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Mechanisms of waterborne Cu toxicity to the pond snail *Lymnaea stagnalis*: Physiology and Cu bioavailability

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ABSTRACT

We examined the mechanisms of toxicity of waterborne Cu to the freshwater pond snail *Lymnaea stagnalis*. The snail is one of the most sensitive species to acute Cu exposure (96 h LC_{50}, LC_{20}: 24.9, 18.0 $\mu g\,l^{-1}$); they are not protected by the water quality criteria of the US EPA. Tissue Na and Ca were also reduced by Cu in the acute exposure. In contrast, during 28 d chronic exposures to Cu in the presence of food, which resulted in higher DOC concentrations, there was no significant mortality but an inhibition of growth, which may reflect a re-allocation of resources to detoxification. Cu detoxification was evidenced in chronic exposure by increases in metallothionein-like protein concentrations and Cu binding to metal-rich granules, decreases in thiobarbituric acid-reactive substances, and changes in the subcellular distribution in the soft tissues. Our results demonstrated that apart from external Cu bioavailability, compartmentalization of metals within the cells can alter toxicity of Cu to the snails.

1. Introduction

Aquatic organisms are often challenged to maintain hyperosmotic internal conditions in freshwaters with relatively low ion concentrations. Maintaining ionic balance is especially important in gastropods that need calcium for shell formation, a process in which carbonic anhydrase plays a key role (Takaichi et al., 2003). Growth of gastropods, due to their high Ca requirement, may be sensitive to metals, for metals are well known to interfere with Ca transport and/or carbonic anhydrase function in other invertebrates (Christensen and Tucker, 1976; Vitale et al., 1999; Skaggs and Henry, 2002). Historically, water quality criteria are designed to protect sensitive species such as rainbow trout, fathead minnow, *Daphnia* or *Ceriodaphnia*, and these are the species which are often used in test protocols (Weber, 1991). Relatively few data are available for freshwater gastropods, though in recent years, pulmonate snails have come under scrutiny.

Pulmonate freshwater snails of the genus *Lymnaea* are found in lentic systems where they play a pivotal role in the consumption and decomposition of aquatic plants and epiphyton (Barnes, 1987). *Lymnaea palustris* was shown to be highly sensitive to Pb (Borgmann et al., 1978; Grosell et al., 2006), and this has been recently confirmed in *Lymnaea stagnalis*, with a Pb EC_{20} less than 4 $\mu g\,l^{-1}$ (Grosell and Bianchard, 2006; Grosell and Brix, 2009). The newly hatched snails exhibited greatly reduced growth. Physiological evidence has demonstrated that Ca influx in the snails is inhibited (Grosell and Brix, 2009), resulting in lower soft tissue Ca concentrations and reduced shell formation. The high Ca uptake by the snails (an order of magnitude higher than in comparably sized fish) likely explains their hypersensitivity to Pb. Similarly, the growth rate of *L. stagnalis* was impaired and the concentration of Ca in the hemolymph was reduced when the animals were exposed to 79 $\mu g\,l^{-1}$ Co, which often acts as a Ca antagonist (De Schamphelaere et al., 2008). *Lymnaea sp.* do not seem to be as sensitive to Cd, often considered to be another Ca antagonist, although high concentrations can inhibit their growth, fecundity and fertility (Cd EC_{50}: 60–142 $\mu g\,l^{-1}$) (Coeurdassier et al., 2003).

Acute and chronic toxicity of Cu have been extensively studied in the Florida apple snail (*Pomacea paludosa*). Survival and growth were reduced by Cu taken up from the soil or Cu leached from soil to the water (Hoang et al., 2008b). Most Cu was located in the soft tissue with a higher percentage accumulated in the viscera than the foot (Hoang et al., 2008a) and among the visceral organs, kidney, liver and stomach/digestive glands showed an increase in accumulation as Cu concentration rose from 8 to 20 $\mu g\,l^{-1}$ (Pyatt et al., 2003). In general, the 96 h LC_{50} increased with the snail's age (Rogevich et al., 2008). No physiological investigations on the toxicity of Cu to this species have been reported so far.

The bioavailability and toxicity of metals to aquatic organisms are dependent on water chemistry factors such as hardness,

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salinity, specific ion levels, pH, alkalinity, complexing agents and dissolved organic carbon (DOC) (Di Toro et al., 2001; Santore et al., 2001). Therefore, the same metal concentration in waters of different chemistry may result in differential toxicity. For example, increasing pH from 5.5 to 8.5 decreased toxicity of Cu to the Florida apple snail, which can be explained by a model prediction of lower Cu-free ions in the water as pH increased above 7 (Rogevich et al., 2008). In addition, Cu toxicity to the snails decreased as dissolved organic carbon (DOC) increased from 0.2 to 3 mg C l^{-1}, but hardness had no effect on Cu toxicity (Rogevich et al., 2008). Likewise, internal metal bioavailability is important because toxicity is not related to the total accumulated metal concentration but rather to the threshold concentration of internal metabolically available metal (Rainbow, 2007). The subcellular partitioning of metals within aquatic organisms can provide valuable information on this aspect.

Our objectives were to examine the mechanisms of acute and chronic toxicity of waterborne Cu to *L. stagnalis*. Mechanisms have been characterized in the context of Cu bioavailability (external and internal) and physiological responses of the snails to Cu (tissue Na, tissue and/or shell Ca levels, growth, feeding rate, cellular oxidative stress and detoxification protein induction).

2. Materials and Methods

2.1. Snail culture

A starter culture of the freshwater great pond snail (*L. stagnalis*) was obtained from a mix of the U.S. and Canadian sources (listed in Acknowledgments). The snails were held in aerated aquaria with a flow-through of dechlorinated moderately hard water (hardness = 123 mg l^{-1} as $CaCO_3$, alkalinity = 95 mg l^{-1}, pH = 8.1; Na = 600 μmol l^{-1}; Ca = 900 μmol l^{-1}; Mg = 300 μmol l^{-1} and Cl = 800 μmol l^{-1}) at 22–24 °C under a photoperiod of 16 h light:8 h dark. Fresh romaine lettuce and carrots were fed to the snails regularly. Snail juveniles (30–40 d post-hatch) were used for experiments after more than 3 generations (each generation ~2 months) had been reared in the laboratory.

2.2. Acute exposure (96 h)—without food

Juveniles (1.9–2.3 cm) were fasted for one day before exposure to avoid the accumulation of feces in the test waters and the binding of Cu to the particles. Different concentrations of Cu (nominal: 0, 20, 40, 60, 80 and 100 μg l^{-1}) were prepared from a primary Cu stock (1 g Cu l^{-1} as $CuSO_4 \cdot 5H_2O$) diluted to 800 ml with dechlorinated freshwater, then left in acid-prewashed 1 L glass beakers for 1 d to equilibrate. Six snails were transferred to each beaker the next day, and each concentration was tested in duplicate at 22 °C. The test beakers were lightly aerated, and food was withheld throughout the 96 h exposure. Water was renewed daily with similarly aged test solutions. Water was sampled regularly and passed through a 0.45 μm Acrodisk in-line-syringe-tip filter and acidified for Cu, Ca, Mg and Na measurements. Survival was checked at 96 h by touching the foot of snails with a dissecting probe. Death was defined by a lack of response to this stimulus. Surviving snails were rinsed with dechlorinated freshwater and stored at −20 °C for later analysis of Cu, Ca and Na concentrations in the soft tissues.

