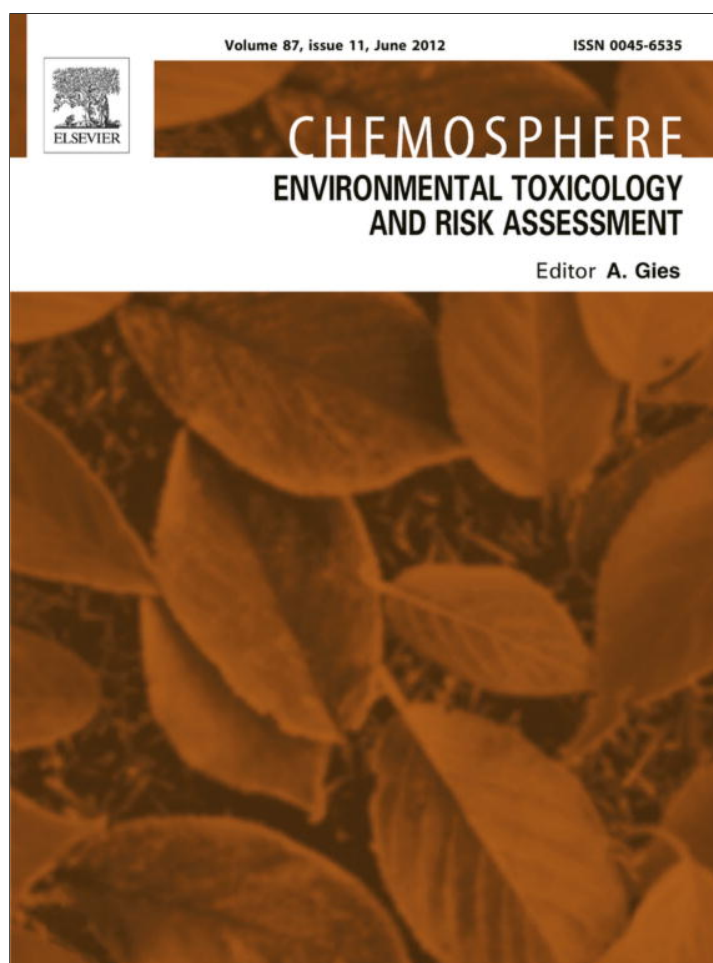


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Use of whole-body and subcellular Cu residues of *Lumbricus variegatus* to predict waterborne Cu toxicity to both *L. variegatus* and *Chironomus riparius* in fresh water

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ABSTRACT

We tested the use of whole-body and subcellular Cu residues (biologically-active (BAM) and inactive compartments (BIM)), of the oligochaete *Lumbricus variegatus* to predict Cu toxicity in fresh water. The critical whole-body residue associated with 50% mortality (CBR₅₀) was constant (38.2–55.6 μg g⁻¹ fresh wt.) across water hardness (38–117 mg L⁻¹ as CaCO₃) and exposure times during the chronic exposure. The critical subcellular residue (CSR₅₀) in metal-rich granules (part of BIM) associated with 50% mortality was approximately 5 μg g⁻¹ fresh wt., indicating that Cu bioavailability is correlated with toxicity:subcellular residue is a better predictor of Cu toxicity than whole-body residue. There was a strong correlation between the whole-body residue of *L. variegatus* (biomonitor) and survival of *Chironomus riparius* (relatively sensitive species) in a hard water Cu co-exposure. The CBR₅₀ in *L. variegatus* for predicting mortality of *C. riparius* was 29.1–45.7 μg g⁻¹ fresh wt., which was consistent within the experimental period; therefore use of Cu residue in an accumulator species to predict bioavailability of Cu to a sensitive species is a promising approach.

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1. Introduction

Exposure metal concentration is often a poor predictor of environmental threats to organisms (Borgmann et al., 1991). This is because toxicokinetics, toxicodynamics, environmental factors (e.g. pH, salinity, and temperature), mechanism of toxic action, and many other factors control the bioavailability and toxicity of metals (Meador et al., 2008). The tissue–residue approach (TRA) provides a link between measures of bioavailability and toxicity. It associates tissue concentration (i.e. residue) of chemicals with adverse biological effects in a dose–response fashion that is used to determine the critical whole-body residue (CBR) (McCarty and Mackay, 1993). The CBR is then used to develop tissue quality guidelines, which are translated into water or sediment guidelines with bioaccumulation factors. This is a complementary regulatory approach to another bioaccumulation-based toxicity model, the Biotic Ligand Model (BLM) which incorporates water quality parameters to make a theoretical calculation of whether a lethal burden of metal will accumulate on an organism's respiratory sur-

face, thereby predicting metal toxicity in different environments (Di Toro et al., 2001). The BLM is originally based on a tissue–residue approach, using short-term metal accumulation in the gill to predict 96-h toxicity (Playle et al., 1993). The BLM-based LA₅₀ (lethal accumulation associated with 50% mortality) is similar to the TRA-based CBR₅₀ (accumulation associated with 50% adverse effects).

