Comparative Biochemistry and Physiology, Part C 154 (2011) 261–267

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

# The toxicity and physiological effects of copper on the freshwater pulmonate snail, *Lymnaea stagnalis*

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ARTICLE INFO

*Article history:*
Received 25 April 2011
Received in revised form 16 June 2011
Accepted 16 June 2011
Available online 23 June 2011

*Keywords:*
Gastropods
*Lymnaea stagnalis*
Copper
Water quality criteria
Ionoregulation
Acid–base balance

ABSTRACT

Several recent studies have demonstrated that the freshwater pulmonate snail *Lymnaea stagnalis* is extremely sensitive to metals (Co, Ni, Pb) in chronic exposures. The objective of the current study was to evaluate the acute and chronic sensitivity of *L. stagnalis* to Cu and investigate the underlying mechanism(s) of toxic action. A 96-h LC50 of 31 μg $L^{-1}$ Cu was estimated indicating *L. stagnalis* was moderately acutely sensitive to Cu relative to other aquatic organisms. However, in a 30-day chronic exposure using juvenile snails an EC20 of 1.8 μg $L^{-1}$ Cu was estimated for snail growth making *L. stagnalis* the most sensitive organism tested to date for Cu. Hardness-based and BLM-based water quality criteria for Cu at the water quality conditions used in this study were 7.8 and 1.5 μg $L^{-1}$, respectively, indicating *L. stagnalis* is significantly under-protected by hardness-based WQC. Investigations into the mechanism(s) of toxic action for Cu were conducted on young adult snails necessitating higher Cu exposures. Exposure to Cu at 12 μg $L^{-1}$ resulted in no detectable effects on hemolymph osmolality, net $Ca^{2+}$ uptake, titratable acid excretion, or ammonia excretion. Exposure to 48 μg $L^{-1}$ Cu was shown to significantly reduce (91%) net $Ca^{2+}$ uptake which is strongly correlated with shell deposition and corresponding snail growth. Snails exposed to 48 μg $L^{-1}$ Cu also exhibited reduced ammonia excretion, a marked hemolymph acidosis, and a compensatory increase in titratable acid excretion. The reduction in net $Ca^{2+}$ uptake was hypothesized to be a secondary effect of Cu-induced inhibition of carbonic anhydrase, but no reduction in carbonic anhydrase activity was detected. Overall, it remains unclear whether inhibition of $Ca^{2+}$ uptake is a direct result of Cu exposure or, along with the other observed physiological effects, is secondary to an unidentified primary mode of toxic action. Given the hypersensitivity of *L. stagnalis* to Cu, further study into the mechanisms of action and effects of varying water chemistry on Cu toxicity is clearly warranted.



## 1. Introduction

An important tenet in establishing water quality criteria (WQC) is that the toxicity data sets utilized to derive these criteria are generally representative of aquatic communities (Stephan et al., 1985). While this has been the goal, most toxicity data sets significantly under-represent key phyla common to many aquatic communities. This appears to be the case even for data rich substances such as divalent metals (Brix et al., 2005). Specific to freshwater, one such under-represented group in toxicity data sets is the gastropods. There are approximately 670 species of freshwater gastropods in North America, 60 of these species are now presumed extinct, 20 species are currently listed as endangered by the US Fish and Wildlife Service and another 290 species are of conservation concern. Hence ~46% of all extant freshwater gastropods are targets for conservation, making them the most imperiled aquatic taxa in the United States (Perez and Minton, 2008).

Despite this, relatively few snail species have been tested for their sensitivity to contaminants and the majority of testing has been limited to acute survival studies. For example, in the recently updated USEPA WQC for Cu (one of the most data rich substances), only 4 snail species have been tested for acute sensitivity and 1 species for chronic sensitivity to Cu (USEPA, 2007). In part, this may be because of the perception based on mostly acute toxicity data that snails are relatively insensitive to many contaminants. This perception is driven by the relatively high number of acute tests with adult snails, and very limited number of chronic studies on sensitive life stages of snails. However, over the last several years there has been an increase in chronic toxicity studies with snails, particularly with the pulmonate snail *Lymnaea stagnalis*. These studies have shown that for several metals (Co, Pb, and Ni) *L. stagnalis* is either the most sensitive or second most sensitive species tested to date (Grosell et al., 2006; De Schamphelaere et al., 2008; Schlekat et al., 2010).

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doi:10.1016/j.cbpc.2011.06.004

These previous studies with *L. stagnalis* all used a similar early life stage test design evaluating survival and growth of juvenile (<7 day old) snails over a 30 day exposure. In each case, growth was the most sensitive endpoint. Investigations into the mechanism of toxic action for Co and Pb (Ni has not been studied) show a strong link between disruption of $Ca^{2+}$ homeostasis and growth effects (De Schamphelaere et al., 2008; Grosell and Brix, 2009). This is perhaps not surprising given that both of these metals are known $Ca^{2+}$ antagonists (Richards and Playle, 1998; Rogers et al., 2003). The objective of the current study was to characterize the acute and chronic sensitivity of *L. stagnalis* to Cu and investigate the physiological mechanisms underlying any observed toxicity. Given that Cu is known to be a $Na^+$ antagonist (Grosell et al., 2002), we hypothesized the mechanism of action and possibly the relative sensitivity of *L. stagnalis* would differ from that observed for Co and Pb.

## 2. Methods and materials

### 2.1. Experimental animals

Adult snails were obtained from an in-house culture maintained in flow through dechlorinated City of Miami tap water ($[Na^+]=1.1$, $[K^+]=0.083$, $[Ca^{2+}]=0.43$, $[Mg^{2+}]=0.13$, $[Cl^-]=1.03$, $[HCO_3^-]=0.68$ mmol $L^{-1}$, [DOC] = 100 μmol $L^{-1}$, pH = 7.8) at 23–25 °C. The culture was fed a mix of lettuce, carrots and sweet potatoes and egg masses were transferred from the main culture tanks to static-renewal nursery tanks for hatching and juvenile growth before they were used in toxicity studies.

### 2.2. Acute and chronic toxicity tests

An acute (96 h) toxicity test was performed using juvenile (7 day old, 8–22 mg ww) snails. Copper exposures were performed in dechlorinated City of Miami tap water under flow-through conditions as described previously (Grosell et al., 2006) with three replicates of 5 snails tested for each Cu treatment. Nominal test concentrations were 0, 20, 40, 80, 160, and 320 μg $L^{-1}$ Cu. Snail survival was monitored daily and water samples were collected at the beginning and end of the experiment for measurement of dissolved (0.45 μM filter) Cu concentrations. Snails were not fed during the Cu exposure.