2.3. Chronic exposure (28 d)—with food

2.3.1. Exposure

Juveniles (size range: 1.2–1.5 cm) were again fasted for a day before exposure. Cu solutions (nominal: 0, 5, 10, 20 and 35 μg l^{-1}) were similarly prepared as in the acute exposure. These concentrations were selected because they are environmentally relevant and the measured concentrations are lower than the 96 h LC_{50} determined in the acute exposure. Ten snails were blotted dry and weighed, then placed into each beaker on day 0. Three replicate beakers were used for survival tests and 2 replicates for bioaccumulation analyses at each concentration, at 21 °C. About 2.5 g lettuce was fed to the snails in each beaker every 2 d when the Cu solution was renewed. As in the acute exposure, water was filtered (0.45 μm) and sampled regularly for analysis of Cu, Ca, Mg, Na and dissolved organic carbon (DOC) during the exposure. Both the samples were taken immediately after water renewal (before food addition) and after food was provided for 2 d. Every week, survival, bulk wet weight, Cu bioaccumulation and feeding rate were measured. One snail was sampled from each bioaccumulation beaker and transferred into clean water for 1 d for rinsing of surface-bound Cu and gut purging. It was then stored at −20 °C for tissue analysis of metals. On day 28, surviving snails were frozen at −80 °C and soft tissue was used for measuring the subcellular Cu distribution and concentrations of metallothionein-like proteins (MTLP) and thiobarbituric acid-reactive substances (TBARS).

For measurement of specific growth rates (SGR), the snails were randomly placed into beakers in groups of 10 for the survival and bioaccumulation tests (a total of 5 beakers each treatment). Total wet weight (soft tissue + shell) was measured in each group every week for calculating the specific growth rate ($n = 5$). SGR in each test beaker was calculated as

$$SGR = 100\% \times [Ln(W_1)\text{-}Ln(W_0)]/t$$

where W_1 and W_0 are the start and end bulk weight (g) in each growth period, respectively, and t is the time interval in days.

2.3.2. Feeding rate

Either one pre-weighed snail (Weeks 1 and 2) or groups of three pre-weighed snails (subsequent weeks) were randomly chosen from each treatment and transferred to each of eight beakers pre-equilibrated with 100 ml of corresponding control or Cu solution for the feeding test. Exactly 1 g of lettuce was fed to the snails in each beaker. Two replicates of beakers with lettuce but no snails were used as the negative control. After 24 h, all snails were transferred back to the original exposure beakers and uneaten lettuce was rinsed in clean freshwater and dried at 65 °C for 2 d. The amount of lettuce eaten was calculated by subtracting the dry weight of uneaten lettuce from the dry weight of the negative control lettuce. Feeding rate was expressed in g lettuce (dry wt.)/g whole body wet weight/d. On day 21, uneaten and dried lettuce from the feeding test was also analyzed for Cu.

2.3.3. The 96 h survival test (without food) after chronic exposure

The snails were observed to be more tolerant to Cu in the chronic exposure (with food) than in the acute exposure (without food) (see Results). To explore the role of food in reducing the toxicity of Cu to the snails, 10 snails from each treatment were exposed to corresponding Cu concentrations (nominal: 0, 5, 10, 20 and 35 μg l^{-1}) in water for 96 h immediately after the 28 d chronic exposure. Food was withheld and water was renewed every day. Survival was recorded at 96 h.

2.3.4. Thiobarbituric acid-reactive substances (TBARS)

Four individuals per treatment were used for this assay, each analyzed separately. Soft tissue was first weighed, then homogenized in 3 ml buffer (20 mM Tris, 2 mM 2-mercaptoethanol and 0.2 mM phenylmethanesulfonylfluoride, pH 8.60) (4 individuals per treatment). Subsequent procedures followed those described in the TBARS Assay Kit (Cayman Chemical Company). The TBARS that were formed under high temperature (90–100 °C) and acidic conditions were measured colorimetrically at 530 nm.

2.3.5. Metallothionein-like proteins (MTLP)

MTLP concentrations in the soft tissue (5 juveniles per treatment) were measured according to the spectrophotometric procedures described in Viarengo et al. (1997). Validation exercises illustrated that this method avoids oxidation of sulfhydryl groups, contamination by low molecular weight thiols and enzymatic protein degradation of the samples, and that the amount of SH-proteins not from metallothionein is minimal. Some caution is needed in interpreting MTLP results since the method may not be as precise as differential pulse polarography (DPP) for measuring metallothionein, Nevertheless, it is widely used as a simple, low-cost and reproducible method for ecotoxicological studies in aquatic species (Rodriguez-Ortega et al., 2002; Falfushynska et al., 2010; Pytharopoulou et al., 2008). Soft tissue was weighed, homogenized in 3 ml buffer (500 mM sucrose, 20 mM Tris, 0.5 mM phenylmethanesulfonylfluoride and 1.3 mM 2-mercaptoethanol, pH 8.60), followed by two sequences of extraction and centrifugation to concentrate the MTLP. The concentration of sulfhydryl groups (which comprise the main functional groups in MTLP) in the homogenate was measured at 405 nm by reaction with 5, 5-dithio-bis 2-nitrobenzoic acid. A calibration curve was established using glutathione standard (Sigma-Aldrich) and the ratio of sulfhydryl to MTLP concentration was determined using the metallothionein-2 standard from rabbit liver (Alexis Biochemicals).

2.3.6. Subcellular Cu distribution

Subcellular fractionation of soft tissue generally followed the procedures of Wallace et al. (2003), except for addition of washing and re-centrifugation in each step. Preliminary experiments proved that these procedures improved the purity of organelle and cytosol fractions. Soft tissue was weighed, homogenized in 3 ml buffer (same buffer as for the TBARS measurement), then centrifuged at 1450 g at 4 °C for 15 min (5 individuals per treatment). The pellet was washed with buffer and re-centrifuged at the same speed. The collected supernatant was centrifuged at 100,000 g, 4 °C, for 1 h for separation of the organelles (ORG) and cytosol. About 0.5 ml KCl (0.15 M) was added to wash the pellet, followed by re-centrifugation at the same speed. Cytosol was then heated at 80 °C for 15 min to denature the

heat-denaturable protein (HDP), which was separated from the metallothionein-like protein (MTLP) by centrifugation at 50,000 g for 10 min. About 2 ml NaOH (1 N) was added to the pellet after the 1450 g spin, then heated at 80 °C for 15–30 min. The mixture was spin at 5000 g for 10 min to collect the metal-rich granules (MRG) and cellular debris (CD). Recovery of Cu from the fractionation was 93.8 ± 5.1% (sum of Cu in each fraction × 100%/Cu in homogenate). Note that some caution is needed in interpreting results with this method, since the data of Braigigand and Berthet (2003) examining Ag concentrations in the MT of the oyster *Crassostrea gigas* indicated that some metal may redistribute to other fractions when the cytosol is heat-denatured.