Dose–response relationships between metal bioaccumulation and biological effects have been observed in fish (Tsai and Liao, 2006), amphipods (Norwood et al., 2003), copepods (Hook and Fisher, 2001) and oligochaetes (Meyer et al., 2002). The underlying principle of TRA is that the CBR is independent of exposure conditions and time (Fisher et al., 1999). This assumption is rarely tested in chronic conditions, especially in water with different hardness. Borgmann et al. (1991) demonstrated that chronic Cd toxicity to *Hyallela* in Lake Ontario (Canada) water with the additions of complexing agents and distilled water was much more constant if toxicity was expressed as a function of Cd bioaccumulated, rather than the concentration added or measured in the water. Furthermore, Meyer et al. (2002) showed that the CBR₅₀ of Cu in *Lumbricus variegatus* under acute exposure was constant in all pH × water hardness combinations. However, whole-body accumulation of metals does not always cause toxicity. Metals may distribute in biologically active pools (organelles, enzymes, proteins) and/or biologically inactive pools i.e., detoxification pools (granules and metallothionein) (Rainbow, 2002). Therefore, toxicity may be

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related to the threshold concentration of internal metabolically available metals (Rainbow, 2007). Couillard et al. (1995) and Perceval et al. (2006) demonstrated that subcellular metal distribution in bivalves can be linked to toxicity and exposure concentrations in freshwater environments. Application of subcellular metal residue analysis to the TRA may further improve its robustness for toxicity assessment (Adams et al., 2011).

While the TRA traditionally establishes threshold CBR values within a single species, a new idea is that it can be extended to develop relationships between metal burden in tolerant metal-accumulating species (biomonitors) and toxicity in metal-sensitive species (Adams et al., 2011). The measured bioaccumulation in the biomonitor could then be used as the surrogate indicator of an effect threshold in metal-sensitive species living in the same habitat. We evaluated these approaches in two benthic species. The oligochaete *L. variegatus* is abundant in aquatic habitats, stores metals in its soft body, and is very resistant, making it a good biomonitor (Xie et al., 2008). *Chironomus riparius* is another dominant benthic organism in polluted areas; while very tolerant to acute metal toxicity (Bechard et al., 2008), on a chronic basis it may be more sensitive due to susceptibility of larval development and growth (Muscatello and Liber, 2009).

The objectives of this study were: first, to test the hypothesis that CBR₅₀ for chronic Cu toxicity is constant in both hard and soft water for *L. variegatus*; second, to evaluate the use of subcellular accumulation as the residue indicator of chronic Cu toxicity in *L. variegatus*; and third to investigate correlations between whole-body Cu accumulation in *L. variegatus* (a metal-accumulator species) and survival of *C. riparius* (a metal-sensitive species) in a chronic co-exposure regime in hard water.

2. Materials and methods

2.1. Animals and acclimation

L. variegatus (Aquatic Foods, Fresno, CA, USA) were acclimated to dechlorinated Hamilton tap water (hard water) (hardness: $120 \pm 3 \text{ mg L}^{-1}$ as CaCO_3 ; Cu: $1 \mu\text{g L}^{-1}$; pH: 7.8–8.0; 20 °C) under a 12 h/12 h photoperiod. After 1–3 weeks in hard water, worms to be used for the softwater experiment were transferred to a mixture of hard and ion-poor water from a reverse osmosis system. Water hardness was gradually reduced over 6 d (3 d 60% hard water followed by 3 d 30% hard water). The organisms were held in the final soft water (hardness: $44 \pm 3 \text{ mg L}^{-1}$ as CaCO_3 ; Cu: $1 \mu\text{g L}^{-1}$; pH: 7.2–7.3; 20 °C) for 2 d. The acclimation did not result into any stress reaction or death. The tanks were aerated and worms were fed ground trout pellets once each week throughout the acclimation and experimental periods.

The *C. riparius* culture was initiated from egg masses acquired from Environment Canada (Burlington, ON, Canada). The culture was held under a 12 h/12 h photoperiod at 20 °C in aerated hard water. Chironomids were fed fish flakes *ad libitum* twice a week.