An early life stage chronic toxicity test (30 days) was performed using newly hatched (≤24 h old) snails. Prior to test initiation, the flow-through system was operated for 5 days without snails and a small piece of sweet potato was introduced to each test container. This allowed a biofilm to be established upon which the juvenile snails could feed. Copper exposures were performed in dechlorinated City of Miami tap water under flow-through conditions with three replicates of 5 snails tested for each Cu treatment. Nominal test concentrations were 0, 2, 4, 8, 16, and 32 μg $L^{-1}$ Cu. Snail survival was monitored daily and at the end of the experiment snails were blotted dry on paper towels after which total body mass (wet weight) was determined to the nearest 0.1 μg on an analytical balance (Mettler, Toledo). Water samples were collected at the beginning of the experiment and weekly thereafter for measurement of dissolved (0.45 μM filter) Cu concentrations. Snails were fed ad libitum a mixture of lettuce and sweet potato during the Cu exposure.

### 2.3. $Ca^{2+}$ flux experiments

Young adult snails used for ionoregulation and acid–base balance studies were not fed for 48 h prior to experimentation. Larger animals were used for physiological studies because many of the measurements were not logistically feasible on newly hatched snails due to their small size. As might be expected, this necessitated the use of higher Cu concentrations to elicit effects. Initial experiments were performed using snails (1.7 g mean ww) exposed to 12 μg $L^{-1}$ Cu, but no significant effects were observed in any of the parameters measured. As a result, a second set of experiments was performed using snails (1.0 g mean ww) exposed to 48 μg $L^{-1}$ Cu. For both exposures, snails were exposed to Cu for 7 days prior to physiological measurements.

Snails used in flux measurements were placed in dechlorinated tap water containing the appropriate Cu concentrations (<0.5, 12 or 48 μg $L^{-1}$). To measure $Ca^{2+}$ uptake and loss rates, 10 snails from each treatment were placed in individual beakers each containing 20 mL of dechlorinated tap water and were allowed to recover from handling for 10 min prior to flux initiation. Each beaker was covered with Parafilm® and gently aerated through polyethylene tubing. At the onset of flux measurements 0.02 μCi $^{45}Ca$ (as $CaCl_2$; Amersham Biosciences) was added to each beaker and a 5 min equilibration period was allowed before water samples were obtained for radioactivity and total ion concentration measurements. This 5 min equilibration period allows for any adsorption of $Ca^{2+}$ (radioactive or not) to occur such that differences between this initial water sample and the final water sample can be ascribed to uptake (or excretion) by the organism. After a total flux time of 2–3 h (exact time for individual snails recorded) a second water sample was obtained from each beaker after which the snails were removed from the flux beaker, rinsed, blotted dry and weighed.

For $Ca^{2+}$ flux measurements, the specific activity of $^{45}Ca$ in the water samples at the beginning and end of the flux period was determined from measured radioactivity and corresponding total ion concentrations. Unidirectional ion uptake rates (nmol $g^{-1}$ $h^{-1}$) were determined from the depletion of radioactivity from the water, the average specific activity of $Ca^{2+}$ over the flux period, the individual snail mass and the time elapsed during the flux period as described previously (Grosell et al., 2000). Net flux rates were determined in a similar manner but simply from the change in total $Ca^{2+}$ concentration from the beginning to the end of the flux period. Unidirectional efflux rates were determined as the difference between influx and net flux.

### 2.4. Acid–base balance experiments

Concurrent with the $Ca^{2+}$ flux experiments, a separate set of young adult snails was used to evaluate potential acid–base balance disturbance from Cu exposure with separate 7-d Cu exposures performed at 12 and 48 μg $L^{-1}$ Cu. Snails were fluxed in dechlorinated tap water containing the appropriate Cu concentrations (<0.5, 12 or 48 μg $L^{-1}$). Ten snails from each treatment were placed in individual beakers each containing 20 mL of dechlorinated tap water and were allowed to recover from handling for 10 min prior to flux initiation. Each beaker was covered with Parafilm® and gently aerated through polyethylene tubing.

Water samples were collected at the beginning and end of each flux period (22 h) for measurement of total ammonia and titratable alkalinity. Samples for total ammonia were frozen at −20 °C and analyzed within 1 week of collection. Titratable alkalinity samples were refrigerated at 4 °C and analyzed within 18 h of collection.

### 2.5. Hemolymph characterization

Osmolality, pH, and total $CO_2$ in extracellular fluids were determined in snails after 7 days exposure to <0.5, 12 and 48 μg $L^{-1}$ Cu. Snails were maintained in individual beakers as for the flux experiments above prior to sampling. A predatory avoidance reflex in *Lymnaea stagnalis* which expels substantial volumes of extracellular fluid in response to stimulation of the foot was utilized to obtain extracellular fluid samples as described in Ebanks and Grosell (2008). In brief, individual snails were gently removed from their exposure medium, carefully blotted dry and held upside down while stimulated to retract into their shell and thereby release extracellular fluid. This procedure results in accumulation of expelled extracellular fluid in the vacated outer shell cavity, which can easily be obtained by a pipette. Collected extracellular fluid was transferred to micro-centrifuge tubes and a subsample was transferred to a separate 0.5 mL tube for immediate pH measurements.

Following pH measurements, the subsamples were used for measurements of total $CO_2$ and osmolality.

### 2.6. Carbonic anhydrase assay

Carbonic anhydrase (CA; EC 2.4.1.1) activity was measured using the electrometric delta pH method (Henry, 1991). The reaction medium consisted of 2.5 mL of buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris base; Sigma Aldrich, MO, USA) kept at 4 °C. The reaction was started by adding 100 μL of $CO_2$ saturated Milli-Q water using a gas tight Hamilton syringe. The reaction rate was measured over a pH change of 0.15 units (+10 mV). To calculate the true catalyzed reaction rate, the uncatalyzed reaction rate was subtracted, and the buffer capacity of the reaction medium was used to convert the rate from mV into mol $H^+$ per unit time. The pH was measured using a PHC4000 combined pH electrode (Radiometer Analytical, Lyon, France) attached to a PHM220 lab pH meter (Radiometer Analytical, Lyon, France). CA assay measurements were performed on snail mantle homogenates (4 mL reaction buffer for 1 g tissue). Mantle tissue (24–49 mg) was homogenized on ice using a motor driven homogenizer, and briefly centrifuged (1 min × 10,000 rpm) to pellet cellular debris. In all cases, duplicate assays were performed with 50 μL of tissue homogenate used per assay. All results were normalized per gram tissue.