2.4. Measurement of metal and DOC concentrations

Cu concentrations were measured in the water, lettuce, soft tissue and subcellular fractions of the snails. Water samples were diluted in 1% HNO_3, whereas other samples were digested in 70% HNO_3 at 65 °C for 1 d, followed by dilution in 1% HNO_3 before measurement by means of a furnace atomic absorption spectrometer (GFAAS, Varian Spectra AA-20 with graphite tube atomizer [GTA-110], Mulgrave, Australia). Samples were also diluted in 1% HNO_3 for Na analysis (water and digested tissue) and 1% HNO_3 with 1% La for Ca (water, digested tissue and shell) and Mg (water) analyses by the flame atomic absorption spectrometer (FAAS, Varian Spectra-220FS, Mulgrave, Australia). Certified reference material (TM15, National Water Research Institute, Environment Canada) was used for internal quality checks and recovery was always within 20%. DOC was measured in 1% HNO_3 by means of a total organic carbon analyzer (Shimadzu TOC-VCPH, Mandel Scientific Company Inc., Guelph, ON, Canada) with 3 min sparge, using the program criterion of agreement of 2 out of 3 injections. Total carbon standard (certified potassium hydrogen phthalate, Nacalai Tesque Inc., Kyoto, Japan) was checked once in every 10 samples.

2.5. Statistical analysis and reporting of results

Measured rather than nominal Cu concentrations have been reported throughout, and all calculations are based on measured values. While measured values were often less than nominal concentrations due to the well-known phenomenon of Cu adsorption to container surfaces, day-to-day variations in concentration did not exceed 4%. Data have been presented as means ± 1 standard error. For the acute test, the 96 h LC_{50} and LC_{20} values with 95% confidence intervals were calculated from observed responses and measured dissolved Cu concentrations using ToxCalc—Toxicity Data Analysis Software v5.0.32 (Tidepool Scientific Software, McKinleyville, USA). One-Way ANOVA was used to test for the effect of Cu among groups at a specific time whereas Two-Way ANOVA was used to test for the main effects of Cu and exposure time, and for interaction between Cu and time for tissue metal concentrations in the chronic exposure. The Tukey *post-hoc* test was employed to identify specific differences. Linear or non-linear regression analyses, as appropriate, were performed to describe relationships between tissue Ca or Na concentrations and Cu exposure concentrations, as well as between Cu concentrations in subcellular fractions, and Cu concentrations in soft tissue. Percentage values of SGR were arcsine-transformed before statistical analysis. Significance of all tests was taken at $p < 0.05$.

3. Results and discussion

3.1. Acute exposure (96 h)—without food

3.1.1. Survival

The 96 h LC_{50} and LC_{20} of Cu were 24.9 μg l^{-1} (95% CI: 19.6–30.4) and 18.0 μg l^{-1} (95% CI: 12.1–22.3), respectively, in water with hardness of 123 mg l^{-1} as $CaCO_3$ (Ca = 924.7 ± 8.4 μmol l^{-1}; Mg = 311.3 ± 1.6 μmol l^{-1} and Na = 641.6 ± 5.1 μmol l^{-1}). Therefore, the snail is more sensitive to Cu than the rainbow trout (96 h LC_{50}: 91 μg l^{-1}) (Taylor et al., 2000) and fathead minnows juveniles (96 h LC_{50}: 60 μg l^{-1}) (Erickson et al., 1997) under similar water chemistry. *L. stagnalis* is also more sensitive than the Florida apple snail at a similar age (30 d old), to Cu (96 h LC_{50}: 44.8–141.7 μg l^{-1}, water hardness about 65 mg l^{-1} as $CaCO_3$) (Rogevich et al., 2008). However, they are less sensitive than the cladoceran *Daphnia magna* (48 h LC_{50}: 16 μg l^{-1} in water hardness of 250 mg l^{-1} as $CaCO_3$) (Canli, 2006). Overall *L. stagnalis* appears to be one of the most sensitive species to acute Cu exposure in moderately hard water. The USEPA (2008) criterion maximum concentration for Cu (water quality criteria for acute toxicity) at this water chemistry was 14.6 μg l^{-1} (as predicted by the HydroQual Inc. Biotic Ligand Model (BLM) version 2.2.3, using the preceding concentrations for Ca, Mg and Na, plus the following additional input parameters: DOC = 1.85 mg C l^{-1} (10% as humic acid); alkalinity = 95 mg l^{-1} as $CaCO_3$; S^{2-} = 5 nmol l^{-1}; SO_4^{2-} = 300, Cl^- = 700 and K^+ = 400 μmol l^{-1}). This water quality criterion is developed for the protection of all aquatic species, but at this concentration, 20% of the snails would have been killed. Therefore, the US EPA ambient water quality criteria (USEPA, 2008) do not protect this species from acute exposure to Cu. However, *L. stagnalis* are well protected by the Environment Canada (2007) CCME guidelines of 3 μg l^{-1} at hardness = 120–180 mg l^{-1} as $CaCO_3$. They would also appear to be protected by new guidelines due to be implemented in the European Union this year, where the predicted no effect concentration (PNEC) has been set to 7.8 μg l^{-1} (pers. comm., K. Lacasse, European Chemical Institute).

3.1.2. Tissue Ca, Na and Cu concentrations

Exposure to increasing concentrations of Cu for 96 h caused an exponential decline in tissue concentrations of both Na and Ca, though with different relationships (Fig. 1, $r^2 \geq 0.97$, $p \leq 0.02$ for both). As in fish, the Na loss (Zahner et al., 2006; Van Genderen et al., 2008) may be caused by the disruption of Na^+/K^+ ATPase activity, leading to a net Na efflux (Playle et al., 1993). A physiological model for Ag (Paquin et al., 2002), a similar Na-specific toxicant, suggests that mortality occurs when an organism has lost approximately 30% of its exchangeable Na pool. However, this does not seem to apply to Cu effects on the snail, as mortality started to occur at 14.7 μg l^{-1} which corresponded to more than a 50% loss of Na (Fig. 1). Tissue Na concentration was a more sensitive indicator of Cu toxicity than tissue Ca concentration, as the average effective concentration of Cu to reduce the tissue Na concentration by 50% was 10.5 μg l^{-1} ($EC50_{Na}$), two-fold lower than that for a comparable reduction of Ca, 24.5 μg l^{-1} ($EC50_{Ca}$), where these EC_{50} values were calculated from the respective regression equations.

