THESIS

EFFECTS OF DIETARY AND AQUEOUS ZINC ON SUBCELLULAR ACCUMULATION AND FEEDING RATES OF MAYFLIES

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ABSTRACT

EFFECTS OF DIETARY AND AQUEOUS ZINC ON SUBCELLULAR ACCUMULATION AND FEEDING RATES OF MAYFLIES:

AN EFFORT TO RECONCILE DISCREPANCIES BETWEEN LABORATORY AND FIELD TOXICITY STUDIES

Although laboratory toxicity tests have shown that mayflies (Ephemeroptera) are highly tolerant to aqueous Zn exposure, (Brinkman and Johnston 2008), field biomonitoring studies have shown marked decreases in mayfly abundance at relatively low concentrations of metals. (Carlisle and Clements 2003; Schmidt et al. 2010). Laboratory testing methodologies used for developing water quality standards rely solely on aqueous exposure and often focus on lethal endpoints. In reality, organisms in the field experience chronic exposures to metals through both aqueous and dietary pathways. To investigate the relative importance of dietary metals exposure, I conducted laboratory toxicity tests where the mayflies *Epeorus albertae* and *Ameletus* sp. were exposed to aqueous (0, 800, 1600 µg/l) and dietary (722 µg/g vs.104 µg/g) concentrations of zinc. Additional aqueous-only trials (0, 800,1600, 3200, 6400 and 12800 µg/l) were ran simultaneously. Subcellular partitioning methods were employed to detect differences in how mayflies compartmentalized dietary and aqueous Zn

exposure. Results were compared to zinc partitioning in organisms collected across a gradient of Zn-contaminated sites in the upper Arkansas River near Leadville, Colorado. Diet was found to significantly influence both total accumulation of Zn and distribution in subcellular fractions in both mayfly genera. Organisms receiving both dietary and aqueous exposure accumulated significantly more Zn than did organisms exposed to only aqueous Zn. Diet also significantly affected feeding behavior and molting frequency. *Ameletus* fed significantly less on algae with high levels of Zn than algae with low levels of Zn.

These findings suggest that traditional laboratory toxicity tests used in establishing water quality criteria significantly under-represent the potential for accumulation of metals and sublethal effects in the field. Tissue concentrations of Zn in *Ameletus* and *Epeorus* after 10 day laboratory exposures were well below those in organisms collected from the Arkansas River, despite lower aqueous concentrations in the field. Addition of dietary exposure improved the realism of our laboratory toxicity tests; however, this alone will not make laboratory tests as sensitive as field studies. These differences in metal uptake between laboratory and field studies suggest that field biocriteria, such as population densities of sensitive mayflies, may be more sensitive and ecologically relevant indicators of stress and recovery in mine-impacted rivers.

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Introduction

Density and diversity of macroinvertebrates observed at metal-contaminated sites are often significantly lower than those expected based on water quality criteria (Warnick and Bell 1969; Winner et al. 1980; Clements 1991; Clements et al. 2000). Acute water quality criteria are almost exclusively developed using short-term laboratory toxicity tests that examine survival as an endpoint and include only aqueous exposure (U.S. Environmental Protection Agency 1985; ASTM 1997). Failure to include dietary exposure and critical sublethal endpoints, such as molting frequency and other developmental bottlenecks, are major shortcomings of toxicity testing methodologies and may result in water quality standards that do not adequately protect organisms in the field (Fisher and Hook 2002). However, given the diverse feeding strategies and diets of aquatic macroinvertebrates, the standardization of ecologically relevant food sources for use in dietary experiments presents a challenge. Seasonal variation of metal concentrations in algae (Anishchenko et al. 2010) further complicates the ability to predict dietary exposure in the field.

The morphological and physiochemical characteristics of digestive systems of aquatic organisms facilitate uptake of toxicants through digestion (Meyer 2005). Diet has been shown to play an important role in metal accumulation in fish (Farag et al. 1999; Long and Wang 2005; Clearwater et al. 2002; Bechard et al. 2008), mollusks (Wallace et al. 2003; Wallace and Luoma 2003) and arthropods (Timmermans et al. 1992; Munger and Hare 1997; Irving et al. 2003; De Schamphelaere et al. 2004; Rainbow et al. 2004; Sofyan et al. 2006;

Conley et al. 2009; Xie et al. 2010). In daphnid species, dietary metals has been shown to influence survival (Sofyan et al. 2006), but also sublethal endpoints such as feeding rate (Taylor et al. 1998; Allen et al. 1995; Sofyan et al. 2006) and reproductive success (De Schamphelaere et al. 2004; Sofyan et al. 2006). In the short time frame of typical laboratory toxicity tests (e.g., 48-96 h), sublethal endpoints might be considered relatively unimportant. However, when extrapolating beyond the limited duration of acute and even chronic laboratory tests, these endpoints have the potential to negatively affect populations in the field.

Microalgae readily accumulates aqueous metals (Vymazal 1984; Chong et al. 2000; Behra et al. 2002; Meylan et al. 2003; Sofyan et al. 2006), thereby increasing risk to primary consumers. For example, decreased survival and fecundity was observed in the mayfly *Centroptilum triangulifer* McDunnough when fed periphyton bathed in selenium (Se) (Conley et al. 2009). Irving et. al. (2003) observed decreased feeding and growth of the mayfly *Baetis tricaudatus* Dodds when fed Cd-contaminated algae. Dietary exposure of mayflies was responsible for increased accumulation of Cd (Irving et al. 2003; Xie et al. 2010), and Se (Conley et al. 2009). While laboratory toxicity tests have shown that mayflies are highly tolerant to aqueous Zinc (Zn) exposure (Warnick and Bell 1969; Brinkman and Johnston 2008), field biomonitoring studies have shown significant decreases in mayfly abundance at relatively low metal concentrations (Carlisle and Clements 2003; Schmidt et al. 2010). Dietary exposure through

algae consumption may be partially responsible for increased sensitivity of mayfly populations in the field (Neptun 2001).

To investigate the relative importance of dietary exposure to Zn, I conducted laboratory experiments with two species of grazing mayflies, *Ameletus* sp. and *Epeorus albertae* McDunnough. Both species were exposed to three sublethal levels of aqueous Zn (0, 800 and 1600 µg/l) which were crossed with two dietary treatments (Zn-contaminated algae, non-contaminated algae). Simultaneously, a traditional aqueous-only toxicity test was conducted exposing organisms to 6 levels of aqueous Zn (0, 800,1600, 3200, 6400 and 12800 µg/l). This experimental design allowed for comparisons between the dietary and aqueous-only experiments at the 0, 800 and 1600 µg/l Zn levels. Dietary treatments were developed by culturing *Scenedesmus* sp. in either Zn-contaminated or uncontaminated growth media. Mayfly feeding rates on contaminated and non-contaminated algae were assessed using photoanalysis and behavioral endpoints. Molting frequency, growth and total Zn accumulation were assessed across treatments, and I also investigated interactions between aqueous and dietary exposure pathways.

Within an organism toxicants associated with cytosolic proteins and subcellular organelles are more likely to elicit a toxic response than fractions associated with connective tissue and metallothionein-like chelating agents (Reinfelder and Fisher 1991; Harrison and Curtis 1992; Meyer 2005; Ng and Wood 2008). Studies conducted with mollusks and insectivorous fish have reported that metals are more readily assimilated by the digestive system when

bound to certain subcellular fractions of food (Rainbow et al. 2004; Bechard et al. 2008). Because of this differential toxicity among compartments and the increased bioavailability of metals bound to specific fractions, I examined total and subcellular concentrations of Zn in organisms from the laboratory experiments and then compared these concentrations to those found in mayflies collected from the Arkansas River, a metal-contaminated stream in Colorado.

Methods

Periphyton Culture

Periphyton-covered cobble substrate was collected from a reach of Clear Creek (Gilpin County), a Colorado stream with high levels of Zn contamination from historical mining activities. Substrate was placed in 1-2 cm water containing Guillard's growth medium (Guillard 1975), fortified with 1.356 millimolar Si using dissolved sodium meta-silicate 9-hydrate in 11.2 cm x 6 cm PVC troughs (Appendix A). Pumps (Taam Inc., Rio 600) provided a recirculating flow of 200 gallons per hour. Periphyton was cultured on 6.25 cm^2 unglazed porcelain tiles (Cinca Tile Co., Fiães, Portugal). Cultures received 12 hour cycles of fluorescent light from wide spectrum plant and aquarium bulbs. Zn (as $ZnSO_4$) was added to growth media of the Zn-contaminated cultures at 1600 µg/l to produce algal tissue concentrations similar to those observed in a survey of Colorado mountain streams (µ=1483.5 µg/g sd=5381 n=227; Travis Schmidt, unpublished research, USGS Central Colorado Assessment Program). Algae was maintained in an exponential growth phase and was used as a food source in toxicity tests for no

more than 3 weeks, after which a new culture was inoculated with tiles from a Zn-contaminated culture. Three weeks prior to each experiment cultures were started using Zn-contaminated (1430 µg/l Zn) and non-contaminated (27.8 µg/l Zn) growth media to provide dietary treatments. Subsequent analyses of these cultures indicated that periphyton communities were dominated by the diatom *Scenedesmus* sp. (Dr. Sarah Spaulding, USGS, Boulder CO).

Several previous studies have examined effects of contaminated periphyton on aquatic insects (Irving et al. 2003;Conley et al. 2009;Xie et al. 2010) and cladocerans (Sofyan et al. 2006). However, these studies relied on equilibration by bathing algae or periphyton mats in a metal solution. In my current study, I cultured algae food sources in Zn-contaminated and non-contaminated media. I conducted studies that determined culturing algae in the presence of contaminants produces a more natural subcellular distribution of toxicants that depurates toxicants more slowly (Appendix A).

Test Organisms

Test organisms were collected from two streams with no history of metal contamination originating from Rocky Mountain National Park, Colorado, USA. *Ameletus* sp. was collected from the Cache La Poudre River (Larimer County) in March, 2009. Historical records describe the dominant species as *Ameletus doddsianus* Zloty; however, the nymphs were too immature to obtain an accurate species identification. *Epeorus albertae* was collected from the Big Thompson River (Larimer County) in August, 2009. The species identity of a subsample of

nymphs and reared adults was confirmed by Dr. Boris C. Kondratieff (Colorado State University, Fort Collins, Colorado). These two taxa were chosen because both are morphologically adapted to scraping periphyton (Merritt 2008). Members of the genus *Epeorus* (Heptageniidae) are dorsoventrally flattened and have a labrum and maxilla well adapted to feeding on thin algal mats while clinging to medium sized substrate in riffle areas. The comb like maxilla of *Ameletus* have pectinate spines that scrape periphyton from substrate in the slow littoral margins of mountain streams. In addition, both genera have been collected at uncontaminated reference sites in the Upper Arkansas River (Clements 2004).

Laboratory Experiment Methods

I conducted an experiment using *Ameletus* nymphs in March of 2009 and *Epeorus* nymphs in August of 2009. Each experiment included a 3 x 3 factorial trial where mayfly nymphs were exposed to 3 aqueous Zn concentrations (0, 800 and 1600 µg/l) crossed with 3 dietary exposure levels including Zn-contaminated algae, non-contaminated algae and no food (aqueous-only). Organisms in the aqueous-only treatments received no algae but were provided bare porcelain tiles as a control. Simultaneously, I conducted an aqueous-only trial at 3200, 6400 and 12800 µg/l Zn. The aqueous only treatments (0, 800, 1600, 3200, 6400 and 12800 µg/l) were representative of traditional laboratory toxicity tests and were conducted on a different diluter than the dietary trials. The 3x3 design allowed for comparisons of feeding rate, molting frequency and Zn accumulation

between the dietary and aqueous-only experiments at the 0, 800 and 1600 µg/l levels. The 3200, 6400 and 12800 µg/l aqueous-only treatments allowed us to make comparisons between accumulation rates of traditional toxicity tests to that of a dietary and aqueous exposure at 1600 µg/l. Each treatment combination was replicated in four experimental chambers.

Experimental methods differed slightly for the two species of mayflies to best simulate their natural microhabitat characteristics. After a 24 hour acclimation period, 12 *Ameletus* were randomly assigned to each of 48 1-L rectangular polypropylene containers equipped with an air stone to provide gentle water circulation. For experiments with *Epeorus*, five individuals were randomly assigned to each of 48 850 ml, circular artificial streams (Brinkman and Johnston 2008) constructed to provide a high velocity flow. Feeding tiles (1-3) placed on the bottom of the containers maintained *ad libitum* feeding conditions and were exchanged every 24 h throughout the experiment. In addition to the feeding tiles, polypropylene mesh and an additional bare tile were added to provide additional substrate for organisms.

Treatment chambers and artificial streams received dechlorinated municipal tap water (Fort Collins, Colorado, USA) from continuous-flow serial diluters (Benoit et al. 1982). The diluters delivered a control concentration as well as two (dietary treatments) or five (aqueous-only treatments) concentrations of metal toxicant with a 50% dilution ratio. A flow splitter allocated each concentration equally among 4 replicate chambers at a rate of 40 ml/min. Food-grade vinyl tubing delivered test solutions to exposure chambers. Metal stock

solutions were prepared by dissolving a calculated amount of metal sulfate salts in deionized water. A concentrated stock solution was delivered to the diluter by a peristaltic pump at a rate of 2.0 ml/min. In dietary trials organisms were allowed to feed *ad lib* for the first 9 days of the 10 days of exposure, allowing 24 hours for expulsion of gut contents before being processed for subcellular fractionation of Zn, as described below.