### 2.7. Analytical chemistry

Water samples for determination of Cu exposure concentrations were passed through a 0.45 μm cellulose nitrate syringe filter (Acro-disc, Pall Life Sciences, MI, USA) and acidified by addition of $HNO_3$ (Fisher Scientific, Trace metal grade) to a final concentration of 1%. Copper concentrations were analyzed by graphite furnace atomic absorption (Varian 220Z, Varian, Walnut Creek, CA, USA).

Concentrations of $Ca^{2+}$ in water samples were determined by atomic absorption spectrophotometry (VarianAA 220FS, Mulgrave, Victory, Australia). Extracellular fluid pH was determined in 20–50 μL subsamples using an Accumet micro pH electrode coupled to a Radiometer 220 pH meter (Radiometer, Copenhagen, Denmark). Concentrations of total $CO_2$ in hemolymph samples were analyzed using a Corning Carbon Dioxide Analyzer 965 (Essex, England). Hemolymph osmolality was measured using a vapor pressure osmometer (Wecor Vapro 5520, Logan, UT, USA). Gamma radioactivity from $^{45}Ca$ was analyzed using an automated scintillation counter (TmAnalytical Beta Tract 6895).

Total ammonia in water was measured by a micro-modified colorimetric method (Verdouw et al., 1978). Titratable alkalinity was measured by double endpoint titration to pH 3.8. Samples were gassed with $N_2$ for 30 min, initial pH recorded and the samples were titrated with acid to pH 3.8, gassed with $N_2$ for an additional 15 min and then titrated back to the initial pH with base. Titration acid and base (0.02 N) was dispensed using 2 mL Gilson microburettes. Acid and base solutions were normalized against each other and all measurements corrected accordingly. Net titratable acid and ammonia were calculated as previously described (Patrick and Wood, 1999).

### 2.8. Data analysis

All analyses were performed on measured test concentrations. Data are presented as mean ± SEM and n = 10 in all cases. The acute toxicity test was analyzed using Probit analysis (Finney, 1971) while the EC20 in the chronic toxicity test was estimated using the linear interpolation method (USEPA, 2002). Statistical evaluation of data from physiological studies consisted of Student's two-tailed t-tests and results were considered statistically different at $p<0.05$. Acute and chronic toxicity tests were analyzed using CETIS (Tidepool Scientific, 2010). All other analyses were performed using SigmaStat (SPSS, 2002).

## 3. Results

### 3.1. Acute and chronic copper toxicity tests

Strong concentration response relationships were observed in both the 96-h acute (Fig. 1) and 30-d chronic Cu toxicity tests (Fig. 2). The estimated 96-h LC50 for *L. stagnalis* was 30.7 (28.9–32.7 95% CI) μg $L^{-1}$ Cu. In the chronic toxicity test, significant effects on snail survival were observed with 100% mortality occurring in the two highest treatments (Fig. 2A). The resulting NOEC, LOEC and EC20 for snail survival in the chronic toxicity test were 4.0, 7.0, and 5.6 (4.8–6.7 95% CI) μg $L^{-1}$ Cu, respectively. Significant growth effects were also observed in the chronic toxicity study with 33, 44, and 83% reductions in snail wet weight relative to the controls observed at 2.3, 4.9, and 7.0 μg $L^{-1}$ Cu (Fig. 2B). The corresponding NOEC, LOEC and EC20 for growth were 2.3, 4.9, and 1.8 (1.3–6.1 95% CI) μg $L^{-1}$ Cu, making snail growth the most sensitive endpoint measured. It should be noted that the estimated EC20 for snail growth is below the lowest Cu treatment tested.

### 3.2. Physiological studies after chronic Cu exposure

Results from the various physiological measurements of snails after 7 days exposure to Cu revealed several disturbances in $Ca^{2+}$ uptake and acid–base balance. Comparison of control responses between the 12 and 48 μg $L^{-1}$ Cu experiments revealed that both $Ca^{2+}$ uptake and titratable acid excretion were significantly higher ($p<0.05$) in the smaller animals (1.7 vs. 1.0 g mean ww) from the 48 μg $L^{-1}$ Cu experiments (Figs. 3 and 4).

No significant effect relative to the control on $Ca^{2+}$ uptake, efflux or net flux was observed in snails exposed to 12 μg $L^{-1}$ Cu for 7 days. In contrast, snails exposed to 48 μg $L^{-1}$ Cu had a statistically significant 50% reduction in $Ca^{2+}$ influx and a statistically insignificant 90% increase in $Ca^{2+}$ efflux ($p=0.197$). The corresponding result of these two effects was a statistically significant 91% reduction in net $Ca^{2+}$ uptake compared to the control.

The acid base balance characterization revealed several non-significant trends in snails exposed to 12 μg $L^{-1}$ Cu (Fig. 4A). Both titratable acid excretion and ammonia excretion trended higher in the Cu exposed animals with the corresponding net acid excretion (sum of titratable acid and ammonia excretion) indicating a strong trend ($p=0.07$). Exposure to 48 μg $L^{-1}$ Cu resulted in statistically significant effects on both titratable acid excretion and ammonia excretion. However, unlike the 12 μg $L^{-1}$ exposure, ammonia excretion was significantly reduced in snails exposed to 48 μg $L^{-1}$ Cu. Because there was an increase in titratable acid excretion and decrease in ammonia excretion, no significant effect was observed on net acid secretion although there was an increasing trend ($p=0.113$).

