were not adequate to calculate the lower 95% CI.

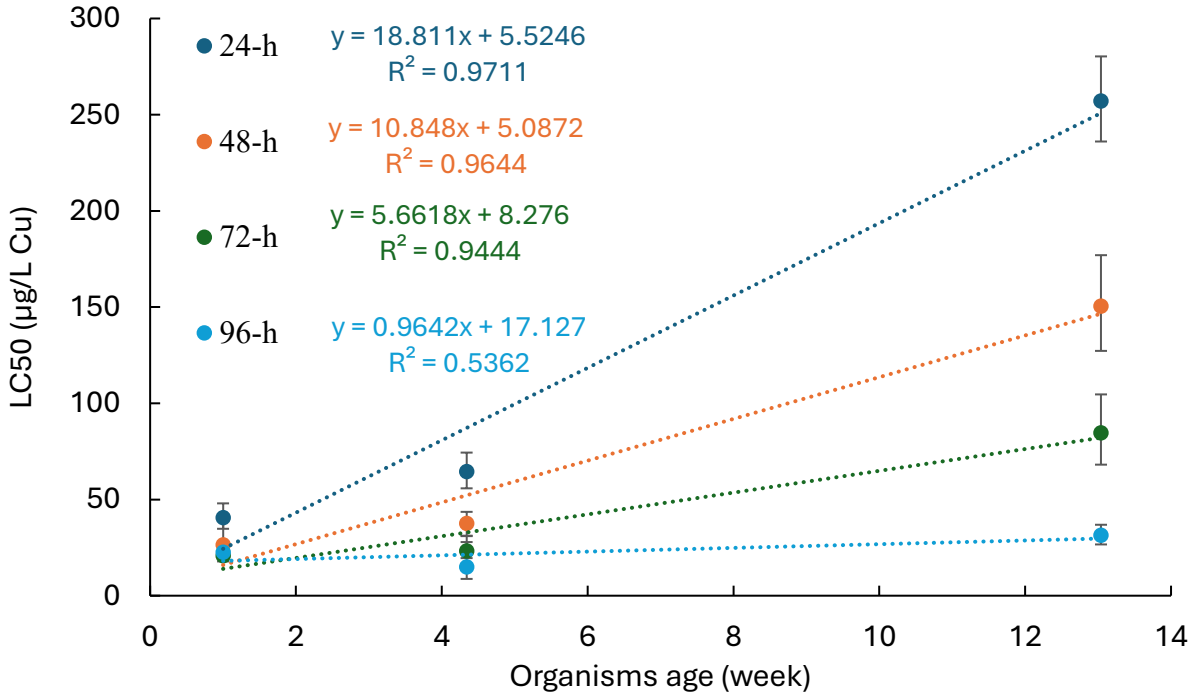

Figure 3.4: Relationships between LC50 and organism age (Error bars represent 95% CI).

Results of the toxicity tests with laboratory-grown and field-collected snails showed that there was no clear difference in Cu sensitivity of laboratory-grown and field-collected snails. For one-week-old snails, the LC50s of the same exposure time for both laboratory-grown and field - collected snails were similar. With three-month-old snails, the 24-h LC50 for field-collected snails (499.13 µg/L Cu) was approximately two times higher than the 24-h LC50 for laboratory-grown snails (257.07 µg/L Cu) (Table 3.3, Figure 3.5). When exposure time was longer, the LC50s were higher for laboratory-grown snails than field-collected snails.