To make comparisons to traditional laboratory toxicity tests, aqueous-only treatments (bare tiles with no periphyton) were conducted simultaneously with the dietary experiments described above for both *Ameletus* and *Epeorus*. In addition to the 0, 800 and 1600 µg/L treatments, this experiment also included 3200, 6400 and 12800 µg/l Zn (n = 4). These higher concentrations are considered sublethal based on standard laboratory toxicity tests; however, these levels are well above those observed at even highly metals-polluted field sites in Colorado (Clements et al. 2000). All aqueous experiments ran 10 days and were conducted in the treatment chambers described above.

Hardness, alkalinity, anions, chloride, sulfates, conductivity, dissolved oxygen, temperature, and Zn concentrations (dissolved and total) were measured every 48h. Exuvium, survival, and emergence were assessed daily while cleaning treatment cages. Presence of organisms on feeding tiles was observed each morning as a behavioral indicator of feeding. Head capsule and body length of organisms in each treatment group were measured using photoanalysis. Immediately before and after the experiment organisms from each replicate were placed in 2 mm of water and photographed adjacent to a

ruler. Mean head capsule and body length were assessed for organisms in each replicate using Image J version 1.40g (Abramoff 2004). Total and subcellular Zn accumulation in mayfly tissues were analyzed at the end of the trials with the procedures described below.

In the dietary trials the dry mass of algae consumed per organism per day was estimated by photoanalysis of feeding tiles. Underwater photographs of feeding tiles were taken before and after mayfly feeding using an Olympus Stylus 850 SW camera (F=4.0. Shutter 1/250) under uniform lighting, distance and shutter speed. Image J version 1.40g (Abramoff 2004) was used to crop, rotate, and convert photos from JPEG to TIFF format. For each cropped photo Matlab R2009b on Fedora Core 10 operating system was used to calculate mean greenness, or the average green saturation of the pixels in a photo based on a 255 RGB color model. The dry mass of algae on each tile was estimated using the following equation based on an index of mean greenness:

Dry Mass (μg) = 0.00005661 X [255-(Mean Greenness)] – 0.00330380;

(R^2 = 0.813, n = 13). Throughout the trials, feeding tiles were placed in treatment cages with no organisms to ensure algae was not being lost to turbulence. No loss of algae was observed.

Field Study

Immediately following laboratory toxicity experiments, *Ameletus* (March, 2009) and *Epeorus* (August 2009) were collected at several sites from the Arkansas River along a known gradient of Zn contamination (Clements 2004).

Sites were located upstream (EF5, AR1) and downstream (AR3, AR5) from the California Gulch Superfund Site near Leadville, Colorado, USA. Dissolved Zn concentrations were taken from a 3 years seasonal (May and August) average from routine biomonitoring (Will Clements, Colorado State University, Personal Communication). Organisms were transported in water collected from each site in insulated coolers with air stones and artificial substrate. Organisms were depurated in site water for 24 hours at the mean temperature of field sites then processed for subcellular distribution of Zn as described below.

Water Quality Assessment

Aqueous Zn and cation samples for both field and laboratory studies were filtered using a 0.45 µm disk filter and preserved with ultrex nitric acid in a 15ml polypropylene centrifuge tube. Analysis for metals and cations was conducted using an Instrumentation Laboratory Video 22 with Smith-Hieftje with background correction. Alkalinity and water hardness were generally assessed by titration using EPA methods 200.7 and 310.7; however, hardness for *Epeorus* trials was determined by Atomic Absorbtion Spectroscopy.

Subcellular Partitioning and Zn Accumulation

To assess Zn compartmentalization, organisms from each treatment were partitioned into subcellular fractions using differential centrifugation (Fig. 1). Fractions included cellular debris and nuclei (Karp 2002)(800 *g* pellet), subcellular organelles including mitichondria (15,000 *g* pellet), heat labile

cytosolic proteins, and heat stable cytosolic proteins. The method also allowed us to differentiate fractions of Zn that were associated with metal-rich granules or loosely bound to the exoskeleton of organisms and cell fragments. The same subcellular fractionation process was conducted to characterize Zn compartmentalization in the algae of dietary treatments (Appendix A).

All organisms from each replicate were processed together in a composite sample to meet minimum tissue requirements. Each composite was rinsed with deionized water, air dried on filter paper for 60 seconds on a Buchner funnel with aspiration, submerged in 3.0 ml of 0.01M Mg EDTA solution and gently agitated for 60 seconds to remove loosely bound Zn. Two ml of EDTA solution were retained in 2 ml polypropylene centrifuge tubes as a measure of Zn loosely sorbed to the surface of an organism (Fraction EXT). Zn associated with the EXT fraction was excluded from the total tissue burden of insects because it likely causes little toxicological affect. Organisms were rinsed with DI water and again dried for 60 seconds on a Buchner funnel. The number of insects was recorded and organism mass was measured using an Ohaus GA200D analytical balance (0.00001g).

A modification of the subcellular partitioning methods developed by Bechard (Bechard et al. 2008), Wallace (Wallace et al. 2003), and Brinkman (Brinkman 2008) was used in this study (Fig. 1). Organisms were homogenized using a Tissue Terror® (Biospec Products Inc., Dremel, Racine WI, USA) homogenizer in 1.7 ml of helium-saturated 0.05M HEPES Buffer (pH 7.4) with 0.2 millimolar phenylmethylsulfonyl fluoride protease inhibitor. Tris buffer was

avoided because of limited buffering capacity at pH7.4 at 4ºC (Sigma-Aldrich 1996). Homogenate (0.5 ml) was transferred to a 1.5 ml polypropylene centrifuge tube representing the "total" metals (Fraction TOT) and was used to calculate percent recovery. Recovery of Zn throughout the subcellular partitioning of insects averaged 100.9% (sd = 10.4; Fig. 2).

Homogenate (1.0 ml) was centrifuged at 800 *g* for 10 minutes at 4º C in a 1.5 ml polypropylene centrifuge tube using an Eppendorf 5415 c centrifuge. The resulting pellet consisted of cellular debris including tissue fragments, membranes, nuclei and metal rich granules. This pellet was held at -20º C until it was resuspended for differentiation of metal rich granules. The supernatant was transferred to a new 1.5 ml polypropylene centrifuge tube and centrifuged at 15,000 *g* for 10 minutes at 4º C. The pellet (Fraction ORG) consisted of subcellular organelles including mitochondria. The supernatant was transferred to a new 1.5 ml polypropylene centrifuge tube, heated to 100 º C for 10 minutes to denature heat-labile proteins, cooled on an ice bath for 10 minutes and centrifuged at 15,000 *g* for 10 minutes. The pellet (Fraction HLP) contains heat-labile cytosolic proteins and the supernatant (Fraction HSP) contained heat-stable cytosolic proteins such as metallothionein, glutathione and phytochelating agents. The pellet representing cell fragments was resuspended in 1M NaOH, repeatedly vortexed while being heated to 80º C for 20 minutes, cooled, and centrifuged at 5,000 *g* for 10 minutes. The supernatant (Fraction CDN) contains resuspended metals not associated with metal rich granules, leaving metal rich granules in the pellet (Fraction MRG).

Throughout the partitioning process all fractions and reagents were chilled on an ice bath to minimize changes in Zn distribution among fractions. Fractions were then dried, digested at 80º C in 0.1ml of concentrated nitric acid for a minimum of 2 hours followed by 0.1 ml 30% hydrogen peroxide and evaporated to dryness. Samples were brought up to 1.5 ml with 10% nitric acid for Zn analysis by flame atomic absorption using an Instrumentation Laboratory Video 22 with Smith-Hieftje background correction.

Statistical Analyses

For each mayfly species the total and subcellular concentrations of Zn, algal consumption, organism presence on tiles and growth were compared across treatments by 2-way ANOVA (PROC GLM in SAS 9.1.3) to test the main effects of dietary and aqueous exposures to Zn and their interaction. I also employed LSMEANS and REGWQ for comparison of treatment means. Confidence intervals and descriptive statistics were calculated using PROC MEAN. All statistical analyses were conducted using $\alpha=0.05$ in SAS 9.1.3. Type III sum of square tables were used if replicates were missing due to emergence, mortality and/or insufficient amounts of sample material. When interactions were found to be insignificant the interaction term was removed and the ANOVA was reanalyzed. Distributions of proportions appeared normal and variances were homogenous so values were not transformed.

Dietary and aqueous trials at 0, 800 and 1600 µg/l Zn were analyzed together as a 3 x 3 factorial for tile occupancy, molting frequency, total

accumulation and Zn accumulation for each subcellular fraction. However, molting frequency and feeding tile occupancy was analyzed with and without the aqueous-only treatments, providing an opportunity to compare traditional toxicity tests with tests including dietary exposure. Statistical analysis of algae consumption included only non-contaminated and contaminated dietary treatments since algae was not present in the aqueous-only treatments. I also compared responses in the Zn-contaminated dietary exposure groups with the non-contaminated dietary exposure groups.

Results

Water Quality

Aqueous Zn concentrations were consistent throughout the experiments and only slight differences were observed between diluters and between the *Ameletus* and *Epeorus* trials (Tables 1 and 2). Water quality conditions showed little variation between diluters and experiments (Table 3).

Mortality and Growth

Because laboratory toxicity tests were conducted using sublethal levels of aqueous Zn mortality was minimal in trials for both species. *Ameletus* mortality averaged 1.0% (sd=3.1) across all 24 dietary treatments and 16.3% (sd=12.0) across all 24 aqueous-only treatments. *Epeorus* mortality averaged 17.2% (sd=15.5) across the 24 dietary treatments and 26.3% (sd=20.4) across the 24 aqueous-only treatments. The use of late instars in the *Epeorus* tests allowed

23.7% (sd=26.4) organisms to emerge where little emergence was observed in the *Ameletus* experiments (1.2% sd=3.4).

Since we were unable to differentiate among individual organisms, the change of average head capsule widths for each replicate was used to measure growth between treatments. Organisms were initially assigned to test chambers in a manner that assured initial sizes were similar across treatments at the beginning of the trials. However, due to bias caused by the loss of larger organisms to emergence, I was unable to assess changes in growth throughout the experiment.

Algal Consumption

Aqueous and dietary Zn exposure, and their interaction, significantly affected algae consumption rates of *Ameletus* (Fig. 3; $F_{5,18}$=12.00; $p_{overall}$ <0.0001). *Ameletus* nymphs consumed significantly more non-contaminated than contaminated algae ($F_{1,18}$=39.68; p<0.0001). Consumption of non-contaminated algae also decreased with aqueous exposure ($F_{2,18}$=5.06; p=0.018). However, this trend was not observed for contaminated algae, resulting in a significant interaction term ($F_{2,18}$=5.10; p=0.0176). Neither aqueous nor dietary exposure to Zn affected consumption of algae by *Epeorus* ($F_{5,18}$=0.72; $p_{overall}$ =0.6160).

The presence of mayflies on tiles was significantly different across treatments for both *Ameletus* ($F_{4,31}$=26.60 p<0.0001) and *Epeorus* ($F_{4,29}$=9.24; p<0.0001. Figure 4). Tile occupancy decreased with dietary exposure but not

aqueous exposure for both *Ameletus* ($F_{2,31}$=51.71; p_{diet} <0.0001, $F_{2,31}$=1.50; $p_{aqueous}$ =0.2398) and *Epeorus* ($F_{2,29}$=18.07; p_{diet} <0.0001, $F_{2,29}$=1.19; $p_{aqueous}$ =0.3200; Fig. 4). Both species occupied feeding tiles over alternative substrate most often in non-contaminated, then contaminated and lastly bare-tile treatments.

When comparing the tile occupancy of the Zn-contaminated and non-contaminated treatments, *Ameletus* ($F_{3,20}$= 2.68; $p_{overall}$ =0.0746) avoided contaminated tiles ($F_{1,20}$=5.21; p_{diet} =0.0335, $F_{2,20}$=1.41; $p_{aqueous}$ =0.2667), but this effect was not significant in *Epeorus* ($F_{3,20}$= 2.17; $p_{overall}$ =0.1227) where no difference was observed ($F_{1,20}$=0.72; p_{diet} =0.4073, $F_{2,20}$=2.90; $p_{aqueous}$ =0.0781). An overall higher occupancy of both feeding tiles and substrate tiles was noticed in *Epeorus,* which was likely due to its niche of clinging to cobble in riffle areas.

Molting Frequency

Zn contamination influenced molting frequency in *Ameletus* ($F_{4,31}$=20.99; $p_{overall}$ <0.0001) but not *Epeorus* ($F_{4,29}$=1.94; $p_{overall}$ =0.1306) and this was entirely the result of dietary exposure (Fig. 5). Molting frequency was heavily influenced by dietary but not aqueous exposure for both *Ameletus* ($F_{2,31}$=40.60; p_{diet} <0.0001, $F_{2,31}$=1.37; $p_{aqueous}$ =0.2679) and *Epeorus* ($F_{2,29}$=3.68; p_{diet} =0.0377, $F_{2,29}$=0.45; $p_{aqueous}$ =0.6450).

When omitting aqueous-only treatments from the analysis, *Ameletus* ($F_{3,20}$=5.56 $p_{overall}$ =0.0061) receiving non-contaminated food sources molted more than those receiving Zn-contaminated food sources ($F_{1,20}$ =14.81; p_{diet}

=0.0010, $F_{2,20}$ =0.92; $p_{aqueous}$ =0.4149). This effect was not significant in *Epeorus* ($F_{3,20}$=0.22; $p_{overall}$ =0.8812, $F_{1,20}$ =0.01; p_{diet} =0.9416, $F_{2,20}$ =0.33; $p_{aqueous}$ =0.7244).