Cu was accumulated significantly in the soft tissues of snails exposed to 14.7 μg l^{-1} (55.5 ± 6.0 μg g^{-1} wet wt) and 26.5 μg l^{-1} (46.6 ± 4.0 μg g^{-1} wet wt) compared to the control animals (13.5 ± 0.9 μg g^{-1} wet wt), however, no statistics can be run for the single surviving snail in 46.8 μg l^{-1} (33.2 μg g^{-1} wet wt)

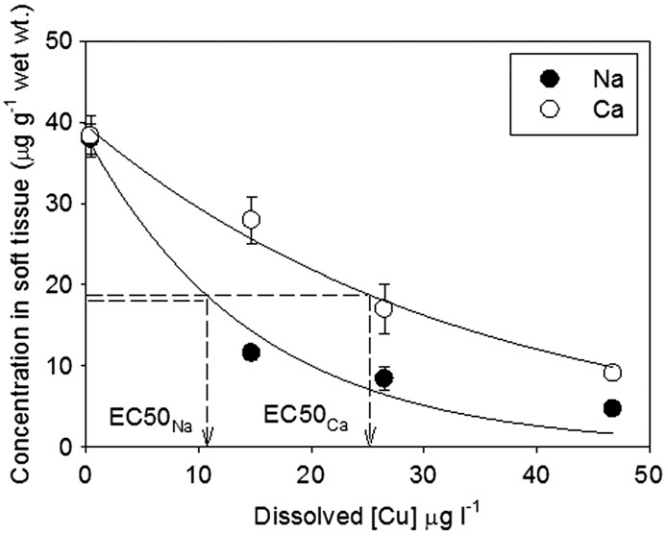

Fig. 1. Soft tissue Ca and Na concentrations (μmol g^{-1} wet wt.) in the snails at 96 h in the acute exposure to Cu. Means ± 1 standard error (n = 2–8). Exponential curves were fitted to the data and tested for significance at $p < 0.05$ (*) by regression analysis. $EC50_{Ca}$ and $EC50_{Na}$: effective concentrations of Cu (μg l^{-1}) to reduce tissue concentrations of Ca and Na, respectively, by 50% (values calculated from regression equations and reported in the text).

(data not shown). The snails accumulated similar levels of Cu in their soft tissues from this 96 h waterborne exposure as in the 18 h diet-borne exposure of Croteau and Luoma (2008). In that study, the snails accumulated about 38 μg Cu g^{-1} tissue wet wt. when exposed to a diet with a Cu concentration (318,000 μg Cu kg^{-1}) which was approximately 4 orders of magnitude higher than the waterborne concentrations used in the present study. These data suggest that the bioavailability of Cu from the diet is far lower than from the water. In general, there was not a strong relationship between tissue Cu burden and toxicity. The apparent disconnect between bioaccumulated Cu and toxicity might be explained if the measured Cu concentrations comprise, in large part, Cu that is non-selectively bound to sites that did not participate in the toxic response. Indeed this is a conclusion which is becoming increasingly drawn in the tissue residue literature (Adams et al., 2011).

3.2. Chronic exposure (28 d)—with food

3.2.1. Survival, growth and feeding rates

Despite the high sensitivity of snails to acute Cu exposure, they were much less sensitive in the chronic exposure, except for sublethal effects observed on growth. Survival of all snails remained high in the chronic exposure to Cu at day 28 (86.7–100%) when food was present, and the 28 d LC_{50} and LC_{20} values were estimated to be higher than the highest Cu concentration tested, 18.2 μg l^{-1} (water chemistry is reported in "no food" and "with food" conditions in Fig. 4). In general, SGR became lower throughout the exposure period (Table 1). In the first week, snails exposed to 11.2 and 18.2 μg l^{-1} Cu had only half the growth rate of the control snails, but they increased growth in week 2 (2-fold faster growth than the control). In Week 3, snails in the highest Cu treatment did not grow significantly among the five replicate beakers. Growth rate became comparable in Week 4 in all treatments. In this study, the snails were fed lettuce every 2 d. Very often, remains of lettuce were observed in between food renewal, therefore, underfeeding is not a factor that would confound the Cu effects on growth. Initial whole body wet weight of the snails at Week 0 was similar among treatments ($p > 0.05$).

Table 1
Specific growth rate (% d^{-1}) and feeding rate (g g^{-1} whole body wet weight d^{-1}) of snails in the chronic exposure to Cu. Mean ± 1 standard error (SGR: $n=5$; feeding rate: $n=8$). ND: feeding rate could not be determined in Week 2. Within the same week, groups sharing the same letter are not significantly different at $p < 0.05$.

Exposure period	Dissolved Cu	SGR	Feeding rate
Week 1	Control—2.3 μg l^{-1}	2.9 ± 0.2^{A}	0.03 ± 0.004^{A}
	4.4 μg l^{-1}	3.3 ± 0.3^{A}	0.03 ± 0.01^{A}
	6.4 μg l^{-1}	2.8 ± 0.2^{A}	0.03 ± 0.01^{A}
	11.2 μg l^{-1}	1.6 ± 0.3^{B}	0.04 ± 0.004^{A}
	18.2 μg l^{-1}	1.5 ± 0.2^{B}	0.03 ± 0.01^{A}
Week 2	Control—2.3 μg l^{-1}	1.3 ± 0.2^{A}	ND
	4.4 μg l^{-1}	1.5 ± 0.1^{A}	ND
	6.4 μg l^{-1}	1.3 ± 0.1^{A}	ND
	11.2 μg l^{-1}	2.4 ± 0.2^{B}	ND
	18.2 μg l^{-1}	2.9 ± 0.1^{B}	ND
Week 3	Control—2.3 μg l^{-1}	0.9 ± 0.2^{AB}	0.02 ± 0.001^{A}
	4.4 μg l^{-1}	0.9 ± 0.2^{AB}	0.02 ± 0.001^{A}
	6.4 μg l^{-1}	1.4 ± 0.2^{A}	0.02 ± 0.001^{A}
	11.2 μg l^{-1}	0.6 ± 0.1^{B}	0.02 ± 0.01^{A}
	18.2 μg l^{-1}	-0.5 ± 0.2^{C}	0.02 ± 0.002^{A}
Week 4	Control—2.3 μg l^{-1}	1.4 ± 0.5^{AB}	0.02 ± 0.002^{A}
	4.4 μg l^{-1}	0.6 ± 0.1^{A}	0.02 ± 0.001^{A}
	6.4 μg l^{-1}	0.8 ± 0.3^{AB}	0.02 ± 0.001^{A}
	11.2 μg l^{-1}	1.1 ± 0.1^{AB}	0.02 ± 0.001^{A}
	18.2 μg l^{-1}	2.1 ± 0.2^{B}	0.01 ± 0.002^{A}

Resulting from the effects on growth in Weeks 1 and 3, the whole body wet weight of snails in 6.4, 11.2 and 18.2 μg l^{-1} Cu was smaller compared to controls after 4 weeks (Fig. 2, Cu effect: $p < 0.01$; Week effect: $p < 0.01$; Cu x Week; $p = 0.38$). Previous studies on other metals have also demonstrated an adverse effect on growth, without mortality. For example, newly hatched snails were 60-fold smaller at the end of a 30 d exposure to 245 μg l^{-1} Pb, with an EC_{20} at 4 μg l^{-1} (Grosell et al., 2006). Growth of 30 d post-hatch snails were also reduced by 40–86% in the first 2 weeks, when exposed to 270 and 860 μg l^{-1} Co, followed by a negative growth in the last 2 weeks (De Schamphelaere et al., 2008). Apparently, the chronic inhibitory effect on growth was less extensive for Cu, compared to Pb and Co. The difference may be explained by exposures at different developmental stages. The newly hatched snails with faster growth rate (4.7–16.8% d^{-1} in the above studies) may be more susceptible to metals.