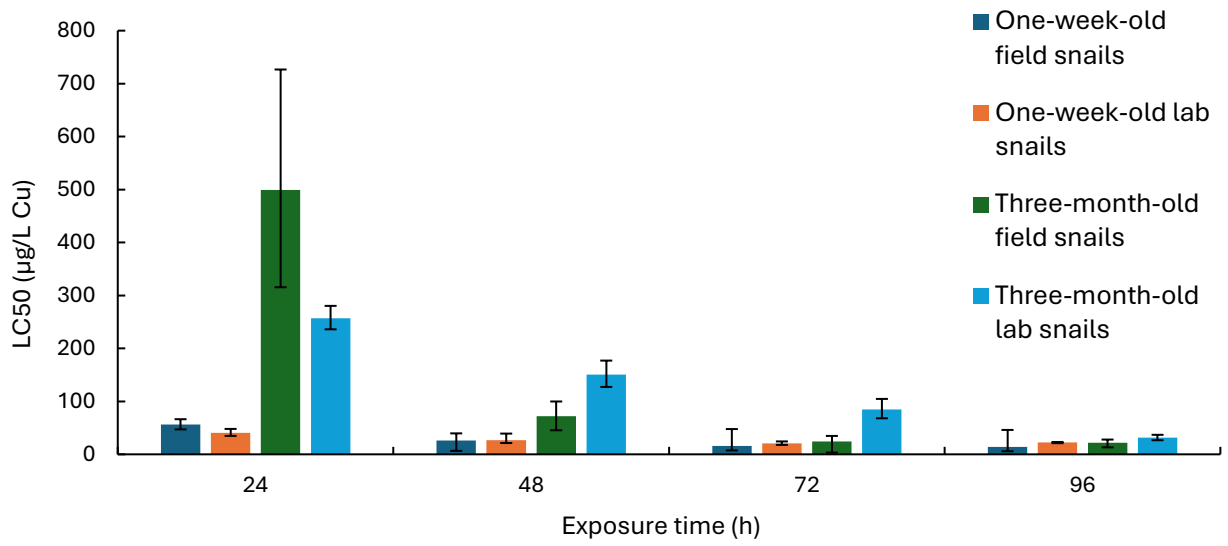

Figure 3.5: Relative sensitivity of laboratory-grown and field-collected *P. trivolvis* to Cu exposure (Error bars represent 95% CIs).

3.5. Discussion

3.5.1. Growth, development, and reproduction of *P. trivolvis*

The present study successfully grew *P. trivolvis* under laboratory conditions. The snails produced eggs after about three months, suggesting that *P. trivolvis* would reach the adult life stage at about three months old. The maximal reproduction rate was 8.1 eggs/adult/week, which is lower than the reproduction found by Norton & Newman (2016) and Norton & Anderson (2025) at 25 eggs /day/pair of snails. Reproduction can be influenced by feeding and water conditions. The snails in the present study were fed with a mixture of spinach and lettuce, which is less diverse than food sources available in the natural environment (Norton & Newman, 2016). This might explain the differences in the snail's reproduction between the studies. The size and

weight of snails in the present study were 7.7 mm in diameter, 6.29 mm in height, and 144 mg in weight, which were smaller than the snails grown by Mischke et al. (2021) that used field water with a more diverse food source. Mischke et al. (2021) reported that snails attained these sizes in about 2 months. Norton & Newman (2016) reported that many environmental conditions, such as water quality and food sources, can affect snail growth and reproduction. The lower diversity of food sources used in the present study might explain the difference in the growth. Although the reproduction rate and growth of the snail in the present study were lower than those in previous studies, the detailed growth models and reproduction rates in the present study have not been published in the literature. The findings of the present study are important for understanding the biology and ecology of this snail species, particularly in commercial aquaculture ponds. While Cu treatment in catfish aquaculture ponds to control harmful algal blooms is applied frequently throughout the year, snails reached the adult life stage in three months. This suggests that snails in aquaculture pond systems can be exposed to Cu throughout their adult lives. This can likely promote the development of Cu tolerance, resulting in less Cu sensitivity when Cu is used to treat the snails. This would result in different Cu sensitivities between laboratory-grown and field-collected snails.