2.2. Cu exposure in hard and soft water

Chronic 28-d exposures (*L. variegatus* alone) at 21–22 °C were conducted in both hard and soft water. About 24 h prior to exposure, worms (5–6 cm) were fasted and exposure solutions were prepared in separate beakers. Silica sand (Ultra Reef Marine Sand, Estes Company Ltd., NJ, USA) was added to each beaker to provide a suitable habitat (350 g in 500 mL solution). It was washed with hard or soft water and impurities were removed prior to use. For the hard water exposures, about 50 worms were put into each beaker with nominal Cu concentrations of 0, 50, 100, 150, 200 and 250 $\mu\text{g L}^{-1}$ (prepared from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). There were 7 beakers of

each concentration – 2 replicates were noted for survival, 2 replicates were used for whole-body bioaccumulation analysis, and 3 replicates were used for subcellular fractionation analysis. Worms were fed once a week with ground trout pellets during the exposure. Water was renewed every 48 h and sampled (Acrodisk 0.45 μm in-line-syringe-tip filter) for Cu, Na, Ca and Mg measurements on 0, 7, 14, 21 and 28 d. Survival was observed at 7, 14, 21 and 28 d, and worms were sampled for bioaccumulation analysis at 4, 7, 14, 21 and 28 d. Prior to sampling, worms were placed in clean hard water for 24 h to purge the food which may have adsorbed Cu. Worms for subcellular fractionation were sampled on 28 d with a minimum of 20 worms forming a replicate due to the larger amount of tissue required for subcellular analyses. Worms were stored at –20 °C (bioaccumulation) or –80 °C (subcellular fractionation) for later analysis.

Methods for soft water exposures were similar, with a few exceptions. The nominal concentrations were 0, 10, 30, 50, 80, 100 $\mu\text{g Cu L}^{-1}$; there were 6 beakers at each concentration (3 for survival/subcellular fractionation; 3 for whole-body bioaccumulation analysis). Worms were sampled for bioaccumulation analysis on days 7, 14, 21, 28 and for subcellular fractionation analysis on day 28, after 24-h gut purging in clean soft water.

A chronic 15-d Cu co-exposure of *L. variegatus* and *C. riparius* was conducted at 21–22 °C in hard water. Egg ropes of chironomids were transferred to a petri dish with hard water for hatching. Within 48 h, 15 first instar larvae were transferred to each beaker with nominal Cu concentrations of 0, 25, 50, 100, 150 $\mu\text{g L}^{-1}$ in hard water. Since preliminary tests showed no detectable predation of each species upon the other, 15 oligochaete worms were also transferred to each beaker. Five beakers were noted for survival, with no replicate at each concentration. Three replicates were used for whole-body bioaccumulation analysis with nominal concentrations of 0, 10, 25, 50 and 100 $\mu\text{g L}^{-1}$. Both species were fed a pinch of Tetramin fish flakes every 2 d because survival of chironomid larvae was higher when they were fed with flakes. *L. variegatus* exhibited similar survival on this food as on trout pellets. Water was sampled for Cu, Ca, Na and Mg regularly throughout the exposure, and renewed at 7 d. Survival was checked at 7 d and 15 d. Death of chironomid larvae was defined by grey colouration of the body or disappearance. Mortality of *L. variegatus* was insignificant at the highest concentration, so it was used as the resistant or accumulator species for predicting survival of chironomids. Samples of worms were collected on days 7 and 15 for whole-body bioaccumulation analysis, after 24-h gut purging.

2.3. Subcellular fractionation

Subcellular fractionation of soft tissue generally followed Wallace et al. (2003) except for the addition of washing and re-centrifugation in each step. In preliminary experiments, this modification increased the purity of organelle and cytosol fractions (see detailed procedures in Ng et al. (2011)). About 20 worms (whole bodies) were weighed and homogenized in 2 mL buffer (25 mM Tris buffer, 0.2 mM phenylmethanesulfonyl fluoride, 2 mM mercaptoethanol, pH 7.2). Part of the homogenate was saved for the Cu recovery test and the remainder was differentially centrifuged. Five fractions were obtained – heat-denaturable proteins (HDPs), metal-rich granules (MRGs), organelles (ORGs), cellular debris (CDs) and metallothionein-like proteins (MTLPs). Overall recovery of Cu was $100.5 \pm 9.4\%$ (sum of Cu in each fraction $\times 100\%$ /Cu in homogenate). MTLP and MRG are generally considered the biologically metal-inactive fractions (BIM) because they bind metals, rendering them inert. HDP and ORG can be inactivated or damaged by metals, and so are considered the biologically metal-sensitive fractions (BAM) (Vijver et al., 2004; Meador et al., 2008). Only Cu distribution in HDP, MLTP, ORG

and MRG is presented since CD represents the broken cellular fragments during fractionation and has no biological significance (not BIM or BAM). Subcellular Cu concentration is expressed in $\mu\text{g g}^{-1}$ whole body fresh weight.

2.4. Metal concentrations in water and tissue analysis

See Supplementary content for detailed description. All metal concentrations in tissue or subcellular fractions were presented in fresh weight, with the assumption that Cu did not affect the water content of the organisms.