Total Tissue Accumulation

Zn concentrations in *Ameletus* ($F_{4,31}$ =29.55 $p_{overall}$ <0.0001) were increased by both dietary ($F_{2,31}$ =18.34 p_{diet} <0.0001) and aqueous ($F_{2,31}$=40.77 $p_{aqueous}$ <0.0001) exposure (Fig 6). This trend was also observed in *Epeorus* ($F_{4,29}$=14.19 $p_{overall}$ <0.0001) where total Zn tissue concentrations were significantly increased by both dietary ($F_{2,29}$ =24.51 p_{diet} <0.0001) and aqueous exposure ($F_{2,29}$ =3.86 $p_{aqueous}$ =0.0326).

Although Zn uptake increased with aqueous concentrations, across the range of all aqueous treatments examined dietary exposure contributed significantly to metal accumulation in both species (Fig. 7). Zn levels in mayflies from the aqueous-only exposures approached those observed in the dietary treatments only at the highest concentrations (6400-12800 µg/l).

Subcellular Distribution

Mayflies accumulated Zn from algae that was cultured in Zn-contaminated media, but also accumulated Zn from non-contaminated algae in the 800 and 1600 µg/l aqueous treatments (Appendix A). This resulted in significantly greater Zn concentrations for a majority of subcellular fractions for both species compared to aqueous-only treatments (Fig. 8 to 12; Tables 1 and 2). For

Epeorus nymphs, diet was a significant exposure route of Zn accumulation in the CDN, ORG, HLP and HSP fractions but was not significant for the MRG fraction (Table 4). Dietary Zn exposure had a significant effect on accumulation in *Ameletus* nymphs for the CDN, MRG, ORG and HSP fractions but was not significant for the HLP fraction (Table 4). For many fractions, aqueous exposure increased Zn accumulation by *Ameletus* (CDN, MRG, HLP, HSP) and *Epeorus* (CDN, HSP).

The subcellular distribution of Zn also differed between species. Averaged across all laboratory treatment groups, the proportion of Zn bound to ORG ($F_{1,67}$=87.04; p<0.0001) and CND ($F_{1,67}$=5.29; p=0.0245) fractions were significantly higher for *Ameletus* compared to *Epeorus* (Fig. 13, Table 5). In contrast the proportions of Zn bound to HLP ($F_{1,67}$=33.24; p<0.0001) and HSP ($F_{1,63}$=53.45; p<0.0001) were higher for *Epeorus* nymphs.

Field Study

Zn concentrations in *Ameletus* and *Epeorus* collected from the Arkansas River increased downstream of California Gulch and were generally greater than levels measured in laboratory experiments (Fig. 14). Zn concentrations in mayflies collected from station AR5 were 2x (*Ameletus*) to 7.4x (*Epeorus*) greater than those in the highest Zn treatments in the laboratory, despite much lower aqueous concentrations in the field. Both *Ameletus* sp. and *Epeorus* sp. collected from the Arkansas River had much higher concentrations of Zn in the HLP fraction than any of the laboratory treatment levels.

Discussion

Algae consumption, feeding behavior, molting frequency, and Zn accumulation in mayflies were significantly influenced by exposure to metal-contaminated periphyton. *Ameletus* sp. and *Epeorus albertae* that were fed contaminated algae consistently accumulated more Zn than organisms in the aqueous-only treatments. Zn accumulation was over five times higher in treatments with a dietary component versus traditional aqueous-only exposure. Specifically, organisms exposed to Zn in the combined diet and 1600 µg/l aqueous treatments accumulated similar amounts of Zn as the 6400 and 12800 µg/l aqueous-only treatments. Zn tissue concentrations in the field, where species are exposed through diet and water, was double that of even the highest laboratory exposure level, despite much lower aqueous concentrations in the field . Dietary exposure of mayfly nymphs clearly influenced Zn accumulation as well as feeding and molting, both critical functions for survival. Thus, dietary exposure should be considered in development of water quality criteria for aquatic organisms. My study demonstrates that the aqueous-only toxicity tests used to set these criteria clearly under-estimates the potential for toxic exposures in the field.

In addition to increasing overall accumulation, dietary exposure to Zn also significantly influenced accumulation in most subcellular fractions in the two mayfly species. In both species, a majority of subcellular fractions (4 of 5) were

significantly influenced by dietary exposure, including fractions (ORG and HLP) most likely responsible for eliciting toxic responses (Reinfelder and Fisher 1991; Harrison and Curtis 1992; Wallace et al. 2003; Cain et al. 2004; Meyer 2005; Cain et al. 2006; Ng and Wood 2008). In both species the ORG fraction was significantly affected by diet but not aqueous Zn exposure. This could imply that Zn accumulated though the diet is assimilated into cellular organelles or that the dietary Zn is preferentially being sequestered into vacuoles. Although *Ameletus* accumulated more Zn per gram of tissue than did *Epeorus*, this genus sequestered most of the Zn in the less toxic, HSP fraction. In general, more of the subcellular fractions of *Epeorus* nymphs were influenced or altered through dietary exposure than through aqueous exposure, while fractions in *Ameletus* were influenced by both routes of exposure. These differences further support field reports that mayfly genera respond differentially to Zn contamination (Clements et al. 2000).

Dietary exposures to Zn significantly altered feeding as determined by presence of nymphs on feeding tiles and molting frequency of both mayfly species. *Ameletus* and *Epeorus* increased molting frequencies when provided a food source, even when that food was contaminated with Zn. Photoanalysis revealed that *Ameletus* nymphs consumed less algae with increased dietary and aqueous exposures to Zn. Specifically, *Ameletus* nymphs consumed less Zn-contaminated algae than non-contaminated algae, regardless of aqueous exposure levels. Consumption of non-contaminated algae by *Ameletus* decreased with higher aqueous concentrations of Zn, which suggests a

palatability, nutritional, or toxic response as aqueous Zn adhered to the surface of the non-contaminated algae. By contrast, *Epeorus* utilized tiles and consumed algae regardless of whether the level of contamination. Others have found changes in critical life history characteristics of mayflies when exposed to metals. For example, Irving et al (2003) found decreases in consumption and growth in *Baetis tricaudatus* when exposed to dietary Cd. Growth and emergence were reduced in *Epeorus* mayflies fed diatoms with elevated levels of Cu or Zn (Hatakeyama 1989). *Baetis tricaudatus* mayflies experienced reduced growth when fed metal-contaminated biofilm (Clements et al. 2002; Carlisle and Clements 2003). I speculate that reduced molting and feeding rates of mayflies will affect energy budgets and could result in reduced growth, survival and fecundity.

Relative to other fractions, the proportion of Zn in the CDN fragments (associated with the exoskeleton, cellular debris and nuclei) steadily increased with laboratory-based aqueous exposure in *Ameletus* across all treatments. My methods were unable to differentiate between Zn bound to cell membranes and Zn bound to connective tissue. Since membranes play an important role in the toxic pathways of many metals (Klaassen 2001; Niyogi and Wood 2004) further refinement of this fraction would prove useful to examining the fate of heavy metals.

My laboratory experiments, even with added Zn exposure through food source, did not reflect the concentration in field populations from the metals-contaminated Arkansas River. Zn tissue concentrations were significantly higher

in the field, even though aqueous Zn concentrations at all Arkansas River sites were well below the lowest aqueous exposures in the laboratory experiments. Total Zn concentrations were 2 (*Ameletus*) to 7.4 (*Epeorus*) times greater in Arkansas River mayflies compared to laboratory exposures. Most subcellular tissue concentrations were higher for Arkansas River mayflies including sensitive fractions such as ORG (*Epeorus*) and HLP (both species). These results show that metals were either more bioavailable in the field or that a 10 d exposure was insufficient to simulate chronic and multi-metal exposure conditions in the natural environment. Regardless of the explanation, my results clearly demonstrate that traditional short-term laboratory studies underestimate potential exposure in the field even with the addition of dietary exposure.

With the exception of station AR5, concentrations of Zn in all subcellular fractions of organisms from the field tended to increase with aqueous concentration. Despite the fact that measured Zn concentrations at station AR5 were nearly half that measured at station AR3, accumulation at AR5 was greater for both species. Recent studies have shown that after removal of historical mining pollution recovery of macroinvertebrates and fish at station AR5 is much slower compared to AR3 (Clements et al. 2010), and that routine physicochemical characteristics (e.g., pH, temperature, water hardness, dissolved organic carbon) cannot account for these differences (Clements et al. 2008). I speculate that non-point sources of Zn contamination at AR5 may increase dietary and aqueous exposure of mayflies to Zn. Residual mine tailings located in the floodplain between stations AR3 and AR5 introduce pulses of Zn

during rain events (Clements et al. 2010). These episodic pulses of Zn are difficult to capture as they likely occurred outside of the sampling schedule. A more frequent sampling schedule timed with these precipitation events may be characterize these episodic pulses. Although these pulses may not elicit significant mortality, because algae rapidly accumulate Zn, dietary exposure of grazing mayflies would remain high.

The primary goal of my research was to compare the relative importance of dietary (via algae) and aqueous exposure to Zn. I also compared the efficacy of different methods for preparing algae for use as a dietary treatment (see Appendix A). It was difficult to produce an uncontaminated dietary treatment because of the high rate at which algae accumulated Zn when placed in aqueous Zn treatments. On day 0 of our experiment the algal concentration of Zn in the non-contaminated treatment was only 14% the level in the contaminated treatment; however, this difference was negligible after 24 hours in the 1600 µg/l Zn treatment chambers. Differences between feeding rates and accumulation between Zn-contaminated and non-contaminated treatments would likely have been even greater if feeding tiles were replaced more frequently. The tendency of metals to rapidly move between water and algae limits the ability to create a contaminant free dietary treatment with an aqueous exposure.

Although total and subcellular metal concentrations in *Ameletus* were much higher in laboratory trials than those measured in *Epeorus*, this trend was reversed in the field. There are a number of factors that may explain this reversal in pattern. First, interspecific differences in metal uptake may have been related

to differences in organism size, both in the laboratory and during field collections. In this study *Epeorus* nymphs in laboratory trials were much larger than *Ameletus* and routinely removed all of the algae from feeding tiles. Consequently, feeding *Epeorus ad libitum* for the duration of the study was difficult with the limited space and time needed to culture contaminated and non-contaminated algae. As such, algal consumption and dietary exposure in *Epeorus* may have been higher with more abundant food resources. During a 3 day period when a surplus of algae covered feeding tiles was available, *Epeorus* did show significant differences between feeding rates of contaminated and non-contaminated algae.

In both the laboratory and the field *Ameletus* sequestered Zn to a greater concentration the HSP fraction which contains metal chelating proteins such as metallothionein. The difference in HSP concentration between species was less in field organisms which were of more similar size. Interspecific differences of Zn tissue concentrations in the field could be attributed to timing of observations and duration of exposure. *Epeorus* nymphs collected in August were likely developing during the high metal concentrations typically observed in late spring and early summer. *Ameletus* nymphs were collected just before the spring pulse of high metal concentrations and this cohort likely developed without exposure to seasonal high metal pulses. Although both genera are morphologically adapted to scraping periphyton, food sources and quality are likely different in each microhabitat and fluctuate seasonally. Contaminants that are bound to certain proteins within natural food sources may be in a more readily bioavailable form

(Harrison and Curtis 1992; Meyer 2005; Ng and Wood 2008; Reinfelder and Fisher 1991). Metals in different biofilms likely bind to different proteins changing concentrations and bioaccumulation efficiencies. The differences in uptake efficiency, depuration rates and subcellular profiles have been found to differ between insect taxa (Buchwalter and Luoma 2005; Buchwalter et al. 2008) and these may help explain some of the interspecific differences observed in my study. However, these factors can not explain differences between the field and laboratory results. Laboratory toxicity tests, even those of complete life cycle durations that include a dietary exposure, will likely continue to underestimate metal exposure in the field.

Although the use of natural periphyton assemblages may have increased ecological realism in our experiments (Xie et al. 2010), we believe that exposing mayflies to a monoculture of *Scenedesmus* sp. reduced variation in nutritional quality and bioavailability. We also believe that exposing organisms to periphyton cultured in Zn has advantages over the traditional approach of bathing periphyton in a Zn solution (Appendix A).

Examination of subcellular concentrations of heavy metals is a useful improvement to assessment of total body burden because it demonstrates how the organism mechanistically responds to metals contamination and has the potential to show which species tend to accumulate metals in the most vulnerable compartments. Although high concentrations of Zn pollution are a primary concern in aqueous ecosystems impacted by historical mining activities in Colorado, Zn is an essential micronutrient for both algae and insects. Non-

essential metals pollutants, such as cadmium, also occur at high levels in mine-contaminated rivers of the west and may be regulated differently. Future studies are needed to examine how subcellular sequestration differs between regulated and non-regulated metals. Some interspecific differences observed in field observations could have been explained had a full suite of metals been included in the present study.

Given the difference in tissue concentrations of Zn observed in the field and the laboratory, continuing these laboratory trials to toxic levels would have likely overestimated a safe concentration for *Ameletus* and *Epeorus*. Numerous biotic and abiotic factors contribute to the marked disparity between toxic concentrations predicted in laboratory studies and those observed in the field. Assessing the relative importance of these factors is challenging because of the difficulty reproducing the complexities of natural ecosystems in the laboratory. We suggest that the inclusion of dietary exposure to contaminants represents an obvious improvement in the ecological realism of laboratory toxicity tests and should be considered in the development and validation of water quality criteria for metals and other contaminants. Other researchers have also shown that sublethal measures associated with providing food, such as quantifying foraging, can represent sensitive endpoints in such studies (Sandheinrich and Atchison 1990).