Croteau and Luoma (2008) reported the normal feeding rate of *L. stagnalis* was 0.145 g g^{-1} soft tissue dry weight d^{-1}. Their feeding rate is comparable (0.02 g g^{-1} whole body wet weight d^{-1}) to our study after converting the unit into per whole body wet weight (assuming wet weight of tissue: wet weight of shell + tissue = 0.7; wet weight of tissue: dry weight of tissue = 6). Diet-borne Cu has been reported to reduce food ingestion rate in *L. stagnalis* (Croteau and Luoma, 2008). In addition, Crichton et al. (2004) demonstrated that the feeding of *L. peregra* was inhibited, following a 48 h exposure to waterborne Cd. In the present study, feeding rate of the snails in all treatments became smaller in Weeks 3 and 4 (Table 1), which may relate to the slower growth in the later period of exposure. Feeding rate could not be determined in Week 2 from one snail in each beaker, therefore, no data have been reported. From Week 3, 3 snails were used in each beaker for feeding rate measurements. In general, feeding rate in snails was not significantly different among treatments ($p > 0.05$). Therefore, feeding rate is unlikely to be the controlling factor for the observed inhibitory effects of Cu on growth. Besides feeding inhibition, reduction in growth may be associated with a reduction in conversion of food energy to biomass (Hansen et al., 2004) and inhibition of digestive enzyme activities (Dedourge-Geffard et al., 2009; Kalman et al., 2009).

3.2.2. Tissue Ca, Na, Cu and shell Ca concentrations

Because of the requirements of shell formation, overall Ca uptake rate in *L. stagnalis* has been reported to be 7–8-fold higher than in trout of similar size, therefore, Ca regulation of the snail may be very sensitive to metal exposure (Grosell and Brix, 2009). However, soft

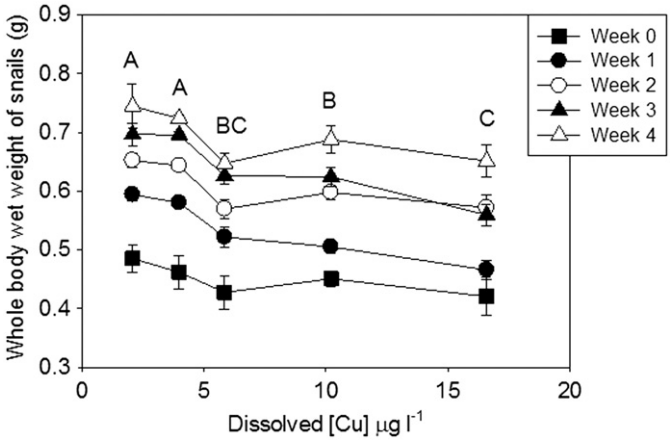

Fig. 2. Whole body wet weight (g) of snails in the chronic exposure to Cu. Means ± 1 standard error ($n=5$). Time, Cu and interaction of Cu and time effects were tested by Two-Way ANOVA, followed by the Tukey post-hoc test. Different letters indicate significant difference between groups over time at $p < 0.05$.

tissue Ca, Na and shell Ca concentrations in the snails during the chronic exposure were not significantly affected by Cu though all concentrations declined over time ($p < 0.01$) (Fig. 3), which may relate to growth dilution. Since changes in condition index were negligible (T. Ng, N. Pais, and C.M. Wood, unpubl. results), there is no evidence that the snails were underfed. In contrast, chronic Pb exposure suppressed net Ca uptake in *L. stagnalis* and resulted in a dose-dependent reduction in soft tissue Ca concentration (Grosell and Brix, 2009); it may thereby reduce $CaCO_3$ precipitation for shell formation. The soft tissue Na concentration was also reduced by Pb, which was interpreted as a compensatory intracellular pH regulation response to systemic alkalosis. Co also reduced Ca concentration in the hemolymph of the snails, which in turn may affect feeding and correlate with growth effects (De Schamphelaere et al., 2008). Overall, our results demonstrated that growth effects of Cu on the snails were not correlated to any effects on feeding and Ca metabolism. Therefore, the toxic mechanism of chronic Cu exposure may be different from Co and Pb.

Cu accumulation in the soft tissue increased as dissolved Cu concentration increased (Fig. 3c). Tissue Cu concentrations in the snails exposed to 11.2 and 18.2 $\mu g\ 1^{-1}$ were significantly higher than the controls ($p < 0.05$). However, Cu accumulation was lower in the chronic exposure ($9.3 \pm 0.5\ \mu g\ g^{-1}$ wet wt. at 18.2 μg Cu 1^{-1}) versus the acute exposure ($55.5 \pm 6.0\ \mu g\ g^{-1}$ wet wt. at 14.7 μg Cu 1^{-1}) under similar Cu exposure concentrations. This may be due to a lower Cu bioavailability or a regulation on Cu uptake and excretion rates by the snails over the chronic exposure period.

3.2.3. Cu bioavailability

Food was present only during the chronic exposures. In the real world, the presence of food is environmentally relevant as it is necessary for normal development and survival. Under conditions of toxicant stress, food may provide both an ion supplement and additional energy (D'Cruz et al., 1998; D'Cruz and Wood, 1998) to the animals, thereby compensating directly for ion losses and for the increased energy expenditures required to maintain ionic balance. In addition, food can increase ion and DOC concentrations in the water, effects that will potentially reduce Cu bioavailability to the snails by competition and complexation reactions (Santore et al., 2001). However, Cu may also bind to lettuce and be taken up by the snails from the diet. There were 34% and 33% increases in Na and Mg concentrations, respectively, in the water when lettuce was added for 2 d (Fig. 4a). DOC also increased about 12-fold during the period (Fig. 4b). DOC and ions may potentially come from the uneaten lettuce, feces of the snails or any dissolved ligands released by the snails during food digestion (De Schamphelaere et al., 2008). The measured Cu concentration in the romaine lettuce in control and low Cu treatments was in agreement with the value obtained by Hoang and Rand (2009) (Fig. 4C). Most of the Cu taken up by the snails was, therefore, probably from the water, except for the highest treatment ($18\ \mu g\ l^{-1}$) where a significantly higher amount of Cu was bound to the lettuce. Croteau et al. (2007) reported that a high percentage of Cu (84%) from the lettuce can be assimilated by *L. stagnalis* but the uptake rate constant from water is still 1000-fold higher than from diet. Therefore, Cu uptake from the diet may not be a major concern in this study.