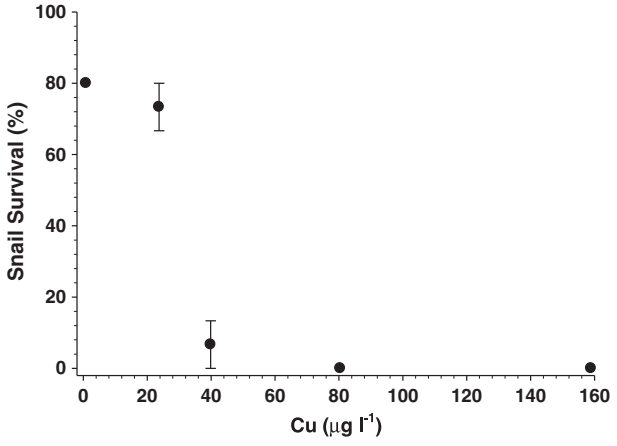


**Fig. 1.** Dose response relationship for 96-h acute toxicity test. Mean ± SEM (n = 3).

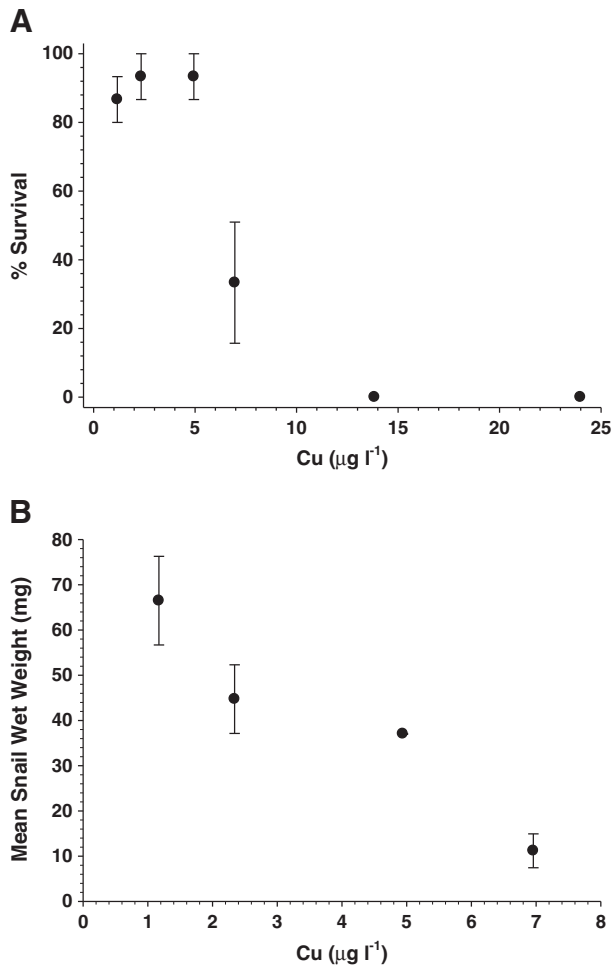


**Fig. 2.** Dose response relationship for 30-d chronic toxicity test A) Survival and B) Growth measured as mean wet weight. Mean ± SEM (n = 3).

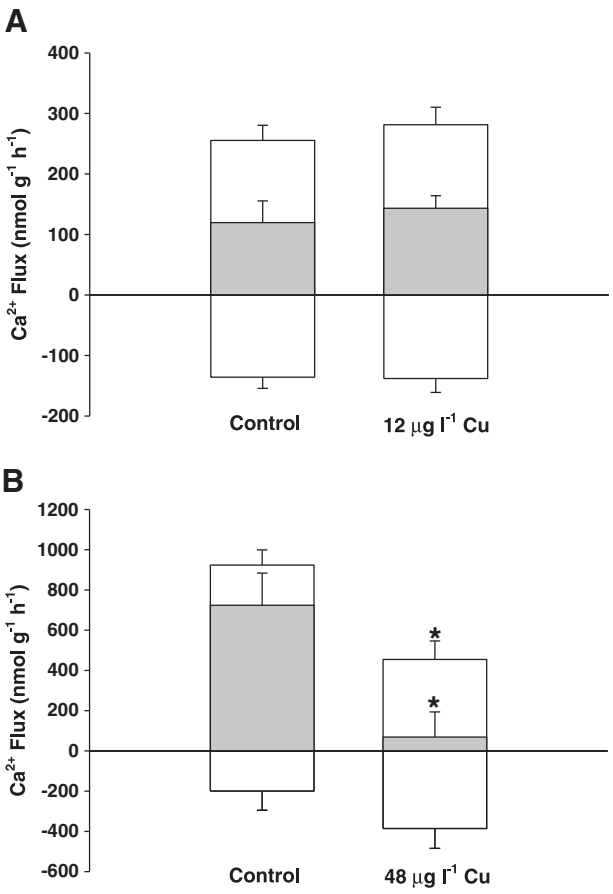


**Fig. 3.** $Ca^{2+}$ influx, efflux and net flux in *L. stagnalis* exposed to Cu for 7 days. A) Experiment with 1.7 g snails exposed to control and 12 μg $L^{-1}$ Cu and B) experiment with 1.0 g snails exposed to control and 48 μg $L^{-1}$ Cu. Positive open bars represent influx, negative open bars represent efflux, and shaded bars represent net flux in nmol $g^{-1}$ $h^{-1}$. Data presented as mean ± SEM (n = 10). * = statistically significant difference from the control ($p \leq 0.05$).

Similar to the above experiments, characterization of snail hemolymph revealed no significant effects on animals exposed to 12 μg $L^{-1}$ Cu, while snails exposed to 48 μg $L^{-1}$ Cu had significantly reduced hemolymph osmolality, total $CO_2$ and pH (Fig. 5). Finally, snails exposed to 48 μg $L^{-1}$ Cu for 7 days exhibited no significant effect on carbonic anhydrase activity in the mantle relative to the controls (Fig. 6).

## 4. Discussion

### 4.1. Acute and chronic sensitivity to Cu

The acute toxicity test with *L. stagnalis* resulted in a 96 h LC50 of 31 μg $L^{-1}$ Cu, indicating this species is of moderate sensitivity and protected by existing hardness and BLM-based WQC for Cu (Fig. 7A) (USEPA, 2007; USEPA, 2009). Previous acute toxicity studies on snails with Cu (n = 6) have resulted in 96-h LC50s ranging from 7 to 34 μg $L^{-1}$ Cu when BLM-normalized to the USEPA default water chemistry (USEPA, 2007) indicating that *L. stagnalis* is among the more insensitive snails to acute Cu toxicity (Arthur and Leonard, 1970; Nebeker et al., 1986; Khangarot and Ray, 1988; Rogevich et al., 2008). The one exception to this is the prosobranch snail *Campeloma decisum* for which a 96 h LC50 of 3573 μg $L^{-1}$ was estimated (Arthur and Leonard, 1970). The adult life stage was tested, which in this snail has a thick operculum that when closed may have allowed it to avoid exposure to elevated Cu concentrations during the test.

Acute Cu toxicity in aquatic organisms is generally the result of inhibition of $Na^+/K^+$-ATPase leading to failure of $Na^+$ homeostasis (Wood, 2001). Grosell et al. (2002) explained the relative sensitivity of aquatic organisms to Cu as a function of body size, which correlates well with $Na^+$ turnover rate. Although we don't have measurements of $Na^+$ turnover in juvenile snails, measurements in 1 g snails suggest that *L. stagnalis* is a typical aquatic organism with respect to $Na^+$ turnover (Grosell and Brix, 2009). Given the size of the test organisms (8–22 mg), the sensitivity of *L. stagnalis* (and most of the other snails tested to date) to acute Cu exposure is approximately an order of magnitude less than would be expected based on the size dependent $Na^+$ turnover model of Grosell et al. (2002). This may be the result of uncertainty in the model as data are limited in this size range. Alternatively, it is known that when *L. stagnalis* retracts into its shell it expels up to ~30% of its extracellular fluid in order to compress its body. Fluid loss is recovered within 8 h, but associated ion loss with hemolymph expulsion requires up to 48 h to recover. The net result is an ~40% loss in hemolymph $Na^+$ that *L. stagnalis* is routinely capable of sustaining (Ebanks and Grosell, 2008). In contrast, Cu-induced loss of 30% extracellular $Na^+$ is considered a lethal threshold in fish (Wood, 2001). Hence, the evolved mechanism of withdrawal into their shell and corresponding high $Na^+$ loss may have led to *L. stagnalis* being particularly resistant to acute Cu-induced $Na^+$ loss.