2.5. Statistical analysis

Data are means \pm 1 SEM unless otherwise specified. Reported water Cu concentrations are measured values. LC_{50} and 95% confidence intervals ($\mu\text{g L}^{-1}$ Cu) were calculated by ToxCalc v5.0.32 (Tidepool Scientific Software, McKinleyville, USA). The critical whole-body (CBR_{50}) or critical subcellular residue (CSR_{50}) was the Cu burden in whole body or subcellular fractions (MRG) of *L. variegatus* that corresponded to 50% reduction in survival of *L. variegatus* in the single species chronic exposure or survival of chironomids in the co-exposure. Regression analyses were performed on relationships between Cu burden and survival, and between Cu in subcellular fractions and whole-body burden. When the regression was significant at $p < 0.05$ or the coefficient of determination (R^2) was greater than 0.6, a goodness-of-fit curve was plotted. CBR_{50} or CSR_{50} were calculated from the regressions of logit mortality against logit Cu burden. Cu burden and survival were corrected for control levels prior to analysis.

One-way ANOVA followed by Tukey's test evaluated differences among CBR_{50} 's of *L. variegatus* in single species chronic exposures, as well as for differences among Cu concentrations on different days within the same subcellular fraction. Student's t-test was used to test for differences between LC_{50} 's of *L. variegatus* in hard and soft water, and between CBR_{50} 's of *L. variegatus* on days 7 and 15 of the co-exposure experiment. Significance of all tests was taken at $p < 0.05$.

3. Results and discussion

3.1. Survival of *L. variegatus*

Measured water chemistry and survival patterns of *L. variegatus* in hard and soft water are reported in Table S1 and Fig. S1. Hard water (average of 117–127 mg L^{-1} as CaCO_3) was about 3-fold harder than soft water (average of 38 mg L^{-1} as CaCO_3). Based on the LC_{50} 's, the worms were 2-fold more sensitive to Cu in soft

water than in hard water in the single species exposure by 28 d (Table 1, $p < 0.01$). Similar positive relationships have been found between water hardness and EC_{50} of fish (Taylor et al., 2000).

3.2. Cu bioaccumulation in *L. variegatus*

Bioaccumulation patterns differed between hard and soft waters. In hard water, Cu bioaccumulation revealed homeostatic control at a more or less constant but elevated level in most exposure concentrations after 14–21 d (Fig. S1), whereas in soft water, bioaccumulation increased linearly over time and *L. variegatus* accumulated more Cu within the same experimental period (Fig. S1). The saturable bioaccumulation patterns in hard water and linear bioaccumulation pattern in soft water were in agreement with whole-body or gill bioaccumulation in chronic studies with rainbow trout (McGeer et al., 2000; Ng et al., 2010). In contrast, a linear bioaccumulation pattern was observed in freshwater snails in hard water (Ng et al., 2011). In that study, the snails did not show significant mortality in any of the Cu concentrations probably due to lower bioavailability of Cu in the presence of food and initiation of detoxification mechanisms in the chronic exposure. In general, bioaccumulation is dependent on water chemistry, exposure concentration and duration. Different water chemistry e.g., increase in calcium and bicarbonate concentrations in hard water, may reduce the Cu bioavailability to the animals (Santore et al., 2001).

3.3. Toxicity and whole-body bioaccumulation relationships

In hard water, survival of *L. variegatus* did not change with bioaccumulation when the bioaccumulation level was low; toxicity increased after the threshold (about 40 $\mu\text{g g}^{-1}$ fresh wt., Fig. 1) was passed. The relationship was in a good agreement with Redeker and Blust (2004) for Cd effects on another oligochaete *Tubifex tubifex*. This phenomenon was also described by Rainbow (2007) in marine invertebrates. When the uptake of metals is lower than the combined rates of excretion and detoxification, toxicity does not ensue, otherwise metals accumulate in the biologically active pools and toxicity occurs when the threshold is exceeded.

In contrast, in soft water, the survival and whole-body Cu bioaccumulation relationships were linear (Fig. 1). Higher bioaccumulation was correlated with higher toxicity, and there was no whole-body bioaccumulation threshold. In soft water, toxicity at the hard water bioaccumulation threshold of 40 $\mu\text{g g}^{-1}$ fresh wt. was prominent (50–60% mortality, Fig. 1), probably due to additional ionic stress in ion-poor water, in combination with Cu toxicity.

Table 1
Mean LC_{50} and 95% confidence intervals ($\mu\text{g L}^{-1}$ Cu) and critical residues ($\mu\text{g g}^{-1}$ fresh wt.) of Cu in *L. variegatus* corresponding to 50% reduction in survival of itself in the single species chronic exposure or 50% reduction in survival of *C. riparius* in the co-exposure. No confidence intervals could be calculated for *L. variegatus* (7, 15 d) and *C. riparius* (15 d). The same letter (a–d) indicates no significant difference between groups at $p > 0.05$. ND: Not determined; NS: Not significant regressions ($p > 0.05$) between whole-body burden and survival or R^2 (coefficient of determination) < 0.6 in the regression.