Despite improvements that can be made to traditional toxicity tests, our understanding of how aquatic insects respond to contaminants will always be limited in the laboratory setting. My study demonstrates that because of these

shortfalls, the negative impacts of metals on field populations of aquatic organisms will typically be under represented. Mayflies that are sensitive indicators of metals pollution in the field and in mesocosm studies (Clements 2004) artificially appear to be metals-tolerant in the laboratory. Metal mixtures that can increase toxicity to these organisms in mine-polluted streams, and factors that ameliorate metals toxicity in the field, such as dissolved organic carbon and calcium or magnesium ions, can be difficult to reproduce. Inclusion of population-level and community-level responses to other natural stressors, such as competition and predation, are also not feasible in traditional laboratory toxicity tests. Species sensitivity distributions used to create water quality standards should be comprised of trials that include sublethal endpoints, community responses, sensitive life stages, multiple stressors and rare species whenever possible. However even improving the realism of laboratory tests may not create truly protective standards. My findings indicate that aquatic biota may receive a higher and more appropriate level of protection where field-derived metrics and biocriteria are considered in determining water quality standards at mine-polluted sites.

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FIGURES AND TABLES FROM THESIS

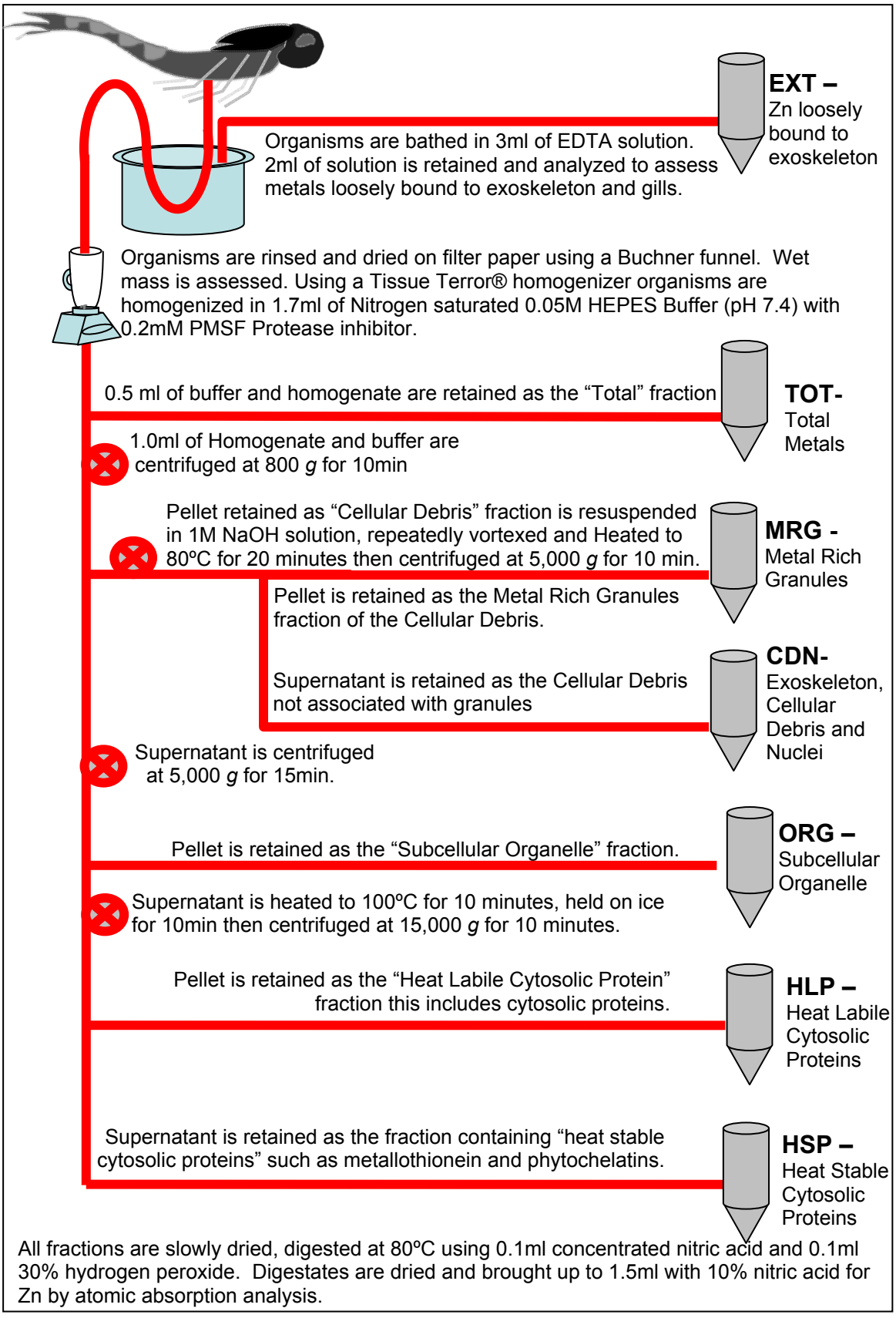

Figure 1 – Flow Chart of Subcellular Partitioning Method.

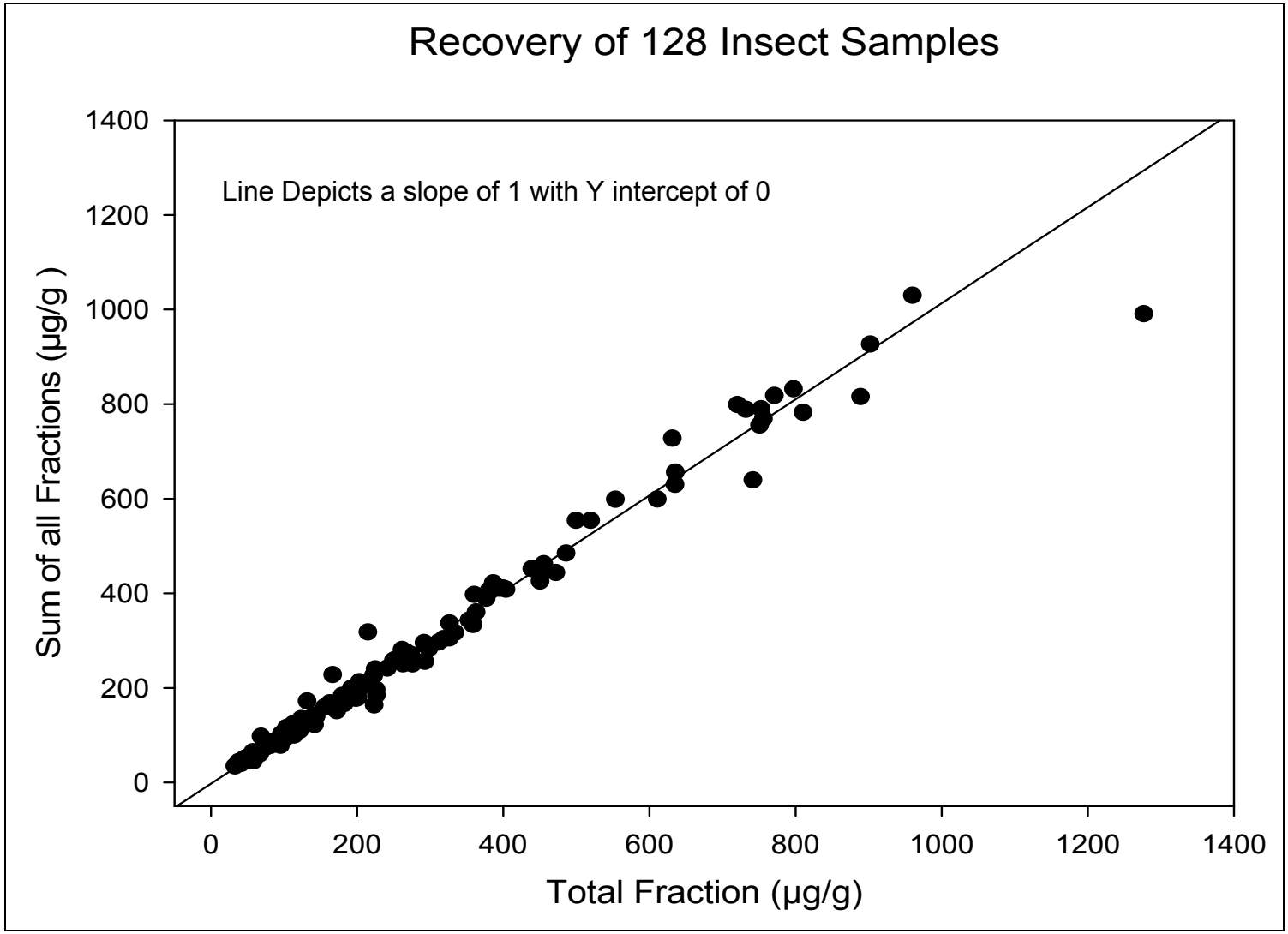

Figure 2 – Recovered Zn as a function of Total Zn. Despite tissue masses from 0.00425g to 0.11725g the recovery of insects averaged 100.9%, (s.d. = 10.4).

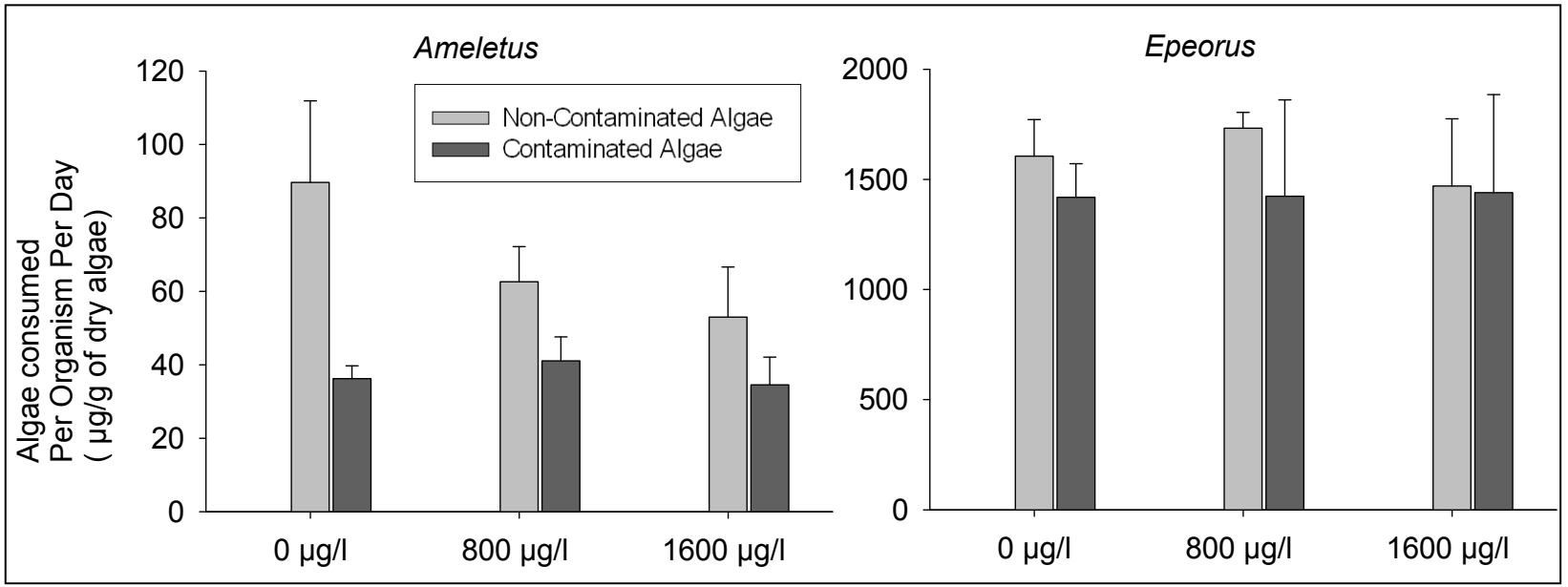

Figure 3 – Feeding rate by *Ameletus* (left) and *Epeorus* (right) nymphs in treatment cages receiving contaminated (dark gray) and non-contaminated (light gray) algae across 3 aqueous Zn concentrations. Error bars denote standard deviation. Note that Y-axes are not the same scale