The chronic toxicity test with *L. stagnalis* resulted in a 30-day EC20 of 1.8 μg $L^{-1}$ Cu. When this result is hardness-normalized to 85 mg $L^{-1}$ (the default hardness used by USEPA for WQC), the corresponding EC20 is 2.6 μg $L^{-1}$ Cu, indicating *L. stagnalis* is the most chronically sensitive aquatic species tested to date (Fig. 7B). Only three other snail species have been chronically tested for Cu sensitivity. Perhaps most interesting

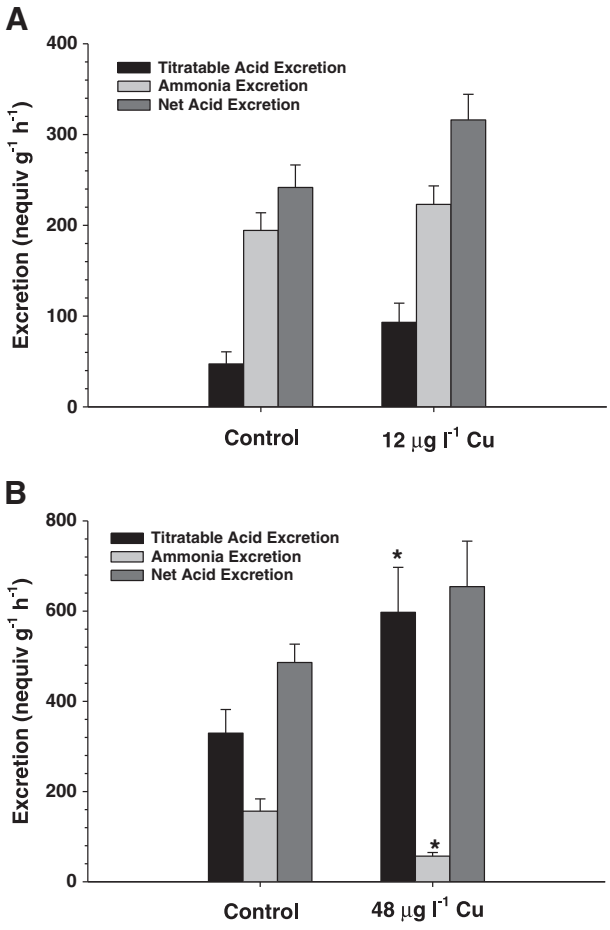


**Fig. 4.** Characterization of acid–base balance in *L. stagnalis* exposed to A) 12 µg $L^{-1}$ Cu and B) 48 µg $L^{-1}$ Cu. Data presented as mean ± SEM (n = 10). * = statistically significant difference from the control ($p \leq 0.05$).

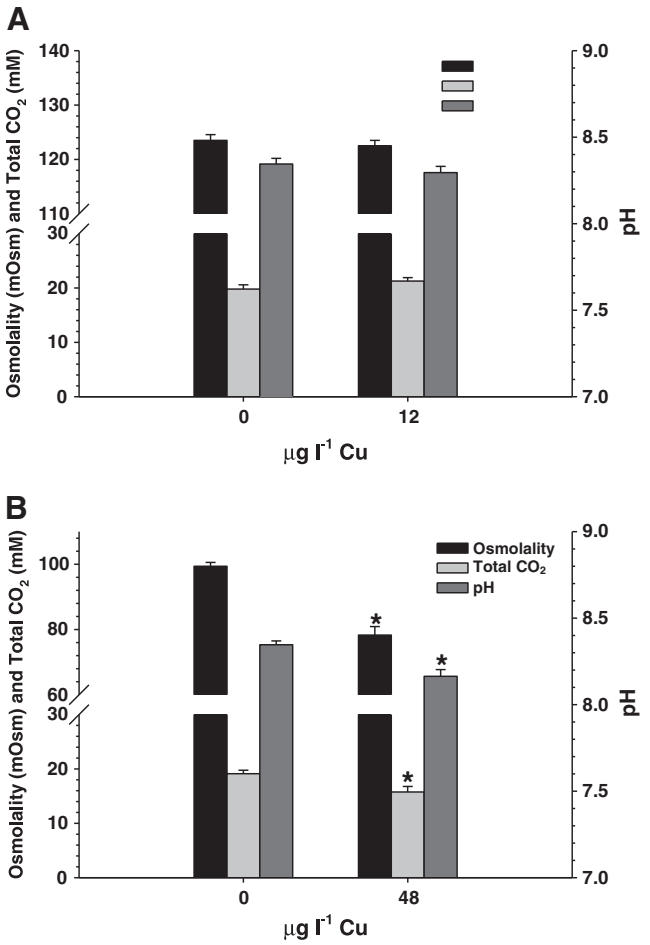


**Fig. 5.** Hemolymph osmolality, total $CO_2$ and pH in *L. stagnalis* exposed to A) 12 µg $L^{-1}$ Cu and B) 48 µg $L^{-1}$ Cu. Data presented as mean ± SEM (n = 10). * = statistically significant difference from the control ($p \leq 0.05$).

are the recent studies by Khangarot and Das (2010) and Das and Khangarot (2011) on the closely related *Lymnaea luteola*. In their first study, these authors exposed <24 h old egg masses to Cu and monitored several developmental endpoints, hatching success and growth of newly hatched snails. They observed significant delays in embryo development at 14 µg $L^{-1}$ Cu and effects on newly hatched snail growth at 8 µg $L^{-1}$ Cu. In their second study, the authors exposed young adult *L. luteola* to Cu for 7 weeks evaluating growth, feeding rates, and reproductive output. Feeding rates and egg production were both significantly inhibited at 10 ug l-1 Cu, while embryo growth within the egg capsule was inhibited at 5.6 ug l-1 Cu, the lowest concentration tested. These studies were performed at a hardness of 230 mg $L^{-1}$ resulting in a hardness-normalized LOEC of 2.4 µg $L^{-1}$ Cu for embyro growth, similar to the hardness normalized EC20 for juvenile snail growth of 2.6 ug l-1 Cu observed in the current study. Two other chronic studies have been performed with Cu on freshwater snails. Arthur and Leonard (1970) estimated an EC20 for survival of 8.8 µg $L^{-1}$ Cu in a 42 day study with *Campeloma decisum*. In a 240 day full life cycle study on *Pomacea paludosa*, Rogevich et al. (2009) observed 100% mortality at 24 µg $L^{-1}$ Cu and no effect on growth at the next lowest concentration tested (17 µg $L^{-1}$ Cu). However two reproductive endpoints were affected with LOECs of 17 and <9 µg $L^{-1}$ Cu (the lowest concentration tested) for hatching success and number of egg masses produced per female, respectively.