	Days	CBR_{50}	CSR_{50}	LC_{50}
Hard water – <i>L. variegatus</i>	7	NS	ND	ND
	14	NS	ND	ND
	21	51.0 (49.9–52.0) ^a	ND	ND
	28	55.6 (37.7–82.6) ^a	ND	61.5 (41.8–77.7) ^c
Soft water – <i>L. variegatus</i>	7	51.8 (25.6–104.9) ^a	ND	ND
	14	43.2 (31.2–59.8) ^a	ND	ND
	21	38.2 (31.7–46.0) ^a	ND	ND
	28	41.2 (34.1–49.9) ^a	4.9 (1.8–11.9)	33.9 (28.7–39.2) ^d
Hard water – Co-exposure	7	29.1 (11.9–53.3) ^b	ND	>93.6 (<i>L. variegatus</i>) 97.9 (78.8–196.1) (<i>C. riparius</i>)
	15	45.7 (6.7–114.9) ^b	ND	>93.6 (<i>L. variegatus</i>) 89.1 (<i>C. riparius</i>)

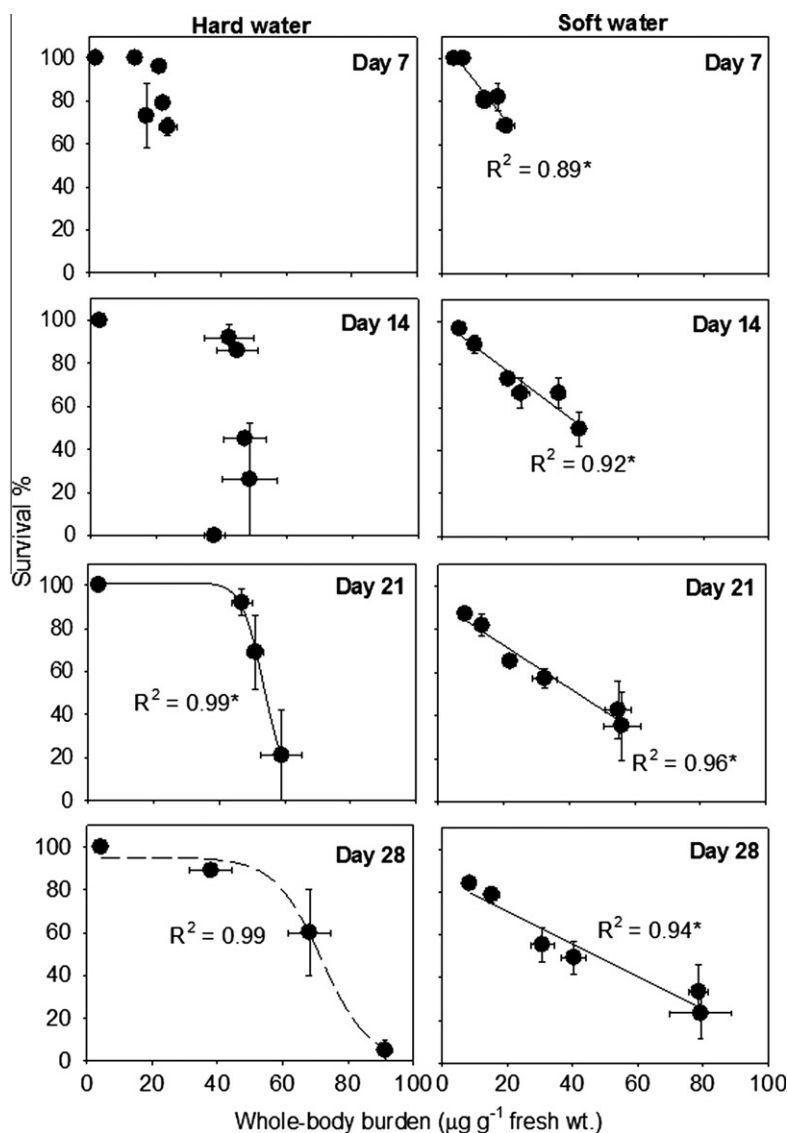


Fig. 1. Correlation between survival and corresponding whole-body Cu burden of *L. variegatus* in hard and soft waters in the chronic exposure. R^2 : coefficient of determination. Asterisk indicates significant regression at $p < 0.05$.