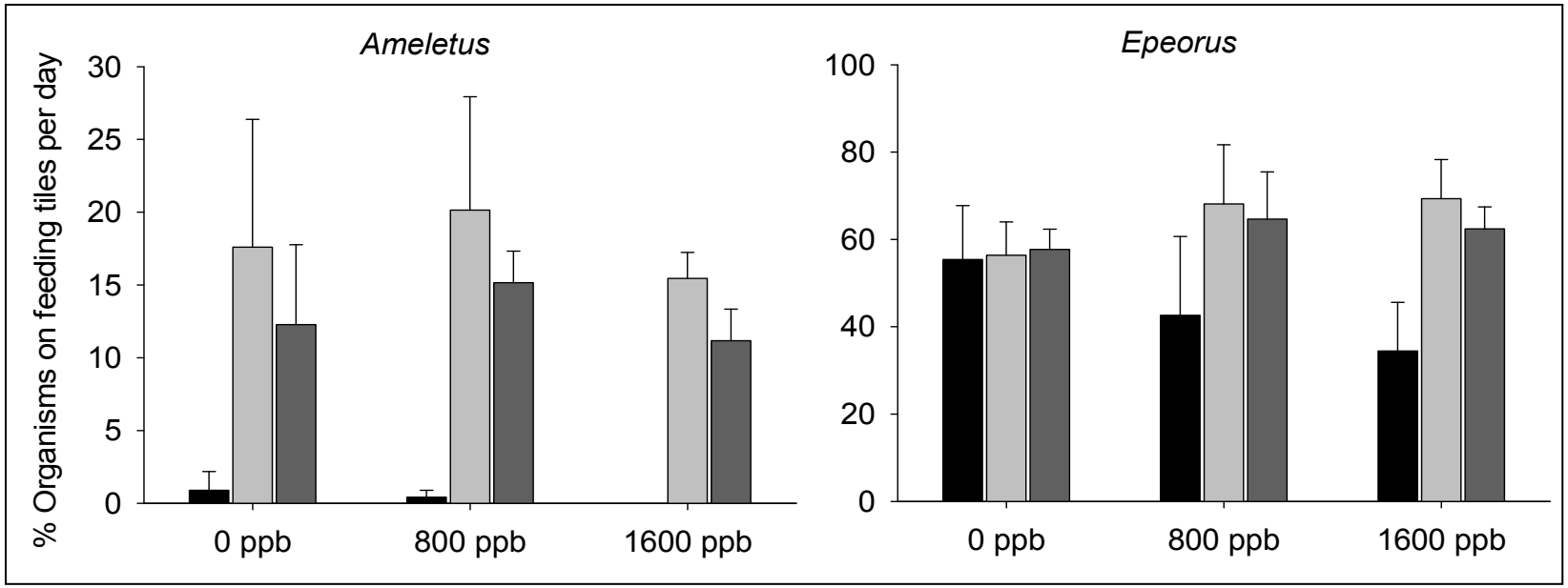

Figure 4 – Proportion of organisms on feeding tiles that have contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation. Note that Y-axes are not the same scale

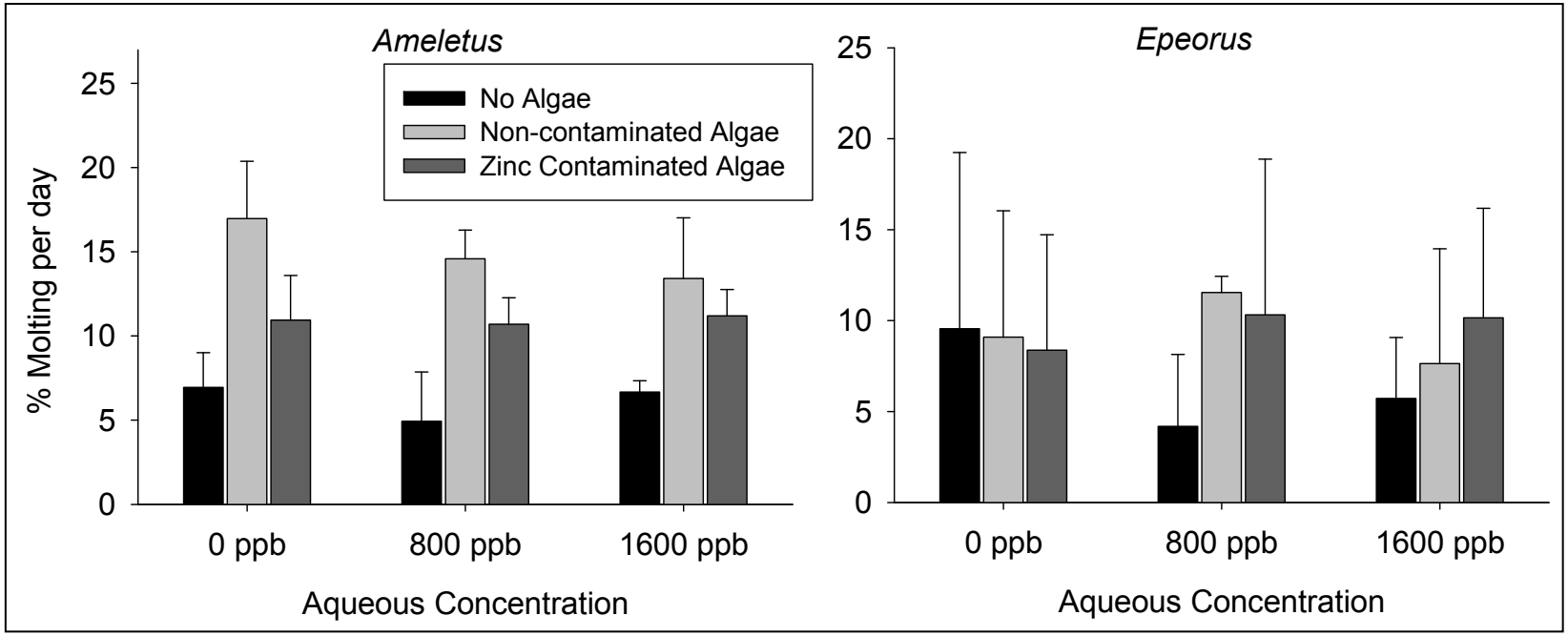

Figure 5 – Molting frequency in treatments that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation.

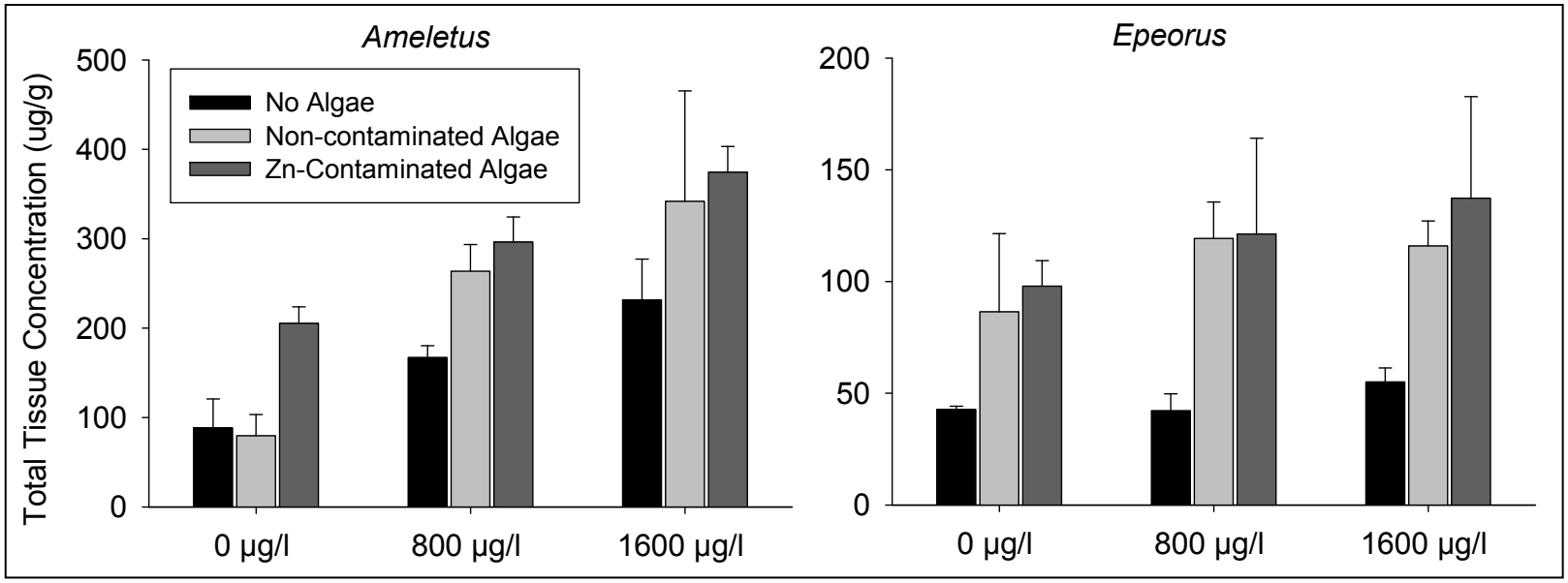

Figure 6 – Total accumulation in *Ameletus* (left) and *Epeorus* (right) nymphs that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. The EXT fraction, representing Zn loosely bound to the exoskeleton, was excluded from these values. Error bars denote standard deviation. Note that Y-axes are not the same scale.

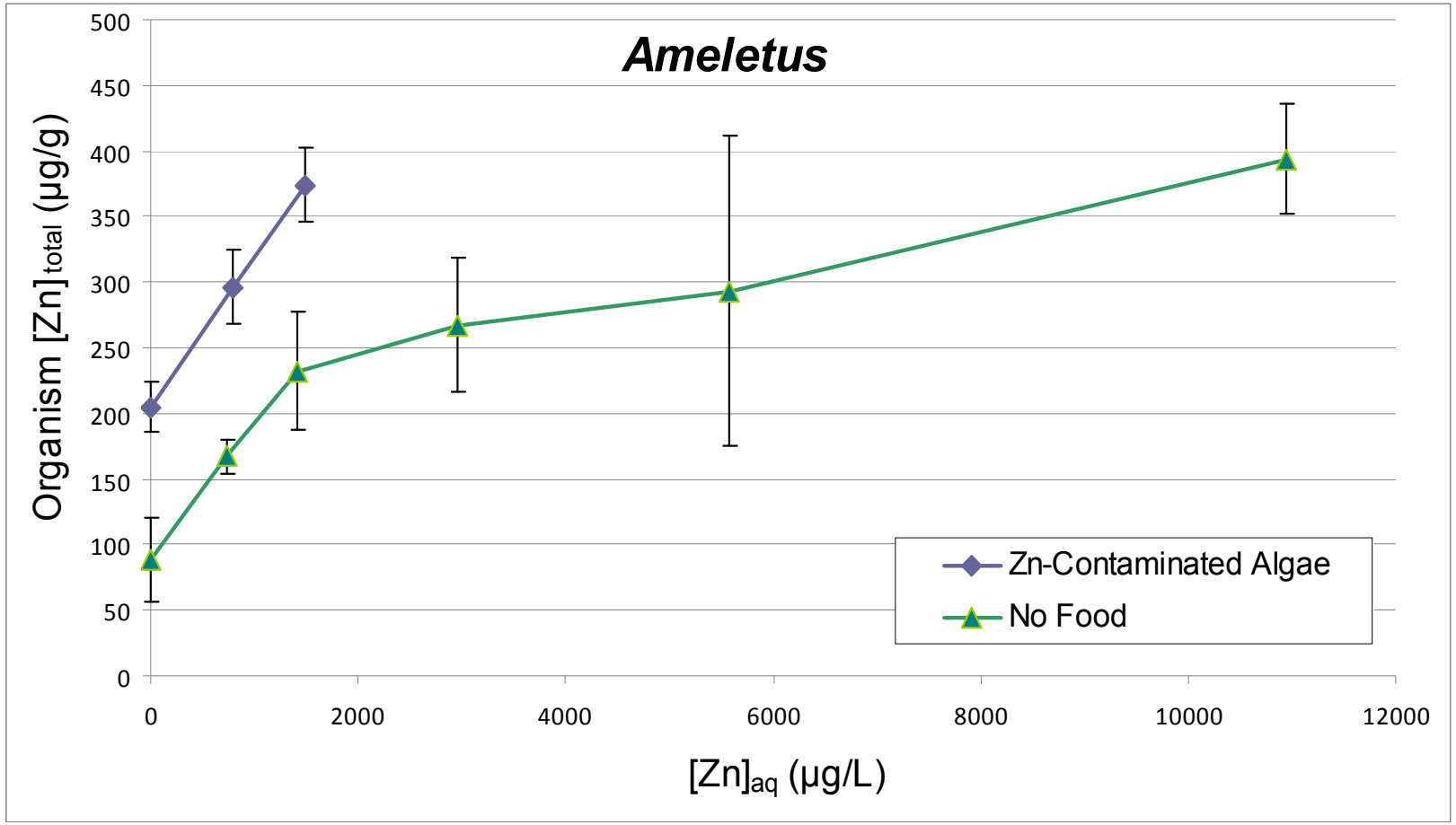

Figure 7a – Total zinc tissue concentrations in *Ameletus* nymphs that received Zn-contaminated algae (diamonds), and no algae (triangles) across aqueous Zn concentrations. Error bars denote standard deviation.