3.5.2. The influence of age on Cu toxicity to *P. trivolvis*

Copper toxicity to *P. trivolvis* was strongly influenced by age, with LC50 values increasing from one-week-old to three-month-old snails. The influence depended on exposure time and on laboratory-grown versus field-collected snails. Among the exposure times, the influence was strongest for exposure at 24 h. The 24-h LC50 for laboratory-grown snails increased from 40.62 µg/L Cu for one-week-old snails to 257.07 µg/L Cu for three-month-old snails, showing over a six-fold increase in tolerance across neonatal to mature snails. As

exposure time increased, the difference in LC50s between age groups decreased, especially at 96 h. The 96-h LC50 increased from 22.60 to 31.43 µg/L Cu. The slope of the relationship between LC50 and organism age also decreased as exposure time increased. The slope of 24-h exposure was 18.811, while it was 0.9642 for 96-h exposure (Figure 3.4).

With field-collected snails, the increase in tolerance with organism age across the neonatal to mature stages was approximately ten-fold, with the 24-h LC50 increasing from 56.09 to 499.13 µg/L Cu. Similar to the laboratory-grown snails, the 96-h LC50s for field-collected snails slightly increased from 14.18 µg/L Cu for one-week-old snails to 21.73 µg/L Cu for three-month-old snails. These results suggest that when the exposure time is longer, Cu tolerance is less dependent on organism age. In aquaculture pond systems, Cu does not persist in the water for long following application. According to Ahmed (2025) and Mcintosh (1975), Cu concentrations in aquaculture ponds decreased significantly after 24-h post-application and to background levels after 48-h post-application. The decrease in Cu concentration and bioavailability in pond water was attributed to Cu adsorption onto phytoplankton and solid particles and to precipitation onto sediment. Therefore, Cu bioavailability post-application is dependent on the phytoplankton and solid particle conditions in the pond. Results of the present study suggest that the influence of organism age should be considered when applying Cu to treat the snails, as it influences Cu bioavailability and toxicity. Specifically, treating the snails at younger life stages would require less Cu than treating them when they are older.

Overall, the observed Cu tolerance in older snails in the present study is consistent with the literature. Previous studies conducted with fish and snails indicated that as age increased, Cu toxicity decreased (McKim, 1977; Rogevich et al., 2008). The tolerance is believed to be due to older organisms' greater capacity than younger organisms to develop metallothionein and

granules to sequester metals (Gibbs et al., 1998; Ng et al., 2011). These defense mechanisms reduce Cu^{2+} levels, thereby lowering toxicity. Although these are known as general detoxification mechanisms, the capacity to develop metallothionein and granules is also species-specific. For instance, with the Florida apple snail, the LC50 for a 96-h exposure differed significantly between the larval and mature life stages (Rogevich et al., 2008). The 96-h LC50 for 2-day-old apple snails was 34.22 µg/L Cu, but 182.00 µg/L Cu for 4-month-old snails. These differences in LC50 values suggest that Florida apple snails have a better defense mechanism against Cu toxicity than marsh ramshorn snails. More research is needed to understand these differences.

3.5.3. Comparison of copper sensitivity of field-collected and laboratory-grown snails

The present study identified the differences between age-dependent laboratory-grown and field-collected snails. One-week-old field snails exhibited Cu LC50 values that were similar to those of laboratory snails (Table 3.3, Figure 3.5), indicating that early life stages may have a similar response to Cu toxicity. These two groups of larval snails were hatched from eggs produced by field-collected adults. Maintaining the larval snails in laboratory water for one week might not be long enough to alter their physiological development or their response to Cu, resulting in similar Cu sensitivity. However, the response was different for older snails. The three-month-old field-collected snails had a higher 24-h LC50 value (499.13 µg/L Cu) than the three-month-old laboratory-grown snails (257.07 µg/L Cu). These results suggest that field snails are less sensitive to Cu than laboratory-grown snails. Field snails are likely exposed to episodic treatments of Cu in commercial catfish ponds, which can induce acclimation to Cu, resulting in less sensitivity to later Cu exposures than laboratory-grown snails that did not experience Cu