CBR_{50} was calculated only for significant relationships and values were comparable (38.2 – $55.6 \mu\text{g g}^{-1}$ fresh wt.) on different days of exposure in hard and soft water (Table 1). In addition, CBR_{50} was comparable between hard and soft water (Table 1) despite the different hardness-dependent patterns of bioaccumulation (Fig. S1). In one chronic study, the measured CBR_{50} of Cd ($23 \mu\text{g g}^{-1}$ fresh wt.) in *T. tubifex* was consistent from 4 to 17 d (Redeker and Blust, 2004). To date, most CBR data are limited to acute studies. For example, (Meyer et al., 2002) reported the 6-h accumulation of Cu (10.7 – $21.4 \mu\text{g g}^{-1}$ dry wt., equivalent to about 1.6 – $3.2 \mu\text{g g}^{-1}$ fresh wt.) in *L. variegatus* was constant across different water hardness and pH combinations, and could be used to predict survival at 48 h. This is essentially a BLM approach. When we analysed our data in a similar fashion, relationships between early Cu burden (7 d) and chronic mortality (at 28 d) (Fig. 2) were qualitatively similar to those when mortality and Cu burden were assessed on the same day (Fig. 1), with the same hardness-dependent patterns. However, the critical body burden were lower than in the same day relationships: the 7-d CBR_{50} 's to predict 28-d mortality were only $11.0 \mu\text{g g}^{-1}$ fresh wt. (95% confidence interval: 9.7 – $12.3 \mu\text{g g}^{-1}$ fresh wt.) and $16.9 \mu\text{g g}^{-1}$ fresh wt. (12.1 – $23.7 \mu\text{g g}^{-1}$

fresh wt.) in soft and hard waters respectively. Overall, this suggests that in soft water, worms sampled starting from 7 d of chronic exposure can be used for tissue residue risk assessment even though bioaccumulation has not yet reached equilibrium (Fig. S1).

The variation of 28 d CBR_{50} (1.3-fold) was less than the variation of 28 d LC_{50} (1.8-fold) between hard and soft waters (Table 1). Our finding is in accord with several other acute and chronic studies, all showing that CBR_{50} varied less than LC_{50} when exposure conditions were altered (Borgmann et al., 1991; Meyer et al., 2002; Penttinen et al., 2008). All of these data support one of the main advantages of the TRA: tissue concentrations observed for toxicity responses are generally less variable than exposure concentrations (Meador, 2006).

3.4. Subcellular Cu bioaccumulation and relationships with toxicity

Distributions of Cu in subcellular fractions are reported in Fig. S2. Note that more Cu was associated with the HDP fraction (part of BAM) at the same whole-body Cu burden in soft than in hard water (e.g., $7.1 \mu\text{g g}^{-1}$ fresh wt. in soft water versus $4.9 \mu\text{g g}^{-1}$

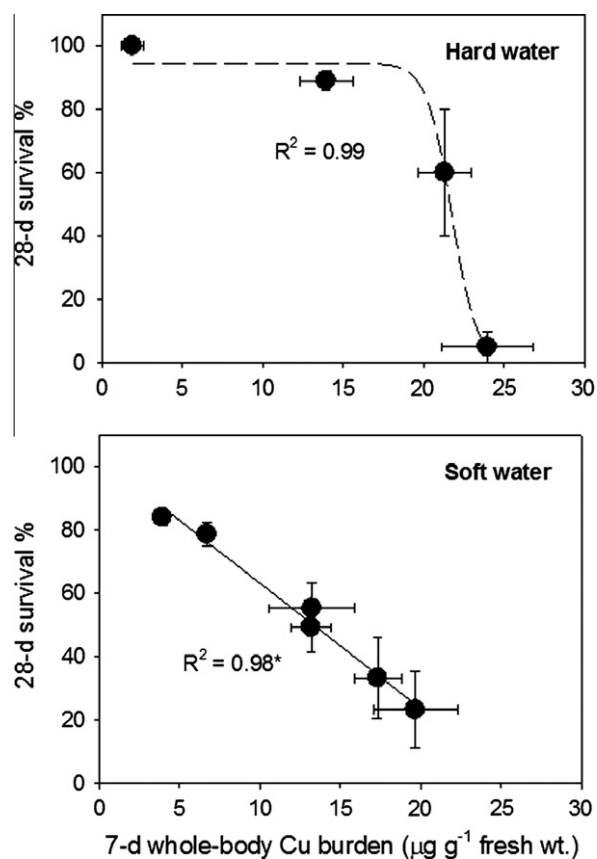


Fig. 2. Correlation between 28-d survival and 7-d whole-body Cu burden of *L. variegatus* in hard and soft waters. R^2 : coefficient of determination. Asterisk indicates significant regression at $p < 0.05$.