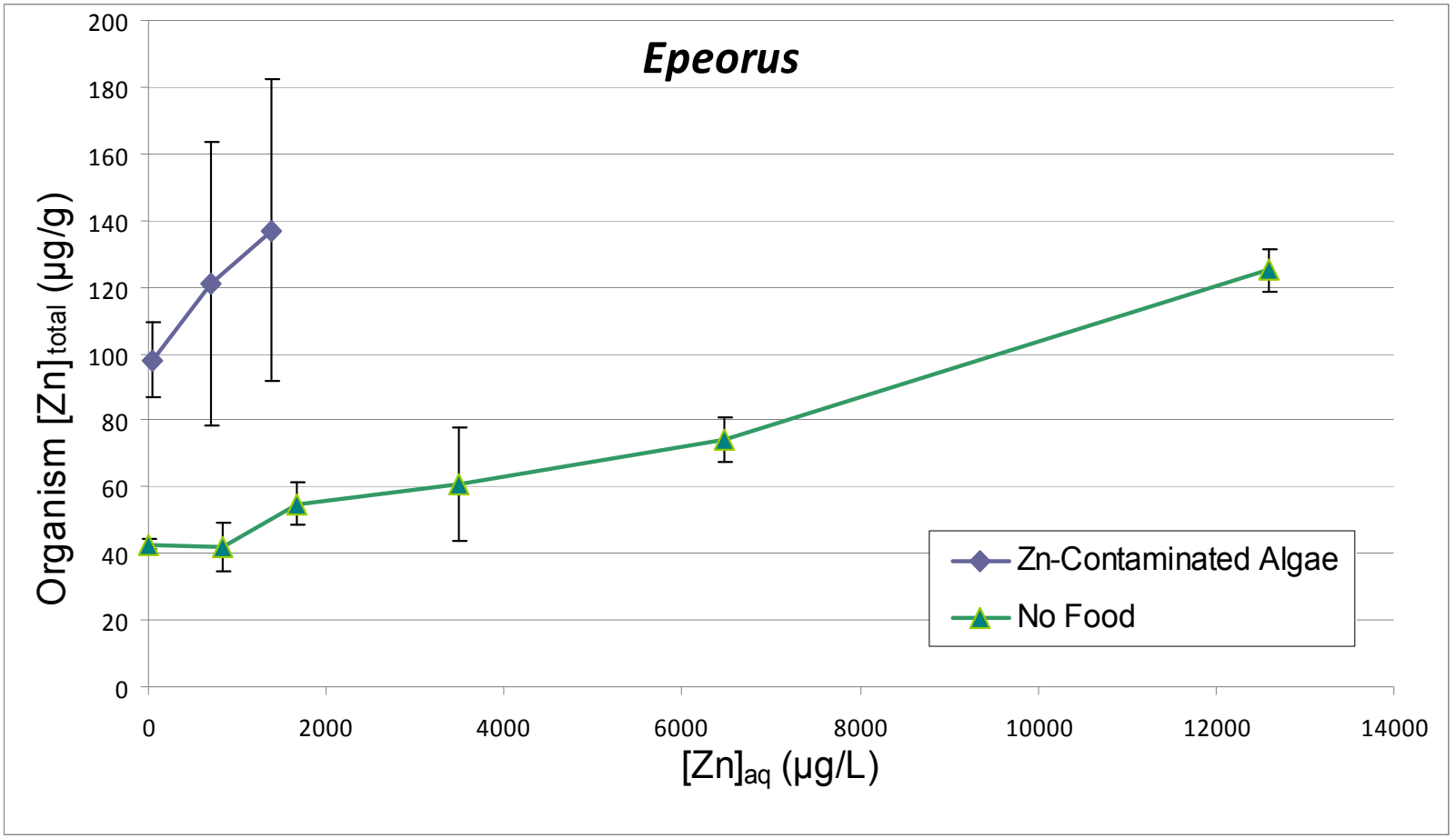

Figure 7b – Total zinc tissue concentrations in *Epeorus* nymphs that received Zn-contaminated algae (diamonds), and no algae (triangles) across aqueous Zn concentrations. Error bars denote standard deviation.

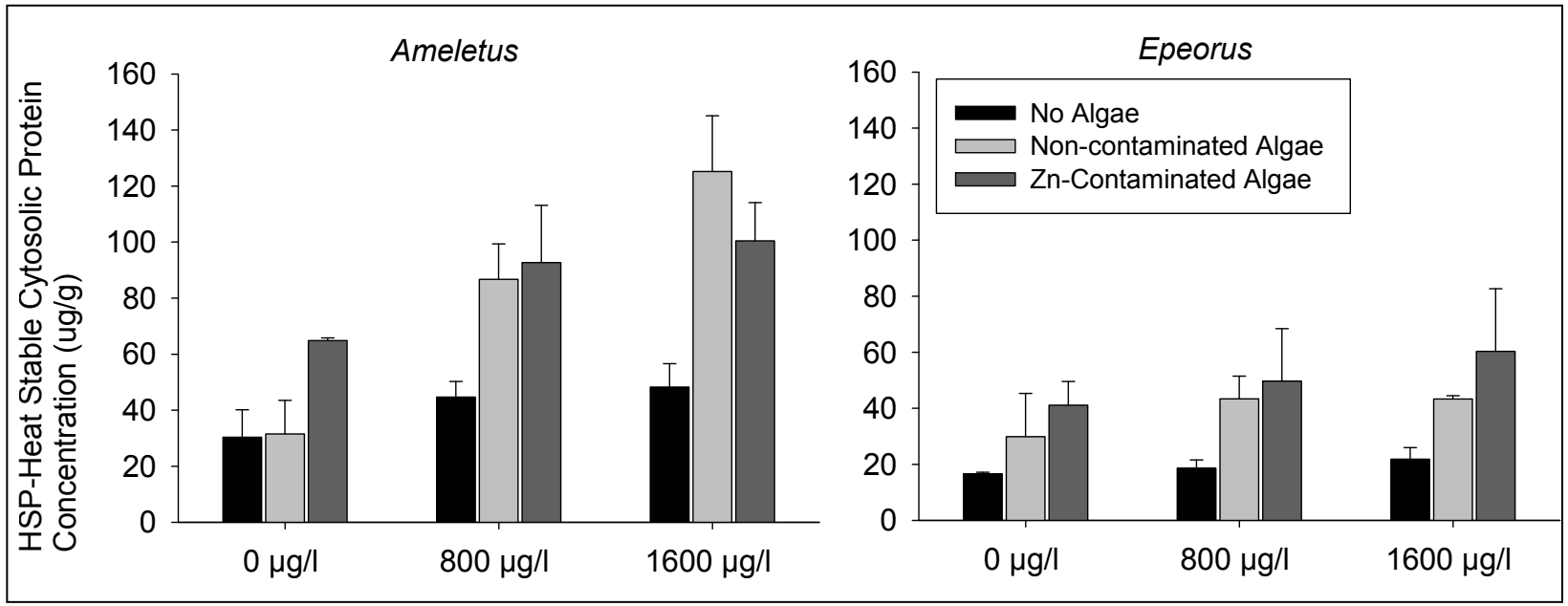

Figure 8 – Zinc concentration in the HSP fraction of *Ameletus* (left) and *Epeorus* (right) nymphs that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation.

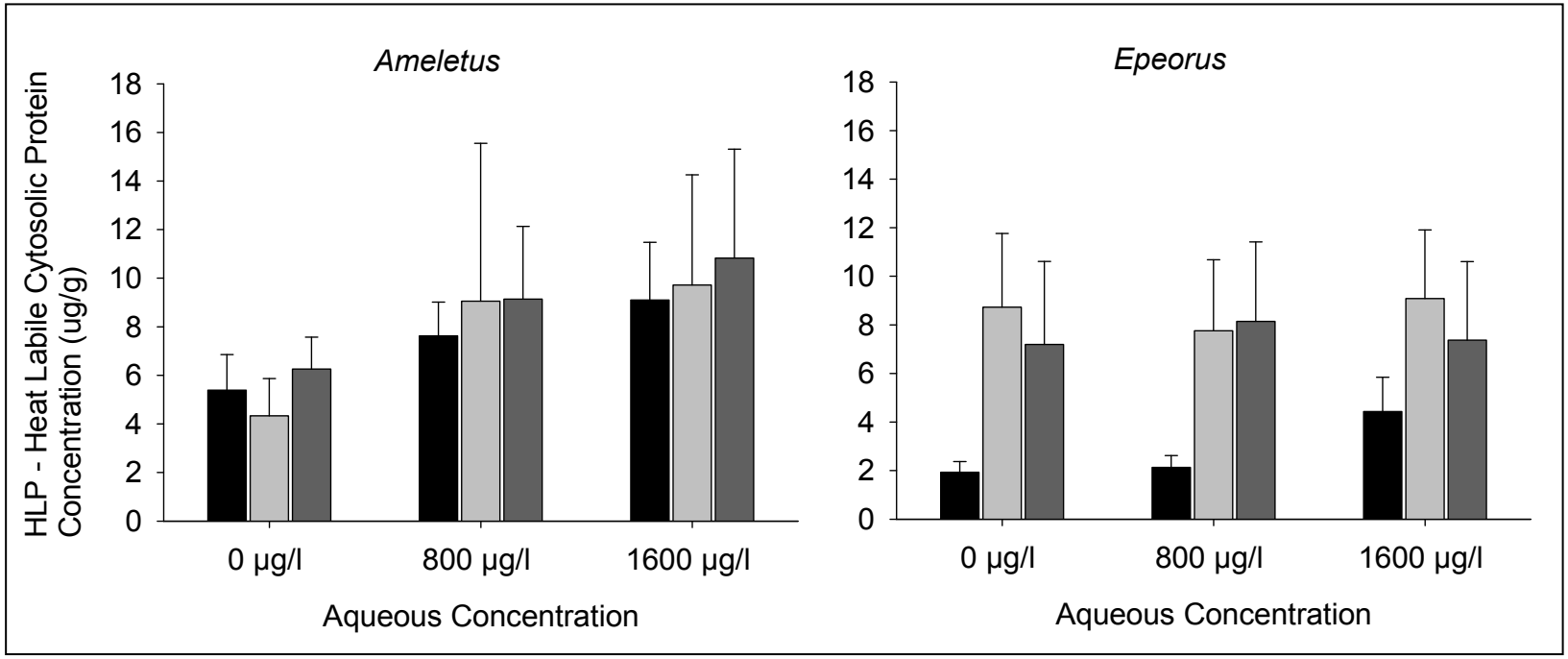

Figure 9 – Zinc concentration in the HLP fraction of *Ameletus* (left) and *Epeorus* (right) nymphs that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation.

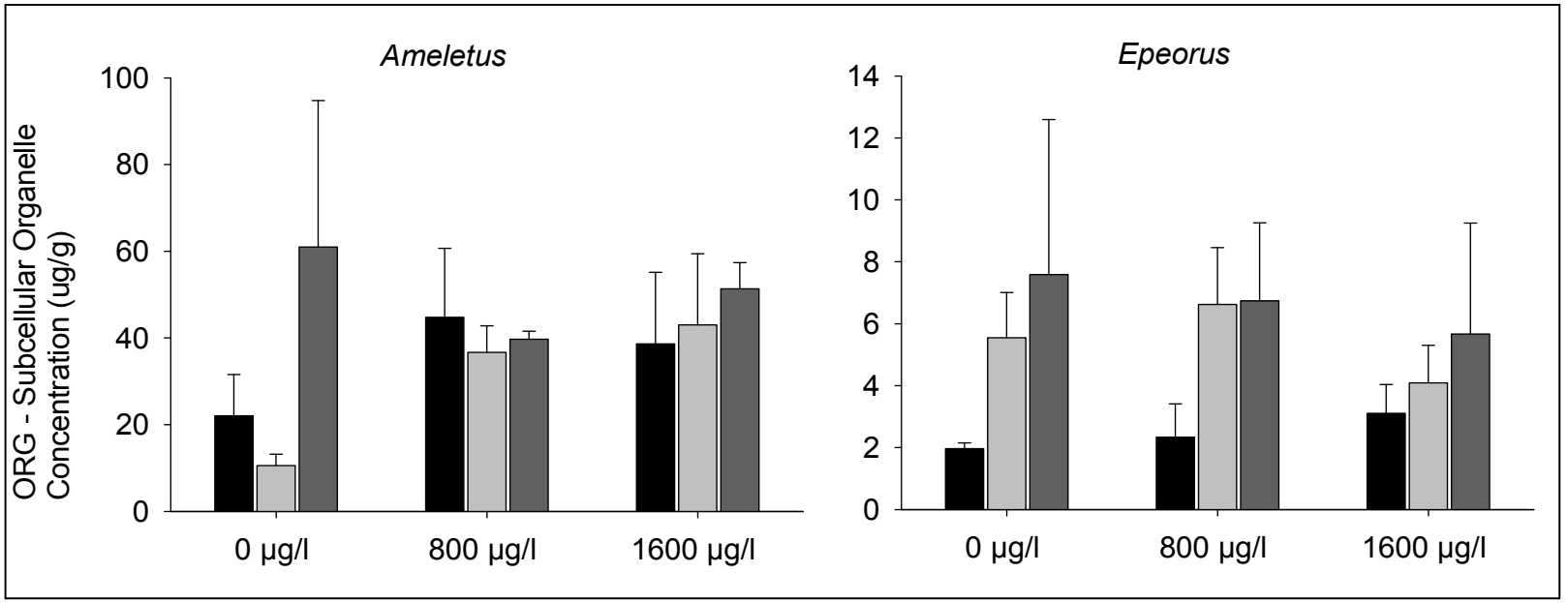

Figure 10 – Zinc concentration in the ORG fraction of *Ameletus* (left) and *Epeorus* (right) nymphs that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation. Note that Y-axes are not the same scale

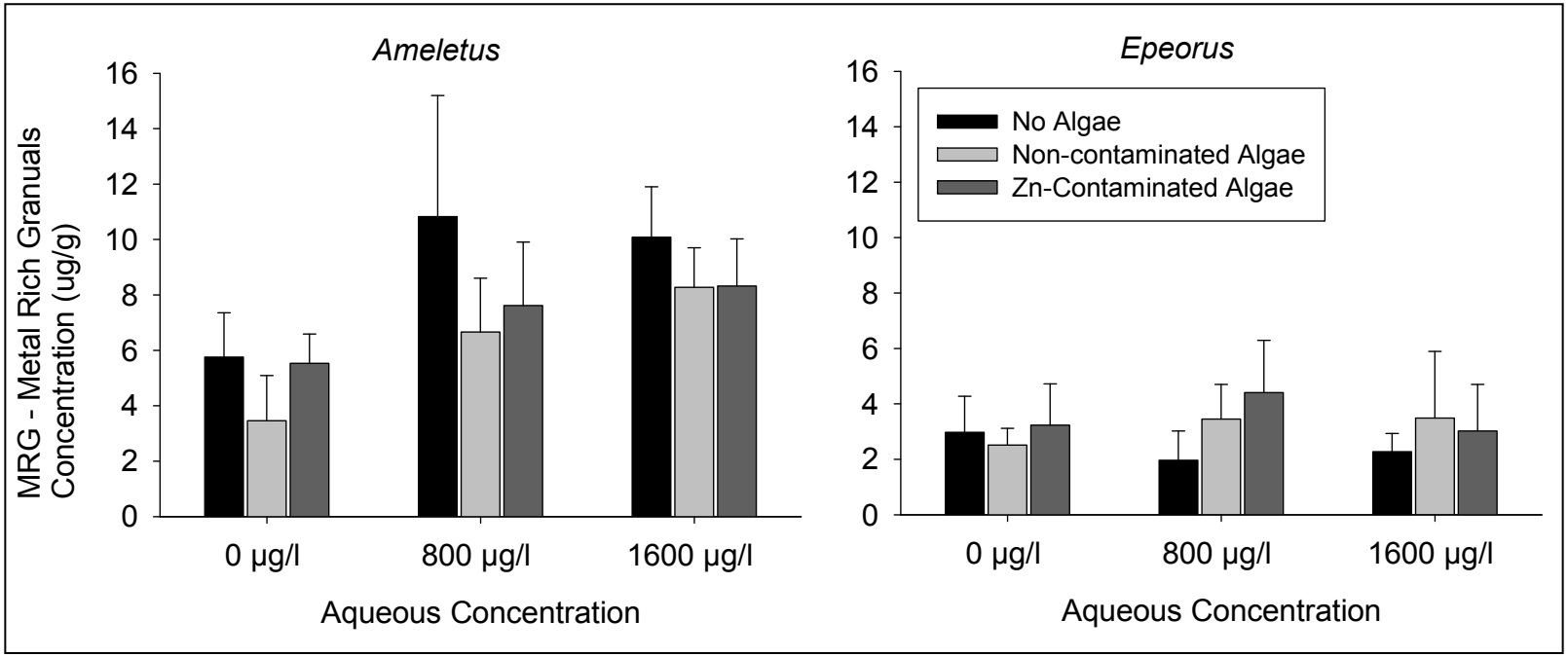

Figure 11 – Zinc concentration in the MRG fraction of *Ameletus* (left) and *Epeorus* (right) nymphs that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation.