exposures during the growing period. These results suggest that although the larval snails were produced by field-collected adults, maintaining them in clean laboratory water for three months, which is the time needed to reach the mature life stage (results of the growth study in this chapter), allowed the snails to adjust their growth and development, resulting in a loss of Cu tolerance. However, it has also been reported that field-collected snails are more sensitive to Cu exposure because they are exposed to trematodes (Richardson et al., 2025). This would be the case if trematodes affect snails' defense mechanism against Cu toxicity, rendering them more susceptible to Cu exposure. Therefore, the interpretation of the relative Cu sensitivity of laboratory-grown and field-collected snails should take pre-trematode exposure and infection into consideration. There was no conclusion about whether the snails used in the present study were impacted by trematodes because this was not the objective of the study. If trematode infection were presented in the snails of the present study, the impacts of trematodes on Cu toxicity would be minor compared to the loss of Cu tolerance of laboratory-grown snails, resulting in a higher Cu sensitivity of laboratory-grown snails than field-collected snails. More research is needed to understand how trematode exposure affects Cu sensitivity of *P. trivolvis*. This is important for determining appropriate Cu doses to treat snails in commercial catfish aquaculture ponds.

3.5.4. Implications for trematode management in catfish aquaculture

The present study found that Cu toxicity to *P. trivolvis* depended on organism age and exposure time. Younger snails were much more sensitive to Cu for short exposure times. Therefore, treating snails at a younger life stage would require less Cu, thereby reducing treatment costs and Cu load in pond systems. The results of field-collected and laboratory-grown snail studies revealed that field snails developed Cu tolerance over time. If Cu doses from earlier

treatments are underestimated, Cu tolerance will develop, requiring higher doses for later treatments. This would increase treatment costs and Cu load into the system, potentially impacting non-target species, such as zooplankton, that are necessary for the system to function. Therefore, it is important to determine appropriate Cu doses to treat snails and avoid ineffective treatments. Since Cu bioavailability is influenced by water quality, incorporating the findings in Chapter 2 into the findings of this chapter would help farmers determine optimal Cu doses to increase treatment efficiency and reduce costs and potential harm to aquaculture pond systems.

3.5.5. Limitations

Although this study found that snail age influenced Cu toxicity, it was conducted under a defined water quality (moderately hard water). The results do not allow prediction of the influence of organism age on Cu toxicity under different water quality conditions. It would be useful to conduct more research to examine the influence of organism age on Cu toxicity across different water quality conditions, and based on the results, develop a multiple linear regression model that includes organism age as a factor. The model would allow the prediction of Cu toxicity to the snails at different life stages and under various water quality conditions. It is also important to understand the Cu sensitivity of younger life stages, such as the embryo, and the contribution of trematode infection to Cu toxicity to the snails. Understanding these unknown factors will help catfish producers determine a suitable treatment method and strategy to control snails and trematode infections in catfish.

3.5.6. Conclusions

This study found that Cu toxicity to *P. trivolvis* was strongly influenced by organism age and growth conditions. Overall, early life stages were more sensitive to Cu, whereas older snails

showed greater tolerance. The snail's sensitivity to Cu was also dependent on exposure time, with shorter exposure times resulting in a clearer sensitivity difference than longer exposure times. This study suggested that snails reached a mature life stage at about 3 months, which was also the age when snails had a clear difference in Cu sensitivity between laboratory-grown and field-collected snails. The results suggest that three months would be sufficient for snails to adjust their Cu tolerance in clean water if they were previously exposed to sublethal Cu concentrations under field conditions. The results of the present study are useful for catfish producers in determining appropriate Cu doses for treating snails, which will help control trematodes infection in catfish.

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Appendix:

Table 3.1: Water quality of toxicity tests with laboratory-grown and field-collected snails (Data are average ± are standard deviation).

