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Use of whole-body and subcellular Cu residues of *Lumbriculus 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 *Lumbriculus variegatus* to predict Cu toxicity in fresh water. The critical whole-body residue associated with 50% mortality (CBR_{50}) was constant (38.2–55.6 μg g^{-1} fresh wt.) across water hardness (38–117 mg L^{-1} as $CaCO_3$) and exposure times during the chronic exposure. The critical subcellular residue (CSR_{50}) 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 CBR_{50} 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.

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 surface, 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 LA_{50} (lethal accumulation associated with 50% mortality) is similar to the TRA-based CBR_{50} (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 CBR_{50} of Cu in *Lumbriculus 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 CBR_{50} 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 ± 3 mg L^{-1} as $CaCO_3$; Cu: 1 μg 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 ± 3 mg L^{-1} as $CaCO_3$; Cu: 1 μg 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 μg L^{-1} (prepared from $CuSO_4.5H_2O$). 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 μg 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 μg 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 μg 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 phenylmethanesulfonylfluoride, 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 × 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