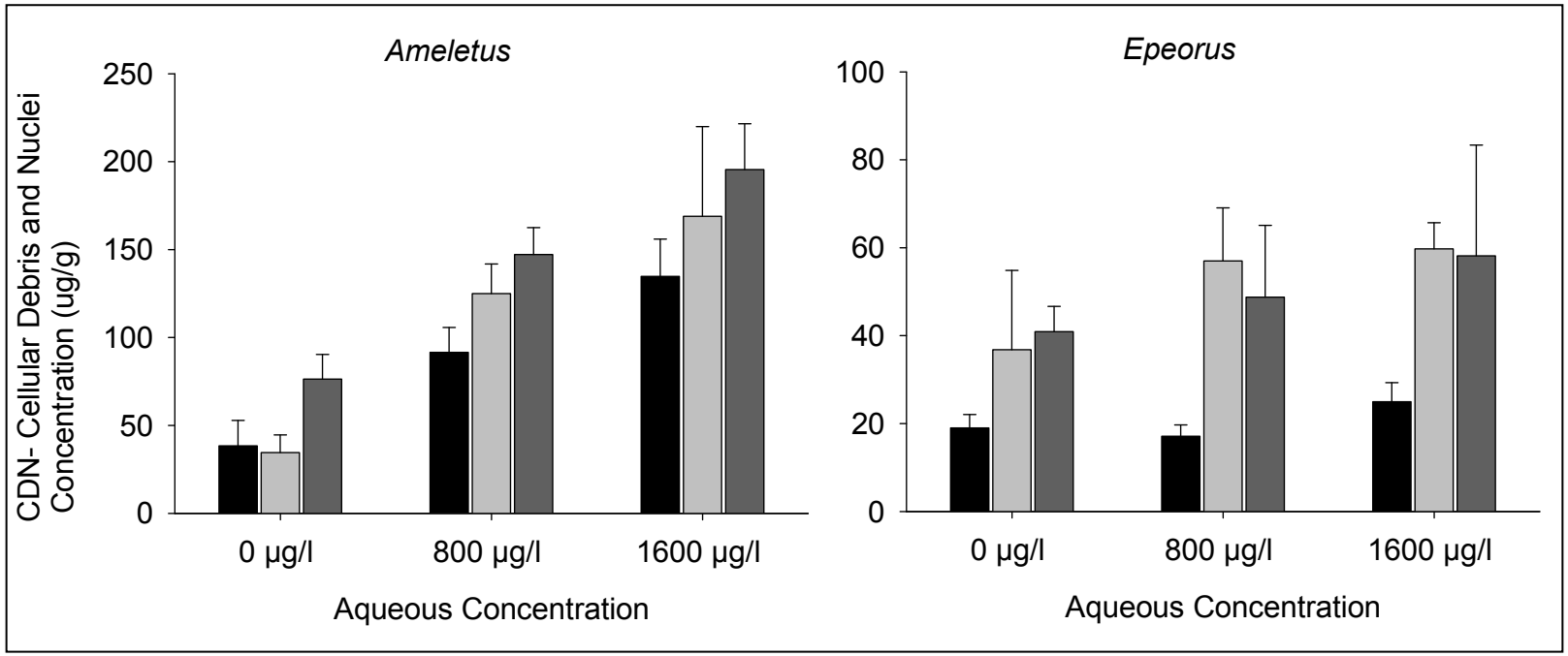

Figure 12 – Zinc concentration in the CDN fraction of *Ameletus* (left) and *Epeorus* (right) nymphs that received contaminated algae (dark gray), non-contaminated algae (light gray), and no algae (black) across 3 aqueous Zn concentrations. Error bars denote standard deviation. Note that Y-axes are not the same scale

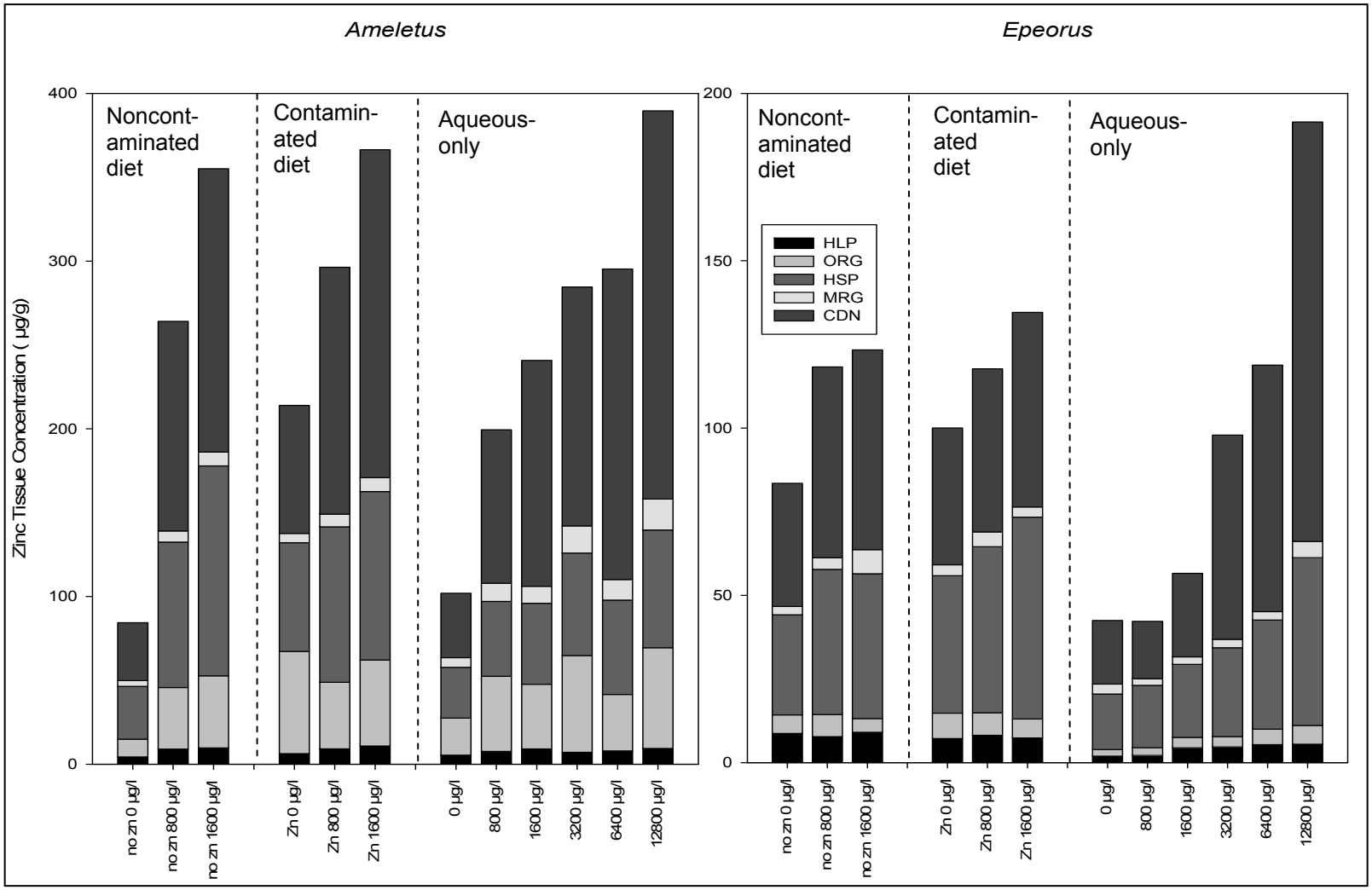

Figure 13 – Summed Zinc concentration and subcellular proportions after 10 day toxicity tests. Notice *Epeorus* (right) is at half the scale of *Ameletus* (left).

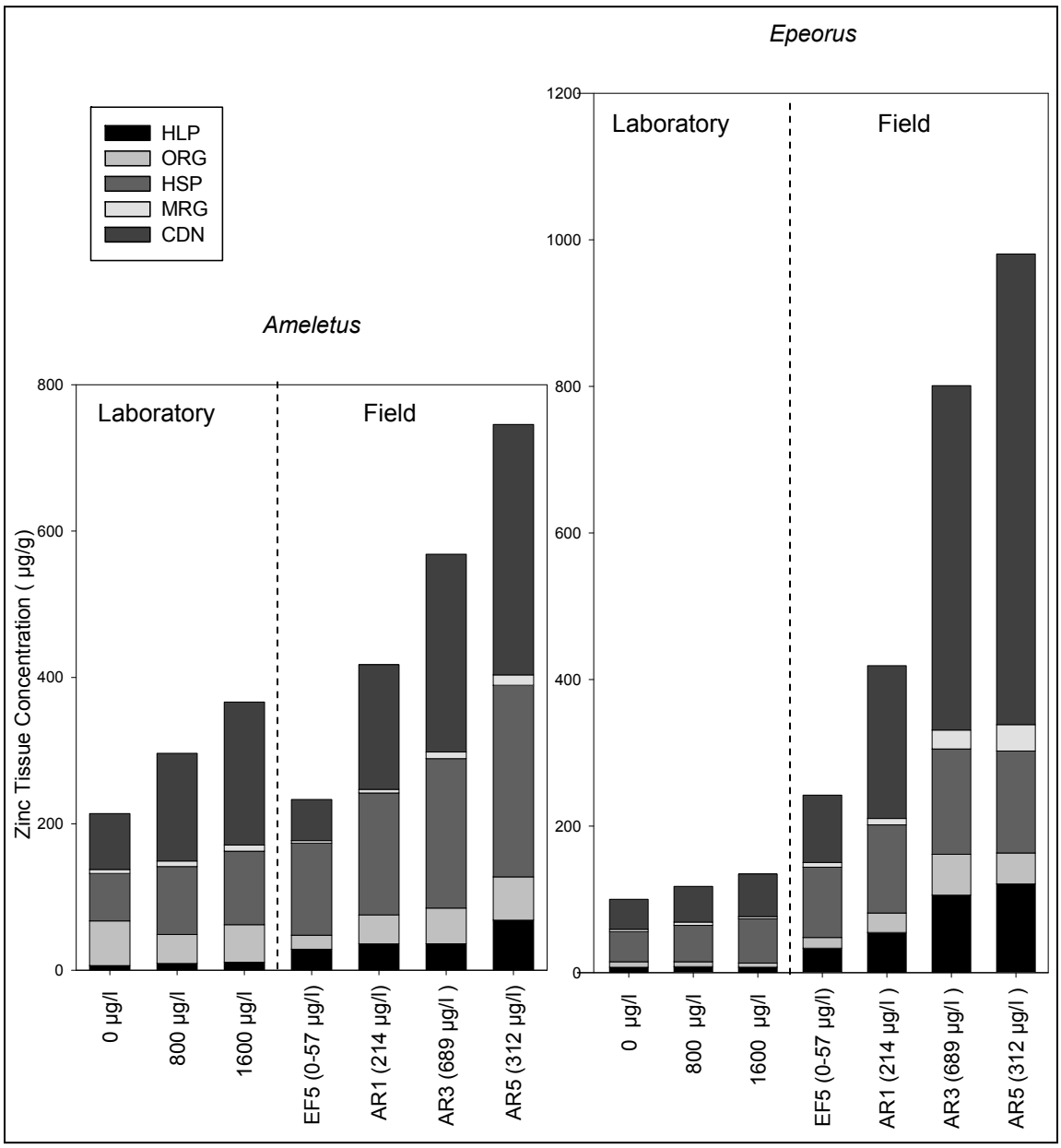

Figure 14 – Summed Zinc concentration and subcellular fractions for organisms collected from the Arkansas River field sites (Field) and organisms from 10 day laboratory experiments receiving Zn contaminated algae and aqueous exposure (Laboratory) .