Type of Experiment	Temperature (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	DOC (mg/L)
One-week-old lab snail	25.17 ± 0.11	8.08 ± 0.08	7.45 ± 0.24	298.46 ± 11.51	92.8 ± 1.79	84.8 ± 3.35	0.49 ± 1.10
One-month-old lab snail	25.16 ± 0.10	7.98 ± 0.03	7.69 ± 0.14	282.5 ± 4.77	98.67 ± 2.07	64.67 ± 1.63	3.01 ± 1.55
Three-month-old lab snail	24.75 ± 0.05	8.09 ± 0.02	8.77 ± 0.15	268.18 ± 6.92	106 ± 2.19	79.33 ± 1.63	1.23 ± 2.27
One-week-old field snail	25.27 ± 0.20	8.06 ± 0.03	7.87 ± 0.20	287.14 ± 3.57	79.33 ± 3.01	60.67 ± 1.63	1.85 ± 1.97
Three-month-old field snail	24.59 ± 0.45	7.92 ± 0.03	8.37 ± 0.17	291.07 ± 3.85	100.00 ± 0.00	62.67 ± 2.07	0.73 ± 1.21

Table 3.2: Nominal and measured Cu concentrations of toxicity tests with laboratory grown and field-collected snails.

Type of Experiment	Nominal Cu (ug/L)	Dissolved Cu (µg/L)	Total Cu (µg/L)
One-week-old laboratory grown snails	0.00	1.09	1.57
	5.00	3.12	4.25
	25.00	13.37	20.21
	50.00	32.48	40.69
	100.00	78.15	93.35
	200.00	163.58	183.18
	400.00	339.46	369.60
One-month-old laboratory grown snails	0.00	0.00	0.00
	5.00	2.97	4.38
	25.00	17.47	11.28
	50.00	48.90	39.82
	75.00	68.45	80.52
	150.00	143.34	168.86
	300.00	301.67	290.94
Three-month-old laboratory grown snails	0.00	4.19	4.39
	5.00	4.32	4.54
	25.00	19.91	21.67
	50.00	44.78	47.30
	100.00	96.08	101.62
	200.00	197.05	210.74
	400.00	347.11	385.25
	0.00	0.00	0.00
	5.00	2.05	2.66
	25.00	19.82	21.66

One-week-old field grown snails	50.00	45.29	45.92
	100.00	87.80	89.09
	200.00	182.34	222.45
	400.00	413.28	432.79
Three-month-old field grown snails	0.00	0.00	0.19
	10.00	3.36	5.02
	50.00	35.16	44.57
	100.00	77.39	95.76
	200.00	166.06	210.69
	400.00	382.17	413.95
	800.00	767.55	786.14