3.2.4. The 96 h survival test (without food) after chronic exposure

A 96 h survival test (without food) on the 28 d surviving snails from each treatment, was run to determine the effect of food on Cu toxicity to the snails. If food provides the protection to the snails (e.g., via DOC or ion supplement), we might expect the survival of snails to fall significantly in the Cu treatments due to

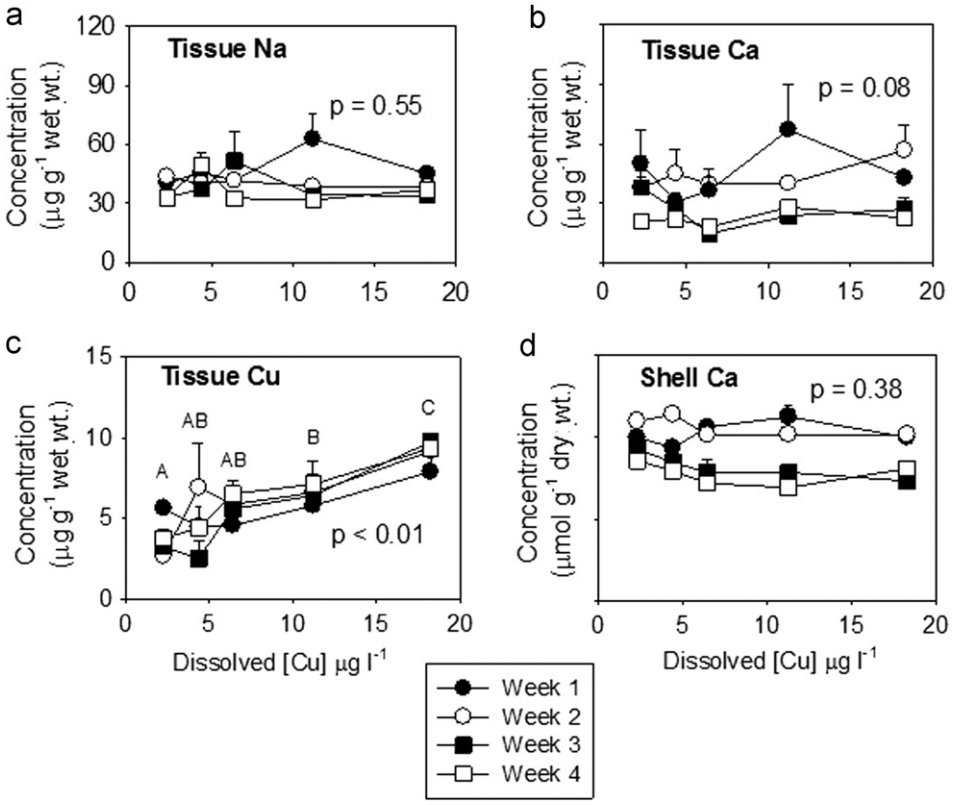

Fig. 3. Concentrations of (a) Na ($\mu mol\ g^{-1}$ wet wt.), (b) Ca ($\mu mol\ g^{-1}$ wet wt.), (c) Cu ($\mu g\ g^{-1}$ wet wt.) in the soft tissues of snails and (d) Ca concentrations in the shell ($\mu mol\ g^{-1}$ dry wt.) of the snails in the chronic exposure to Cu. Means $\pm$ 1 standard error (n=2–8). Time, Cu and interaction of Cu and time effects were tested by Two-Way ANOVA, followed by the Tukey post-hoc test. Different letters indicate significant difference between groups over time at $p < 0.05$.

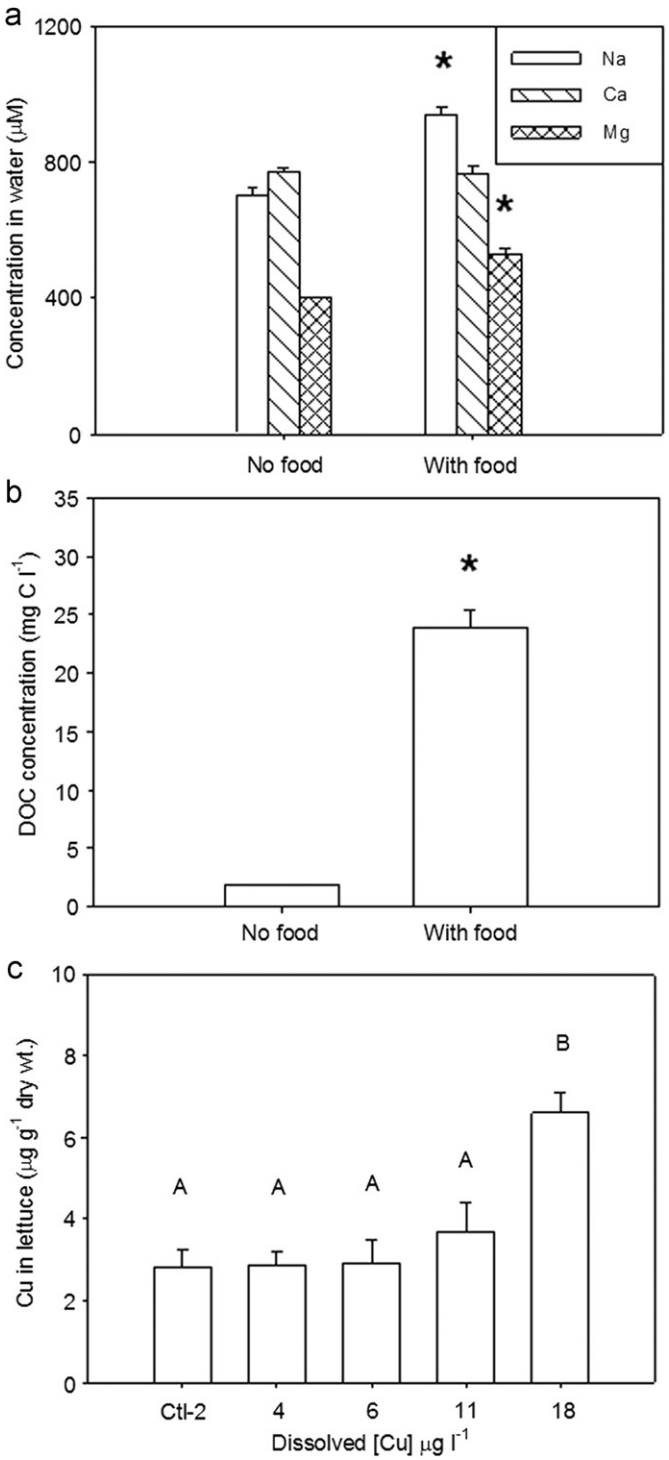

Fig. 4. Concentrations of (a) ions (Ca, Mg and Na, μmol l^{-1}), (b) dissolved organic carbon (DOC, mg C l^{-1}) in the water with and without food and (c) Cu burden of the lettuce after 2 d. Means ± 1 standard error (ion concentrations: $n=15$; DOC, $n=15$; Cu on lettuce: $n=8$). Effect of food was tested by One-Way ANOVA (> 2 groups), followed by the Tukey post-hoc test or Student's *t*-test (2 groups). Asterisks and different letters indicate significant difference between groups at $p<0.05$.

absence of food in this test. However, if they have already developed internal Cu detoxification mechanisms such as metallothioneins or metal-rich granule formation, the survival could remain similar after 96 h. The results demonstrated that survival was 90–100%, with no significant effects of Cu observed, so only the latter conclusion can be drawn. Therefore, detoxification mechanisms that have been triggered by the animals, perhaps resulting in a different compartmentalization of Cu within the cells (internal bioavailability) can alter toxicity of Cu to the snails.