The result for *L. stagnalis* also indicates that it is protected by the BLM-based chronic WQC of 1.5 µg $L^{-1}$ but significantly under-protected by the hardness-based WQC of 7.8 µg $L^{-1}$ (USEPA, 2007; USEPA, 2009). Under-protection by the hardness-based WQC (7.8 µg $L^{-1}$) is of particular concern as we observed an 83% reduction in snail growth at 7.0 µg $L^{-1}$ and 100% mortality at 13 µg $L^{-1}$ Cu. While the BLM-based criterion is sufficiently low to protect snails, the regulatory reality is that currently, no State has adopted the BLM-based WQC as their standard and Cu is currently regulated using the hardness-based WQC throughout the United States.

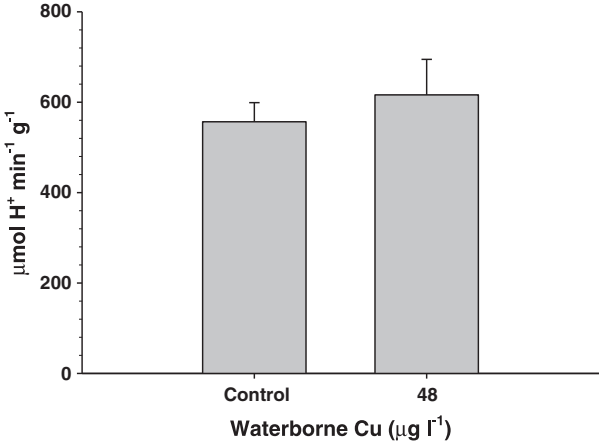


**Fig. 6.** Carbonic anhydrase activity in the mantles of *L. stagnalis* after exposure to 48 mg $L^{-1}$ Cu for 7 days. Data presented as mean ± SEM (n = 6). * = statistically significant difference from the control ($p \leq 0.05$).

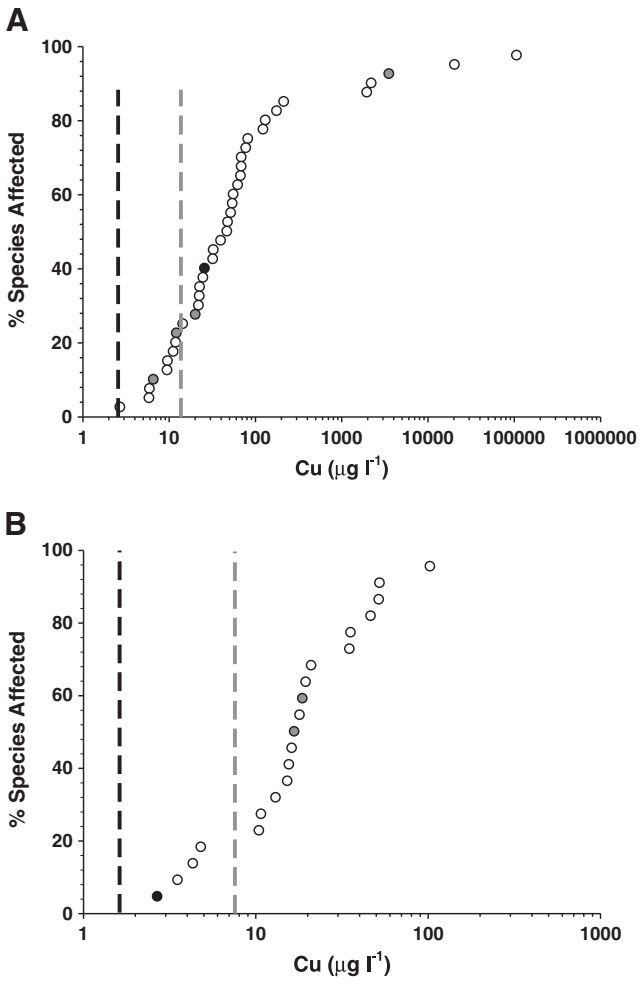


**Fig. 7.** Species sensitivity distributions for Cu A) acute and B) chronic. Data based on USEPA (2007). All acute data normalized using the biotic ligand model to default water quality parameters described in USEPA (2007). All chronic data normalized to 85 mg $L^{-1}$ hardness. Black circle indicates *L. stagnalis*, gray circles indicate other gastropods, open circles indicate all other aquatic species. Black dashed lines indicate BLM-based USEPA water quality criteria and gray dashed line indicates hardness-based USEPA water quality criteria.

### 4.2. Physiological effects of Cu on snails and mechanism of action

In addition to characterizing the relative sensitivity of *L. stagnalis* to Cu, a second objective was to investigate the physiological effects of Cu on this species and through this information potentially identify the toxic mechanism of action. Because of the difficulty in performing many of the desired physiological experiments on juvenile snails, we performed these experiments on young adult animals. This necessitated using higher Cu concentrations than those observed to inhibit growth in juveniles as our initial experiments at 12 µg $L^{-1}$ Cu on adult snails indicated little or no physiological disturbance. It is assumed that physiological disturbances observed at 48 µg $L^{-1}$ Cu in adult snails are similar to those that would be observed in juvenile snails at much lower concentrations, but we currently have no data to support this assumption and so these results should be treated with caution with respect to extrapolating to juvenile snails.

Several physiological disturbances were observed in these experiments. Most straightforward was the 20% reduction in hemolymph osmolality. Although hemolymph $Na^+$ and $Cl^-$ were not directly measured, this reduction was most likely the result of a decrease in hemolymph $Na^+$ (and possibly $Cl^-$) as no other constituents in the snail hemolymph could account for such a significant decline in osmolality (De With and Van der Schors, 1984). This is also consistent with the known etiology of Cu toxicity in fish, where it has been shown to inhibit $Na^+/K^+$-ATPase activity thereby reducing or eliminating the gradient for $Na^+$ uptake (Wood, 2001). With a reduced ability to actively take up $Na^+$ from the environment, freshwater aquatic organisms are unable to combat diffusive $Na^+$ loss and extracellular $Na^+$ concentrations decline accordingly.