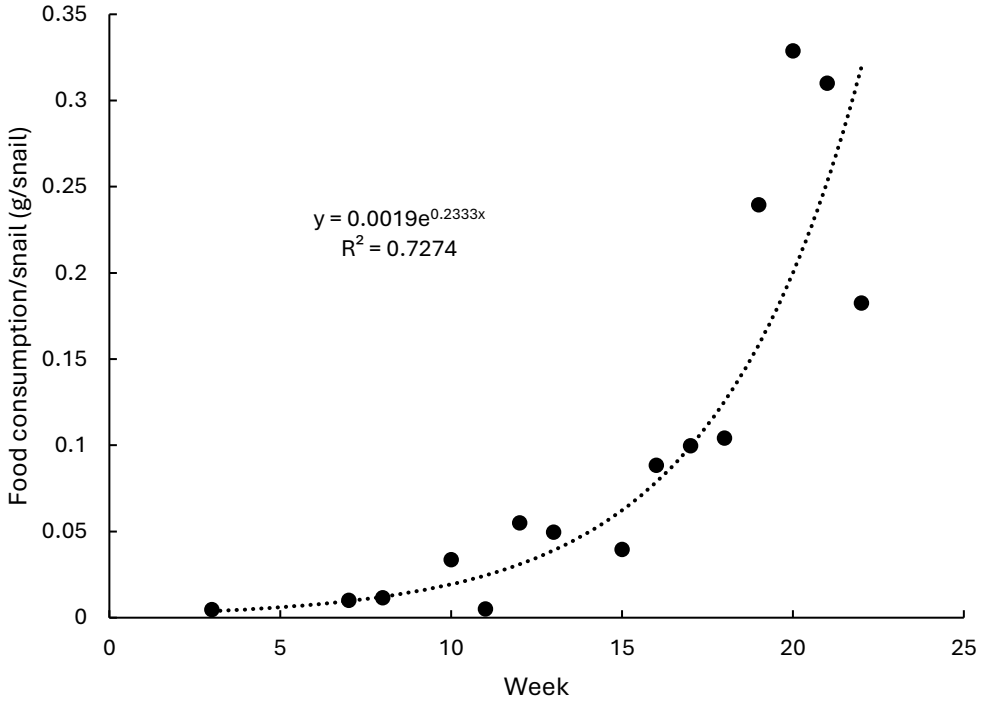

Figure 3.1: Food consumption of *P. trivolvis* during the growth study over the course of 22 weeks.

Overall Thesis Conclusions

The focus of this study was to understand the influence of water quality on Cu toxicity to *P. trivolvis* and based on the results, to develop a multiple linear regression model that can be used to determine appropriate Cu doses to treat *P. trivolvis* in aquaculture pond systems. The thesis includes two main objectives. The first objective determined the influences of exposure time and water quality parameters, including hardness, pH, and DOC on Cu toxicity to *P trivolvis*, which was presented in Chapter 2. The second objective, presented in Chapter 3, was to understand the relative sensitivity of snails to Cu at different life stages and the potential Cu tolerance of snails due to repeated Cu applications in aquaculture systems over time by comparing the toxicity of Cu to snails at different ages, as well as field-collected and laboratory-grown snails.

The results of the first research objective showed strong influences of hardness, pH, and DOC on Cu toxicity to *P. trivolvis*. As the concentration of water quality parameters increased, the LC50 increased, which indicates a decrease in toxicity in accordance with the literature. The results allowed development of five multiple linear regression models to predict Cu toxicity using hardness, pH, DOC, and exposure time with 70-94% variation explained by the models. The models did not work with high hardness and pH levels, suggesting that other Cu species, such as $Cu(OH)^{+}$ and hardness ions might contribute to the toxicity. More research should be conducted to understand the increased Cu toxicity to the snails in water with high pH and hardness.

Research of the second objective identified the influence of age on Cu toxicity to *P. trivolvis* and potential Cu tolerance in field-collected snails. In general, as the organism's age increased from one-week-old to three-months-old, Cu toxicity decreased by a seven-fold

difference for the 24-h LC50. These results agree with the literature that suggests that as age increases, snails have a greater ability to produce metallothionein and granules to sequester metals making them non-bioavailable. Additionally, the study identified potential Cu tolerance in field-collected snails. The results showed a two-fold tolerance for the 24-h LC50 in the three-month-old field-collected snails compared to the three-month-old laboratory-grown snails. These results showed that these snails may have some levels of Cu tolerance for the first 24 hours but may lose this tolerance afterwards.

The findings of this study showed the importance of considering water quality in commercial catfish ponds and the organism's age when treating the ponds with Cu, because the ponds can vary greatly with their hardness, pH, and DOC. These findings also suggest a consideration of the residency time of Cu in commercial catfish ponds. Because the response of the snails changed greatly with exposure time, the 24-h exposure was strongly influenced by water quality, and the least influence was observed when exposure time was prolonged. The results suggest that the 24-h LC50 model to be more applicable than longer exposure time models. Additionally, the findings showed that older organisms were more tolerant to Cu than younger organisms, especially with field collected snails that might have previously experienced episodic exposures of Cu leading to potential Cu tolerance. More research should be conducted and consider other water quality parameters, other Cu species, and other ages, and to form stronger MLR models, as well as potential trematode exposures affecting Cu tolerance in field-collected snails.

The overall results of this study have implications for determining appropriate Cu doses to treat *P. trivolvis* that help avoid overdosing, which would increase treatment costs and Cu load into the pond systems or underdosing resulting in decreasing treatment efficiency.