3.2.5. TBARS and MTLP concentrations

A general pathway of chronic toxicity for many metals is the increased generation of reactive oxygen species, which modulate the occurrence of cell damage via lipid peroxidation (Gutteridge, 1995; Regoli et al., 2002). Metabolic products of lipid peroxidation that are thiobarbituric acid-reactive, are commonly used as a biomarker of oxidative stress e.g., malondialdehyde (MDA) (Almroth et al., 2008). To provide direct evidence for reduced oxidative stress and the development of Cu-detoxification mechanisms in the Cu-exposed snails from the chronic exposure, cellular endpoints—TBARS (an indicator of oxidative stress) and MTLP concentrations (a protein for detoxification) were measured at the end of the 28 d chronic exposure. A trend of lower TBARS concentration in the snails was observed in the higher Cu concentrations (Fig. 5a, $r^2=0.89$, $p=0.02$), and TBARS was significantly lower in the 11.2 and 18.2 μg 1^{-1} treatments compared to the control. These results suggest that there was no significant

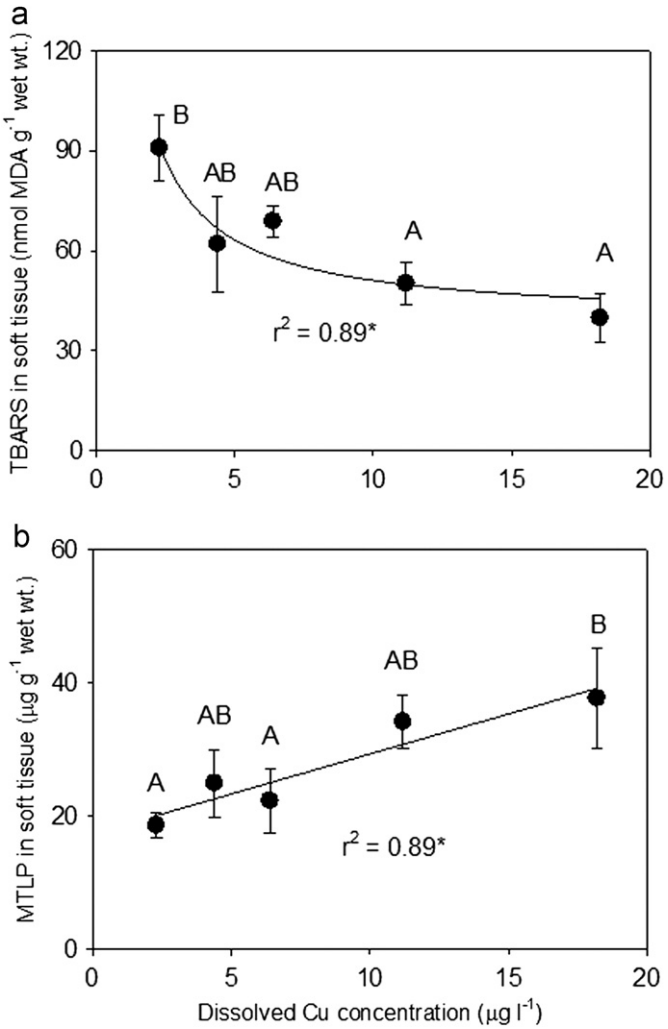

Fig. 5. (a) Thiobarbituric acid-reactive substances (nmol MDA g^{-1} wet wt) and (b) metallothionein-like protein concentrations (MTLP, μg g^{-1} wet wt) in the snail soft tissue at 28 d in the chronic exposure to Cu. Means ± 1 standard error (TBARS: $n=4$; MTLP: $n=5$). Exponential or linear curves were fitted to the data and tested by the regression analysis at $p<0.05$ (*). Differences between groups were tested by One-Way ANOVA, followed by the Tukey post-hoc test. Different letters indicate significant difference between groups at $p<0.05$.

oxidative stress caused by prolonged Cu exposure. This may be explained by the trend of higher MTLP concentrations and higher percentage of Cu associated with the metal-rich granule fraction (MRG) in the high Cu treatments (see below). Metallothionein induction is a common detoxification mechanism for metals in aquatic invertebrates (Amiard et al., 2006). For example, *L. stagnalis* induced metallothionein when they were exposed to high Cd concentrations (Leung, 2003) The MTLP concentration was higher in snails exposed to higher Cu concentrations (Fig. 5b, $r^2=0.89$, $p=0.02$), with significantly higher MTLP in the highest Cu treatment than in the snails exposed to 4.4 μg Cu 1^{-1} and control. In addition to MTLP, there is abundant evidence that snails can develop granules to sequester metals in biologically inactive forms (Howard et al., 1981; Dallinger and Berger, 1997; Desouky, 2006).

3.2.6. Subcellular Cu distribution

Changes in subcellular metal distribution can provide evidence of metal detoxification through altered intracellular partitioning and bioavailability. For example, in the marine snail, *Thais clavigera*, an increasing proportion of Cd was found in the MTLP with increasing time of exposure to dietary Cd (Cheung et al., 2006). Furthermore, there was an increase of granules or a marked upregulation of elemental P and S which binds metals with high affinity in the granules of *L. stagnalis* when they were exposed to Al, Cd and Zn (Desouky, 2006). In the present study, distribution of Cu in the subcellular fractions of the snails was in this order: ORG (32–43%) > MTLP (20–33%)~CD (16–33%) > HDP (3–8%) > MRG (2–6%) (Fig. 6). In the Cu-exposed snails, a significantly higher percentage of Cu was associated with MRG (Fig. 6b) and a lower percentage was associated with the HDP (Fig. 6c) and CD (Fig. 6e). In agreement with Desouky (Desouky, 2006), elemental composition of granules may have changed in response to Cu exposure, to increase the binding of Cu to the granules. Although the percentage of Cu in the MTLP of the Cu-exposed snails was not significantly changed (Fig. 6a), the Cu concentration in this fraction increased as total Cu burden in the soft tissue increased (Fig. 7b), coincident with the increase in MTLP concentration in Cu-exposed snails (Fig. 5b). The same trend was observed for the increase in Cu concentration in the MRG with increase in Cu total burden (Fig. 7a). Thus, both MTLP and MRG are important detoxification mechanisms as they reduced the association of Cu to HDP which is a metal-sensitive fraction. In fact, MTLP and MRG may be inter-related as metals bound to MTLP in the cytoplasm may be redistributed to the granules