With respect to acid–base balance, we observed a significant increase in apparent titratable acid excretion, reduction in hemolymph total $CO_2$ and pH, and reduction in ammonia excretion. It is possible that the increase in apparent titratable acid excretion is actually an increase in titratable base uptake, as the two processes cannot be distinguished via titratable alkalinity fluxes. However, given that hemolymph total $CO_2$ and pH decreased it seems more likely that the observed effect is an increase in titratable acid excretion as a compensatory response to the observed acidosis. If correct, the observed reduction in ammonia excretion is unlikely to be the result of unfavorable gradients as increased acid excretion would facilitate ammonia excretion via proton trapping (Wilkie, 1997). Instead, the observed reduction in ammonia excretion might be the result of reduced protein catabolism and subsequently reduced ammonia generation. Finally, as *L. stagnalis* typically excretes ~30% of its nitrogen as urea (Bayne and Friedl, 1968; Friedl, 1974), it is possible that copper exposure induced an increase in the relative amount of urea excretion and that total nitrogen excretion was unaffected.

Perhaps most interesting was the observed reduction in $Ca^{2+}$ uptake by adult snails exposed to 48 µg $L^{-1}$ Cu. To the best of our knowledge, this is the first demonstration of $Ca^{2+}$ uptake inhibition by Cu in an aquatic organism, as Cu typically inhibits $Na^+$ uptake (Wood, 2001; Grosell et al., 2002; Grosell and Wood, 2002). We have previously demonstrated with Pb that inhibition of $Ca^{2+}$ uptake is strongly linked to inhibition of snail growth (Grosell and Brix, 2009) and so understanding the mechanism underlying this effect may ultimately explain the hypersensitivity of *L. stagnalis* to Cu. There are two possible explanations for the inhibition of $Ca^{2+}$ uptake by Cu in *L. stagnalis*.

First, it is possible that *L. stagnalis*, and perhaps snails in general, accomplish $Ca^{2+}$ uptake via proteins that are unusually sensitive to Cu compared with other aquatic species that have been studied (e.g., fish). In fish, $Ca^{2+}$ uptake across the apical membrane is believed to occur passively via the epithelial calcium channel (ECaC), a voltage independent channel, and is then actively transported across the basolateral membrane by a $Ca^{2+}$-ATPase (Flik and Verboost, 1993; Shahsavarani et al., 2006). In *L. stagnalis*, it is believed that $Ca^{2+}$ uptake is also accomplished partly by a basolaterally located $Ca^{2+}$-ATPase (Ebanks et al., 2010a). However, the mechanism for apical entry of $Ca^{2+}$ differs substantially from fish. Specifically, $Ca^{2+}$ appears to cross the apical membrane via a voltage dependent calcium channel and possibly via an electrogenic $Ca^{2+}/2H^+$ exchanger with an apical $H^+$-ATPase providing the electrogenic motive force (Ebanks et al., 2010a). Given these differences between fish and snails, it is certainly possible that the inhibition of $Ca^{2+}$ by Cu is the direct result of inhibition of proteins found on the apical membrane.

Alternatively, the inhibition of $Ca^{2+}$ uptake may be a secondary effect in response to the direct effect of Cu on some other protein or system in the snail. We considered this possibility and hypothesized that inhibition of $Ca^{2+}$ uptake may have been the result of a direct inhibition of carbonic anhydrase (CA) in the snail mantle. Carbonic anhydrase in the snail mantle hydrates $CO_2$ to form $HCO_3^-$ and $H^+$. The $HCO_3^-$ is utilized for shell formation ($CaCO_3$) which also results in the production of an additional $H^+$ (Ebanks et al., 2010b). Hence, inhibition of CA would not only eliminate the need for $Ca^{2+}$ for shell growth, but would also significantly reduce the availability of $H^+$ to drive $Ca^{2+}$ uptake across the apical membrane. While this hypothesis seemed plausible, we were unable to detect any inhibition of CA in the snail mantle of Cu-exposed animals.

These results suggest that inhibition of CA is not the primary mechanism of action of Cu toxicity. However, the method for assaying CA activity requires a substantial dilution (4-fold) of the tissue homogenate with a buffer solution which may have resulted in Cu repartitioning to the buffer solution sufficiently to not inhibit CA during the assay. Further investigations into this possibility are clearly needed.

## 5. Conclusions

This study aimed to characterize the acute and chronic sensitivity to Cu of the freshwater pulmonate snail *L. stagnalis* and to gain an understanding of the toxic mechanism(s) of action. We demonstrated that *L. stagnalis* is of moderate acute sensitivity to Cu, similar to what has been observed for other snails. With respect to chronic Cu toxicity, *L. stagnalis* appears to be the most sensitive species tested to date and is significantly under-protected by the current hardness-based WQC. These results add to the increasing data set showing *L. stagnalis* to be an exceptionally sensitive aquatic organism to a number of different metals (Grosell et al., 2006; De Schamphelaere et al., 2008; Schlekat et al., 2010).

Regarding the underlying mechanisms of this hypersensitivity to Cu we made several important observations. Surprisingly, we observed a significant reduction in $Ca^{2+}$ uptake as a result of Cu exposure, along with a marked acidosis and apparent compensatory response in the form of increased titratable acid excretion. Ammonia excretion was also reduced suggesting a reduction in protein catabolism in the snails. While inhibition of $Ca^{2+}$ uptake is likely linked to reduced shell formation and therefore growth, it is unclear whether reduced $Ca^{2+}$ uptake is the result of direct inhibition by Cu or secondary to the actual direct mechanism of toxicity. Hence, the mechanism of Cu toxicity to *L. stagnalis* remains unclear, but given their hypersensitivity and under protection by current WQC, further study into the mechanism of toxicity is clearly warranted.

## Acknowledgment

KVB was supported by a University of Miami Maytag Fellowship and NSF Graduate Research Fellowship.

## References

Arthur, J.W., Leonard, E.N., 1970. Effects of copper on *Gammarus pseudolimnaeus*, *Physa integra*, and *Campeloma decisum* in soft water. J. Fish. Res. Board Can. 27, 1277–1283.

Bayne, R.A., Friedl, F.E., 1968. The production of externally measurable ammonia and urea in the snail, *Lymnaea stagnalis jugularis* Say. Comp. Biochem. Physiol. 25, 711–717.

Brix, K.V., DeForest, D.K., Burger, M., Adams, W.J., 2005. Assessing the relative sensitivity of aquatic organisms to divalent metals and their representation in toxicity data sets compared to natural aquatic communities. Hum. Ecol. Risk Assess. 11 (6), 1139–1156.

Das, S., Khangarot, B.S., 2011. Bioaccumulation of copper and toxic effects on feeding, growth, fecundity and development of pond snail *Lymnaea luteola* L. J. Hazard. Mat. 185, 295–305.