fresh wt. in hard water at about $40 \mu\text{g g}^{-1}$ body burden). Since higher toxicity occurred at the same whole-body burden in soft water (Fig. S1), the subcellular metal burden appears to be a better predictor of toxicity than whole-body metal burden. How fast the concentration of metals reaches threshold in the BAM also depends on the capacity of the organisms to translocate the incoming metals from BAM to BIM, as well as the ability of the organisms to excrete the metal (Adams et al., 2011). Our results demonstrate a faster net transport of Cu to the HDP and a similar rate of translocation to the BIM fraction in soft water (slower rate to MTLP, faster rate to MRG), compared to hard water (Fig. S2). The rate of Cu binding to MTLP is associated with the induction rate of metallothionein (Pedersen and Lundebye, 1996). The faster rate of Cu entry into *L. variegatus* in ion-poor soft water (due to less competition with cations) may far exceed the rate of metallothionein production. In hard water, there was a negative relationship between Cu in HDP or MTLP + MRG and survival (Fig. 3, $p \leq 0.04$), but in soft water, only a negative relationship between Cu in MRG and survival (Fig. 3, $p = 0.01$). In view of the time to store metals in equilibrium within subcellular compartments, the value of the use of a critical concentration of metal in a subcellular component (cf. total body concentration) increases even more as acute toxicities turn into chronic ones. This is particularly important when ecotoxicologists need to extrapolate results on metal toxicity from laboratory to field.

In hard water, since survival of the available worms for subcellular analysis was higher than 50%, no reliable CSR_{50} was determined. In soft water, CSR_{50} in MRG was about 10-fold less than CBR_{50} (Table 1). This value however, indicates bioavailability of Cu in a subcellular fraction that was correlated with the overall

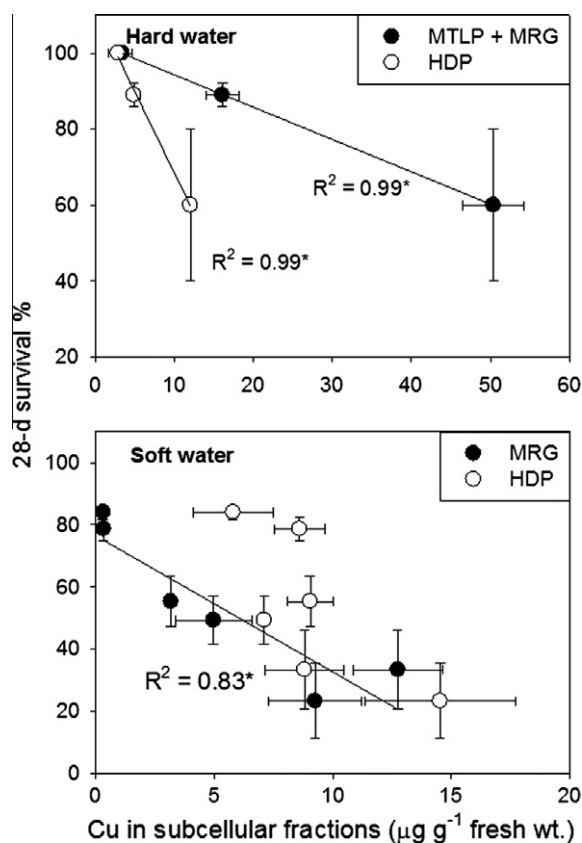


Fig. 3. Correlation between 28-d survival and Cu in subcellular fractions of *L. variegatus* in hard and soft waters in the chronic Cu exposure. R^2 : coefficient of determination. Asterisk indicates significant regression at $p < 0.05$. MTLP: metallothionein-like proteins; MRG: metal-rich granules; HDP: heat-denaturable proteins.

toxicity, but not Cu accumulation in the metal sensitive compartments (i.e. BAM). According to Redeker and Blust (2004), the TRA concept only holds when the formation of inert complexes is limited (i.e. most of the tissue metal is biologically reactive) or when the reactive pool is proportional to the total metal concentration. The present data suggest this concept should be re-evaluated. Metal measurements in BIM, rather than in BAM, may be an alternative approach to indicate bioavailability on a cellular level.

3.5. Co-exposure toxicity and bioaccumulation relationships

The co-exposure test was terminated at 15 d to ensure that control survival did not drop below 80%. Whole-body Cu concentrations in the chironomids appeared to have stabilized by this time (i.e. similar or lower than 7-d levels) (Fig. 4). *L. variegatus* mortality was unchanged from control values at all waterborne Cu concentrations at 7 and 15 d of the co-exposure. Therefore the LC_{50} was greater than the highest measured Cu concentration in water, $93.6 \mu\text{g L}^{-1}$ (Table 1). *L. variegatus* was clearly less sensitive to Cu in this co-exposure than in the single-species exposure in hard water. In accord, *L. variegatus* also had a lower Cu bioaccumulation compared to the single-species exposure at a similar Cu concentration (co-exposure: $19.9 \pm 4.4 \mu\text{g g}^{-1}$ fresh wt. at $76 \mu\text{g L}^{-1}$ Cu; single-species exposure: $68.2 \pm 6.7 \mu\text{g g}^{-1}$ fresh wt. at $66 \mu\text{g L}^{-1}$ Cu). One possible explanation is that excretion and moult shed off by the chironomid larvae during development reduced the bioavailability of Cu (Postma et al., 1996; Groenendijk et al., 1999); another is that the more bioavailable Cu in the water was taken up by the chironomids, leaving mainly a less bioavailable fraction. Both scenarios would result in lower Cu bioaccumulation and toxicity to *L.*