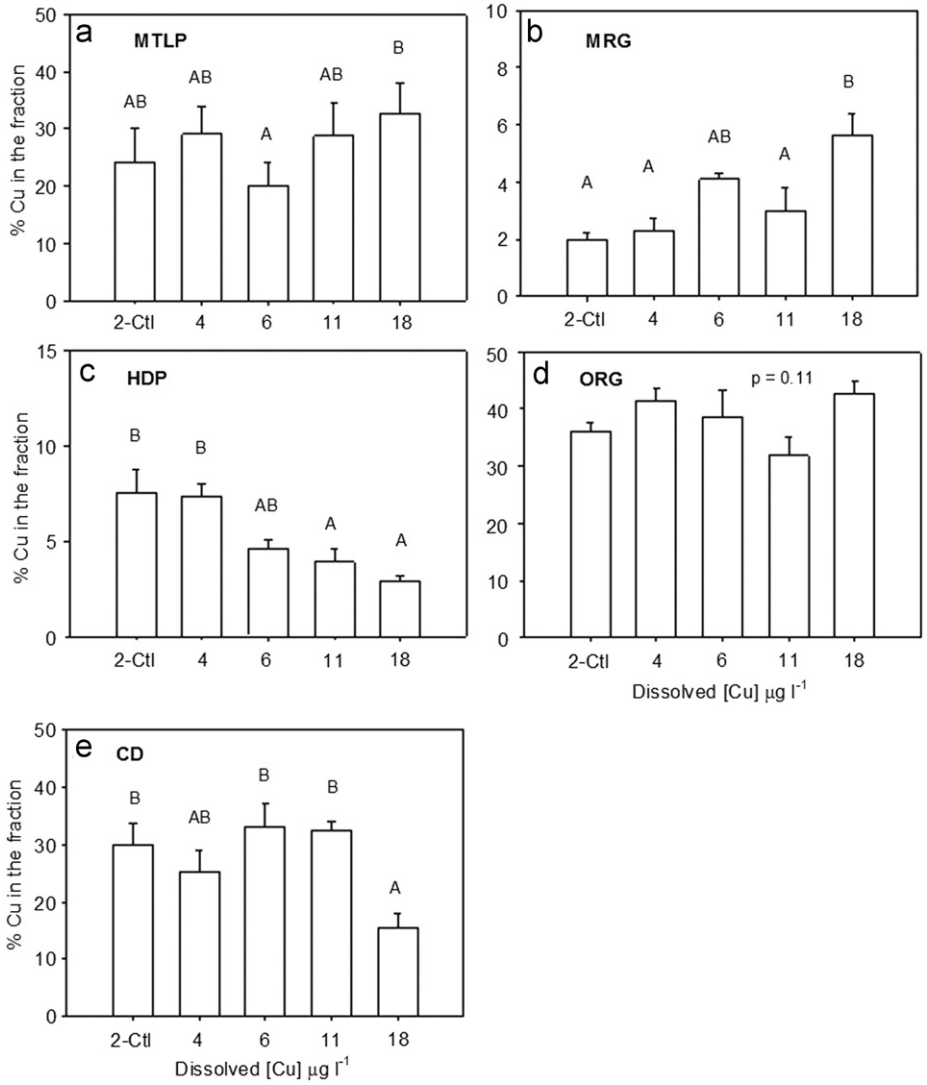

Fig. 6. Subcellular Cu distribution (%) in the soft tissue of snails in the chronic exposure to waterborne Cu on 28 d. Means ± 1 standard error ($n=5$). Differences between groups were tested by One-Way ANOVA, followed by the Tukey post-hoc test. Different letters indicate significant differences between groups at $p<0.05$. (a) MTLP: metallothionein-like protein; (b) MRG: metal-rich granules; (c) HDP: heat-denaturable protein; (d) ORG: organelles; and (e) CD: cellular debris.

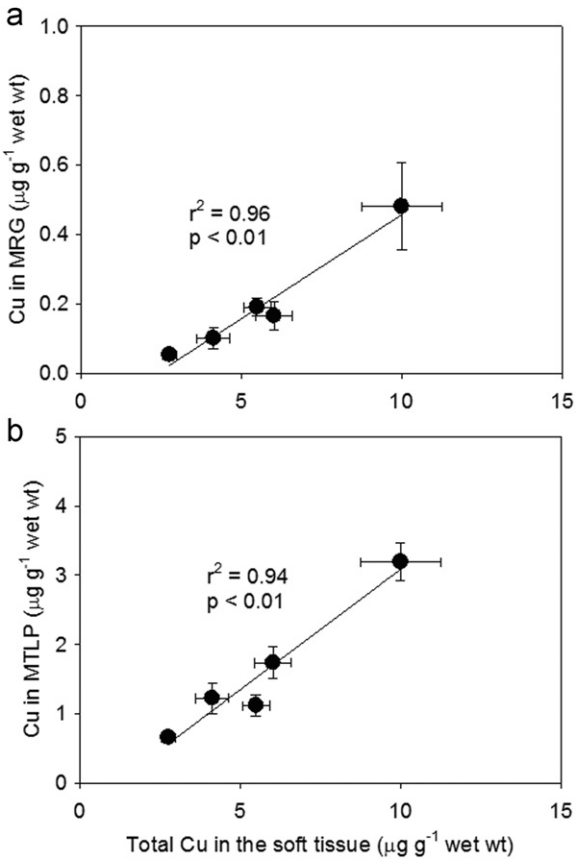

Fig. 7. Correlations of subcellular Cu concentration in (a) MRG and in (b) MTLP ($\mu g\, g^{-1}$ wet wt) with total Cu concentrations in soft tissue ($\mu g\, g^{-1}$ wet wt) after 28 d of chronic exposure to Cu. Means $\pm$ 1 standard error (subcellular: $n=5$; total Cu burden: $n=2$–8). The data were fitted with linear regression lines and tested for significance at $p<0.05$. MTLP: metallothionein-like protein and MRG: metal-rich granules.

for lysosomal degradation and excretion (Hopkin, 1989; Dallinger et al., 2000). Therefore, our results suggest that Cu associated with HDP does not correspond to toxicity when the level is below the threshold (Fig. 6C, Cu in HDP of control snails). MT (Fig. 6A) and MRG (Fig. 6B) reduced the amount of Cu binding to HDP by maintaining it below the threshold level, as the tissue concentration increased. Overall, the well-developed detoxification mechanisms in the snails may suggest that a significant amount of energy is relocated from normal physiological functions such as growth and reproduction, to detoxification (De Coen and Janssen, 2003).

4. Conclusions

The pond snail *L. stagnalis* is very sensitive to acute Cu exposure and not protected against acute toxicity by the current ambient water quality criteria of the USEPA (2008). Toxicity correlates with adverse effects on Na and Ca regulation. However, in the long-term chronic exposures, with the presence of food, the snails were less sensitive, and mechanisms of Cu detoxification were induced. Although there were no observed effects on survival, growth inhibition occurred. This was not related to any effects on feeding or Ca metabolism, in contrast to the mechanisms of effects caused by Pb and Co in early hatched juveniles. We hypothesize that the growth reduction caused by Cu is a result of reallocation of energy and resources to detoxification from growth. Thus, the adverse effect on growth may be an indirect, rather than a direct effect of Cu on physiology.

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