De Schamphelaere, K.A.C., Koene, J.M., Heijerick, D.G., Janssen, C.R., 2008. Reduction of growth and haemolymph Ca levels in the freshwater snail *Lymnaea stagnalis* chronically exposed to cobalt. Ecotoxicol. Environ. Saf. 71, 65–70.

De With, N.D., Van der Schors, R.C., 1984. Urine composition and kidney function in the pulmonate freshwater snail *Lymnaea stagnalis*. Comp. Biochem. Physiol. 79A (1), 99–103.

Ebanks, S.C., Grosell, M., 2008. Fluid and osmolyte recovery in the common pond snail *Lymnaea stagnalis* following full-body withdrawal. J. Exp. Biol. 211, 327–336.

Ebanks, S.C., O'Donnell, M.J., Grosell, M., 2010a. Characterization of mechanisms for $Ca^{2+}$ and $HCO_3^-/CO_3^{2-}$ acquisition for shell formation in embryos of the freshwater common pond snail *Lymnaea stagnalis*. J. Exp. Biol. 213, 4092–4098.

Ebanks, S.C., O'Donnell, M.J., Grosell, M., 2010b. Acquisition of $Ca^{2+}$ and $HCO_3^-/CO_3^{2-}$ for shell formation in embryos of the common pond snail *Lymnaea stagnalis*. J. Comp. Physiol. B 180, 953–965.

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, London.

Flik, G., Verboost, P.M., 1993. Calcium transport in fish gills and intestine. J. Exp. Biol. 184, 17–29.

Friedl, F.E., 1974. Nitrogen excretion by the fresh water pulmonate snail, *Lymnaea stagnalis jugularis* Say. Comp. Biochem. Physiol. A 49, 617–622.

Grosell, M., Brix, K.V., 2009. High net calcium uptake explains the hypersensitivity of the freshwater pulmonate snail, *Lymnaea stagnalis*, to chronic lead exposure. Aquat. Toxicol. 91 (4), 302–311.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Grosell, M.H., Hogstrand, C., Wood, C.M., Hansen, H.J.M., 2000. A nose-to-nose comparison of the physiological effects of exposure to ionic silver versus silver chloride in the European eel (*Anguilla anguilla*) and the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 48, 327–342.

Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp. Biochem. Physiol. C 133, 287–303.

Grosell, M., Gerdes, R.M., Brix, K.V., 2006. Chronic toxicity of lead to three freshwater invertebrates — *Brachionus calyciflorus*, *Chironomus tentans*, and *Lymnaea stagnalis*. Environ. Toxicol. Chem. 25 (1), 97–104.

Henry, R.P., 1991. Techniques for measuring carbonic anhydrase activity *in vitro*. In: Dodgson, S.J., Tashian, R.E., Gros, G., Carter, N.D. (Eds.), Carbonic Anhydrases: Cellular Physiology and Molecular Genetics. Plenum, New York, pp. 119–131.

Khangarot, B.S., Das, S., 2010. Effects of copper on the egg development and hatching of a freshwater pulmonate snail *Lymnaea luteola* L. J. Hazard. Mat. 179, 665–675.

Khangarot, B.S., Ray, P.K., 1988. Sensitivity of freshwater pulmonate snails, *Lymnaea luteola* L., to heavy metals. Bull. Environ. Contam. Toxicol. 41, 208–213.

Nebeker, A.V., Stinchfield, A., Savonen, C., Chapman, G.A., 1986. Effects of copper, nickel and zinc on three species of Oregon freshwater snails. Environ. Toxicol. Chem. 5, 807–811.

Patrick, M.L., Wood, C.M., 1999. Ion and acid–base regulation in the freshwater mummichog (*Fundulus heteroclitus*): a departure from the standard model for freshwater teleosts. Comp. Biochem. Physiol. A 122, 445–456.

Perez, K.E., Minton, R.L., 2008. Practical applications for systematics and taxonomy in North American freshwater gastropod conservation. J. N. Am. Benthol. Soc. 27 (2), 471–483.

Richards, J.G., Playle, R.C., 1998. Cobalt binding to gills of rainbow trout (*Oncorhynchus mykiss*): an equilibrium model. Comp. Biochem. Physiol. C 119, 185–197.

Rogers, J.T., Richards, J.G., Wood, C.M., 2003. Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 64 (2), 215–234.

Rogevich, E.C., Hoang, T.C., Rand, G.M., 2008. The effects of water quality and age on the acute toxicity of copper to the Florida apple snail, *Pomacea paludosa*. Arch. Environ. Contam. Toxicol. 54, 690–696.

Rogevich, E.C., Hoang, T.C., Rand, G.M., 2009. Effects of sublethal chronic copper exposure on the growth and reproductive success of the Florida apple snail (*Pomacea paludosa*). Arch. Environ. Contam. Toxicol. 56, 450–458.

Schlekat, C.E., Van Genderen, E.J., De Schamphelaere, K.A.C., Antunes, P.M.C., Rogevich, E.C., Stubblefield, W.A., 2010. Cross-species extrapolation of chronic nickel Biotic Ligand Models. Sci. Tot. Environ. 408, 6148–6157.

Scientific, Tidepool, 2010. CETIS — Comprehensive Environmental Toxicity Information System. McKinleyville, California.

Shahsavarani, A., McNeill, B., Galvez, F., Wood, C.M., Goss, G.G., Hwang, P.P., Perry, S.F., 2006. Characterization of a branchial epithelium calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 209, 1928–1943.

SPSS, 2002. SigmaStat 3.0. SPSS, Chicago, Ilinois.

Stephan, C.E., Mount, D.I., Hansen, D.J., Gentile, J.H., Chapman, G.A., Brungs, W.A., 1985. Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. Duluth, U.S. Environmental Protection Agency, Environmental Research Laboratory. 98 pp.

USEPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. U.S. Environmental Protection Agency, Cincinatti, Ohio. 350 pp.

USEPA, 2007. Aquatic Life Ambient Freshwater Quality Criteria — Copper. Environmental Protection Agency, Office of Water, Washington, D.C., U.S. 204 pp.

USEPA, 2009. National Recommended Water Quality Criteria. Washington, D.C., United States Environmental Protection Agency. 22 pp.

Verdouw, H., van Echteld, C.J.A., Dekker, E.M.J., 1978. Ammonia determination based on indophenol formation with sodium salicylate. Wat. Res. 12, 399–402.

Wilkie, M.P., 1997. Mechanisms of ammonia excretion across fish gills. Comp. Biochem. Physiol. A 118, 39–50.

Wood, C.M., 2001. Toxic responses of the gill. In: Schlenk, D.W., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, Volume 1 — Organs. Taylor and Francis, Washington, D.C, pp. 1–89.