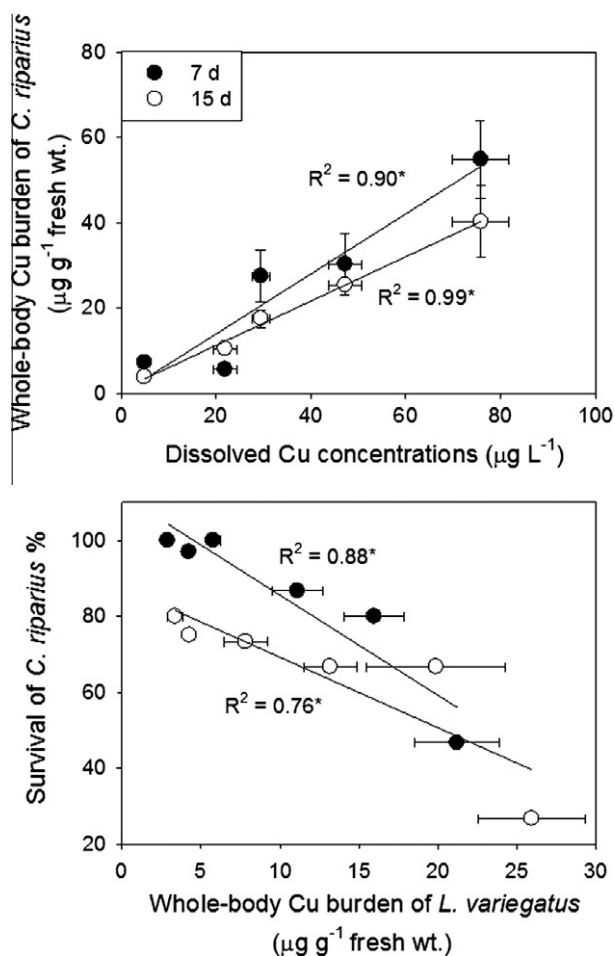


Fig. 4. Cu accumulation by *C. riparius* and correlation of survival of *C. riparius* and whole-body Cu burden of *L. variegatus* in the co-exposure. See Supplementary content for additional information. R^2 : coefficient of determination. Asterisk indicates significant regression at $p < 0.05$.

variegatus. Indeed, this result nicely illustrates the point that laboratory single-species toxicity tests may overestimate or underestimate biological community responses if aquatic ecosystem physical, chemical or biotic factors mitigate toxicity e.g., bioavailability of chemicals (USEPA, 1999).

The toxicity of Cu to chironomid larvae was comparable between 7 and 15 d (Table 1), in accord with the stabilization of the whole-body Cu burden. Most of the surviving larvae were in the fourth instar or pupated at the end of the exposure. Therefore, Cu had significant effects only on survival of first and second instar larvae in the first week; these are often more sensitive to metals (USEPA, 2000).

The 15 d LC_{50} of *L. variegatus* ($>93.6 \mu\text{g L}^{-1}$, 7 and 15 d) was clearly higher than that of *C. riparius* (7 d: $97.9 \mu\text{g L}^{-1}$; 15 d: $89.1 \mu\text{g L}^{-1}$) because worms did not die, whereas only 27% of midge larvae survived at the highest concentration ($93.6 \mu\text{g L}^{-1}$ Cu) (Table 1). *C. tetanus* was reported to be more resistant to Cu (LC_{50} : $54 \mu\text{g L}^{-1}$) than *L. variegatus* (LC_{50} : $35 \mu\text{g L}^{-1}$) in a flow-through 10-d toxicity test (Phipps et al., 1995), however no confidence intervals were given and the developmental stage of chironomids was unknown in that study.

To our knowledge, this is the first study to evaluate the relationship between the whole-body burden of a metal accumulator species (no significant mortality) and the survival of a metal-sensitive species in an experimental co-exposure. Survival of the chironomids was negatively correlated with Cu bioaccumulation by

L. variegatus ($p < 0.05$, Fig. 4). The regression line shifted to the left as exposure time lengthened. The CBR_{50} in *L. variegatus* (predictive of 50% mortality in *C. riparius*) was independent of exposure period ($p = 0.59$, Table 1). Future investigations should examine relationships under different experimental conditions and species with wider variation in sensitivity. Based on the current data, it is promising that the tissue metal residue of the biomonitor species can be used to predict metal toxicity to a sensitive species in the same environment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.chemosphere.2012.01.018](https://doi.org/10.1016/j.chemosphere.2012.01.018).

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