Ameletus sp.

	Target aqueous treatment [Zn]	Mean aqueous treatment [Zn]	Total Zn in mayfly tissue REGWQ	Proportion of Zn in the MRG fraction REGWQ	Proportion of Zn in the ORG fraction REGWQ	Proportion of Zn in the HLP fraction REGWQ	Proportion of Zn in the HSP fraction REGWQ	Proportion of Zn in the CDN fraction REGWQ
Zn contaminated foodsource	0 µg/l	3.6 µg/l (-2-11)	205.4 µg/g (184.2-227.2) B C D	0.028 (0.025-0.030) A	0.300 (0.175-0.479) A	0.032 (0.026-0.039) A	0.336 (0.290-0.382) A B	0.389 (0.292-0.461) A B
	800 µg/l	787.2 µg/l (757-807)	296.3 µg/g (257.0-317.2) A B C	0.028 (0.018-0.033) A	0.149 (0.122-0.192) B	0.033 (0.026-0.039) A	0.315 (0.295-0.345) A B	0.554 (0.465-0.743) A B
	1600 µg/l	1488.2 µg/l (1422-1531)	374.5 µg/g (342.4-405.6) A	0.023 (0.019-0.026) A	0.140 (0.128-0.160) B	0.030 (0.020-0.044) A	0.275 (0.237-0.328) B C	0.532 (0.496-0.590) A B
non-contaminated foodsource	0 µg/l	-1.25 µg/l (-6-1)	79.7 µg/g (45.0-96.7) D	0.051 (0.019-0.088) A	0.141 (0.102-0.184) B	0.058 (0.046-0.091) A	0.390 (0.331-0.421) A	0.458 (0.371-0.655) A B
	800 µg/l	776.8 µg/l (716-802)	263.6 µg/g (224.9-295.7) A B C	0.025 (0.020-0.030) A	0.139 (0.130-0.143) B	0.033 (0.014-0.052) A	0.329 (0.303-0.367) A B	0.475 (0.433-0.518) A B
	1600 µg/l	1483 µg/l (1393-1537)	342.0 µg/g (171.1-451.0) A B	0.031 (0.020-0.056) A	0.136 (0.129-0.152) B	0.031 (0.024-0.036) A	0.323 (0.313-0.343) A B	0.561 (0.474-0.758) A B
no foodsource	0 µg/l	1.6 µg/l (-2,4)	88.3 µg/g (44.4-121.5) D	0.060 (0.041-0.090) A	0.212 (0.190-0.235) A B	0.057 (0.043-0.090) A	0.300 (0.276-0.340) B C	0.372 (0.329-0.400) B
	800 µg/l	735.6 µg/l (638-787)	167.2 µg/g (150.5-178.8) C D	0.056 (0.026-0.088) A	0.222 (0.159-0.303) A B	0.039 (0.031-0.050) A	0.225 (0.189-0.258) C D E	0.459 (0.409-0.530) A B
	1600 µg/l	1417 µg/l (1251-1476)	231.7 µg/g (165.8-270.1) B C	0.042 (0.040-0.045) A	0.155 (0.111-0.205) A B	0.039 (0.024-0.057) A	0.201 (0.177-0.222) D E	0.562 (0.495-0.623) A B
	3200 µg/l	2966 µg/l (2820-3090)	267.0 µg/g (195.6-304.6) A B C	0.063 (0.030-0.147) A	0.201 (0.168-0.220) A B	0.025 (0.022-0.028) A	0.212 (0.172-0.248) D E	0.500 (0.464, 0.539) A B
	6400 µg/l	5578 µg/l (5010-5980)	292.7 µg/g (188.3-461.4) A B C	0.041 (0.019-0.054) A	0.130 (0.068-0.186) B	0.031 (0.013-0.054) A	0.200 (0.153-0.267) D E	0.598 (0.488-0.719) A
	12800 µg/l	10942 µg/l (9580-12160)	393.8 µg/g (332.8-424.8) A	0.045 (0.025-0.092) A	0.156 (0.086-0.292) A B	0.024 (0.017-0.033) A	0.182 (0.148-0.236) E	0.593 (0.489-0.676) A

Table 1 – Observed aqueous treatment Zn concentrations, total tissue concentrations and Zn bound to subcellular fractions of *Ameletus* nymphs. Multiple comparisons by REGWQ compare all treatment levels together for each species. 95% confidence intervals are provided in parentheses. The EXT fraction was not considered in the total or the proportions because Zn loosely sorbed to the exoskeleton is not likely to elicit a toxic response.

Epeorus albertae

	Target aqueous treatment [Zn]	Mean aqueous treatment [Zn]	Total Zn in mayfly tissue REGWQ	Proportion of Zn in the MRG fraction REGWQ	Proportion of Zn in the ORG fraction REGWQ	Proportion of Zn in the HLP fraction REGWQ	Proportion of Zn in the HSP fraction REGWQ	Proportion of Zn in the CDN fraction REGWQ
Zn contaminated foodsource	0 µg/l	51.8 µg/l (-2-96)	97.9 µg/g (84.1-109.1) ABCDE	0.031 (0.015-0.038) AB	0.072 (0.038-0.115) A	0.070 (0.044-0.111) AB	0.411 (0.390-0.432) A	0.416 (0.344-0.472) A
	800 µg/l	705.2 µg/l (606-786)	121.3 µg/g (59.5-158.8) ABC	0.039 (0.020-0.048) AB	0.057 (0.051-0.068) A	0.072 (0.038-0.097) AB	0.415 (0.368-0.457) A	0.417 (0.390-0.444) A
	1600 µg/l	1373 µg/l (1201-1521)	137.3 µg/g (79.6-179.2) A	0.022 (0.014-0.028) B	0.042 (0.027-0.067) A	0.055 (0.049-0.062) AB	0.450 (0.397-0.495) A	0.431 (0.375-0.486) A
non-contaminated foodsource	0 µg/l	38.4 µg/l (-1-101)	86.5 µg/g (43.1-115.2) ABCDE	0.035 (0.020-0.050) AB	0.072 (0.059-0.098) A	0.111 (0.081-0.160) A	0.357 (0.242-0.409) A	0.426 (0.328-0.486) A
	800 µg/l	700.6 µg/l (606-779)	119.3 µg/g (101.7-141.1) ABC	0.029 (0.020-0.040) AB	0.056 (0.043-0.066) A	0.069 (0.036-0.102) AB	0.366 (0.348-0.376) A	0.479 (0.457-0.511) A
	1600 µg/l	1374.2 µg/l (1200-1527)	115.9 µg/g (99.8-125.4) ABCD	0.056 (0.035-0.106) AB	0.033 (0.021-0.039) A	0.073 (0.054-0.105) AB	0.353 (0.300-0.390) A	0.484 (0.450-0.502) A
no foodsource	0 µg/l	1.8 µg/l (-1-9)	42.7 µg/g (41.6-43.7) E	0.071 (0.047-0.094) A	0.046 (0.042-0.051) A	0.046 (0.037-0.054) B	0.391 (0.389-0.393) A	0.446 (0.407-0.485) A
	800 µg/l	840 µg/l (821-877)	42.2 µg/g (33.7-51.0) E	0.045 (0.028-0.075) AB	0.053 (0.035-0.070) A	0.050 (0.043-0.056) B	0.443 (0.392-0.502) A	0.407 (0.371-0.435) A
	1600 µg/l	1668.5 µg/l (1622-1734)	55.1 µg/g (46.0-59.4) DE	0.042 (0.025-0.059) AB	0.055 (0.038-0.070) A	0.077 (0.054-0.094) AB	0.384 (0.352-0.352) A	0.443 (0.403-0.497) A
	3200 µg/l	3494 µg/l (3380-3680)	61.1 µg/g (49.0-73.1) CDE	0.041 (0.039-0.042) AB	0.049 (0.042-0.056) A	0.076 (0.074-0.078) AB	0.434 (0.434-0.435) A	0.400 (0.390-0.410) A
	6400 µg/l	6472 µg/l (6230-6760)	73.7 µg/g (63.9-77.6) BCDE	0.033 (0.019-0.047) AB	0.058 (0.048-0.069) A	0.070 (0.052-0.089) AB	0.417 (0.376-0.500) A	0.422 (0.348-0.459) A
	12800 µg/l	12605 µg/l (12040-13260)	125.4 µg/g (120.4-132.5) AB	0.038 (0.020-0.048) AB	0.043 (0.029-0.057) A	0.042 (0.027-0.054) B	0.388 (0.378-0.404) A	0.489 (0.461-0.518) A

Table 2 – Observed aqueous treatment Zn concentrations, total tissue concentrations and Zn bound to subcellular fractions of *Epeorus* nymphs. Multiple comparisons by REGWQ compare all treatment levels together for each species. 95% confidence intervals are provided in parentheses. The EXT fraction was not considered in the total or the proportions because Zn loosely bound to the exoskeleton is not likely to elicit a toxic response.

	Ameletus sp.		*Epeorus albertae*	
	Diluter A (Dietary)	Diluter B (Aqueous-only)	Diluter A (Dietary)	Diluter B (Aqueous-only)
Hardness	43.45 mg/l (sd=1.15 n=30)	46.39 mg/l (sd=4.83 n=24)	45.65 mg/l (sd=0.62 n=3)	44.81 mg/l (sd=1.65 n=2)
Alkalinity	34.82 mg/l (sd=1.69 n=30)	34.653 mg/l (sd=2.113 n=30)	35.13 mg/l (sd=1.56 n=30)	35.37 mg/l (sd=0.79 n=24)
Conductivity	88.8 µs/cm (sd=4.56 n= 30)	93.274 µs/cm (sd=20.76 n= 30)	86.92 µs/cm (sd=5.73 n= 36)	94.0 µs/cm (sd=9.36 n= 30)
pH	7.38 (sd= 0.09 n= 30)	7.35 (sd= 0.049 n= 30)	7.87 (sd= 0.2484 n= 36)	7.75 (sd= 0.2368 n= 30)
Temp	11.87˚C (sd=0.131 n=30)	10.73˚C (sd=1.76 n= 30)	11.68˚C (sd=0.406 n= 36)	10.75˚C (sd=0.280 n= 30)
Disolved Oxygen	9.199 mg/l (sd=0.184 n= 30)	9.257 mg/l (sd=0.064 n= 30)	8.88 mg/l (sd=0.311 n= 24)	8.99 mg/l (sd=0.3367 n= 18)
Sulfate	7.193 mg/l (sd=0.972 n=6)	9.9 mg/l (sd=2.915 n=5)	12.16 mg/l (sd=0.23 n=3)	12.15 mg/l (sd=0.049 n=2)
Chloride	1.41 mg/l (sd=0.564 n=8)	1.51 mg/l (sd=0.037 n=6)	2.59 mg/l (sd=0.059 n=3)	2.65 mg/l (sd=0.049 n=2)
Ca	15.10 mg/l (sd=0.53 n=9)	15.16 mg/l (sd=0.59 n=5)	16.22 mg/l (sd=0.230 n=3)	15.92 mg/l (sd=0.467 n=2)
Mg	1.88 mg/l (sd=0.09 n=9)	1.89 mg/l (sd=0.063 n=5)	1.25 mg/l (sd=0.01 n=3)	1.23 mg/l (sd=0 n=2)
K	0.71 mg/l (sd=0.168 n=9)	0.66 mg/l (sd=0.040 n=5)	0.53 mg/l (sd=0.015 n=3)	0.51 mg/l (sd=0 n=2)
Na	3.60 mg/l (sd=.265 n=9)	3.7 mg/l (sd=0.310 n=5)	2.43 mg/l (sd=0.042 n=3)	2.36 mg/l (sd=0* n=2)

Table 3 – Observed physical chemistry means (standard deviations and sample size) for water quality in laboratory toxicity tests. Most of the variation associated with Alkalinity and conductivity was associated with the 6400 and 12800 µg/l aqueous-only treatments.

		Overall Model	Diet F_2=	*p* =	REGWG	Aquious F_2=	*p* =	REGWG	Interaction F_4=	*p* =
Ameletus sp.	TOT	**$F_{4,31}$=29.55 *p* <0.0001**	**18.34**	**<0.0001**	Zn Cl None	**40.77**	**<0.0001**	Hi Low Cont	*	*
	EXT	**$F_{4,31}$=4.78 *p*= 0.0040**	**5.60**	**0.0084**	None Cl Zn	**3.96**	**0.0294**	Hi Low Cont	*	*
	CDN	**$F_{4,30}$=42.77 *p* <0.0001**	**14.07**	**<0.0001**	Zn Cl None	**74.05**	**<0.0001**	Hi Low Cont	*	*
	MRG	**$F_{4,30}$=8.60 *p* <0.0001**	**5.05**	**0.0129**	None Zn Cl	**12.06**	**<0.0001**	Hi Low Cont	*	*
	ORG	**$F_{8,26}$=3.61 *p*= 0.0061**	**5.34**	**0.0114**	Zn None Cl	2.16	0.135	Hi Low Cont	**3.37**	**0.0238**
	HLP	**$F_{4,30}$=3.36 *p*= 0.0219**	0.56	0.5778	Zn Cl None	**6.30**	**0.0052**	Hi Low Cont	*	*
	HSP	**$F_{8,22}$=24,19 *p* <0.0001**	**43.34**	**<0.0001**	Zn Cl None	**40.05**	**<0.0001**	Hi Low Cont	**8.46**	**0.0003**
Eperous albertae	TOT	**$F_{4,29}$=14.19 *p* <0.0001**	**26.57**	**<0.0001**	Zn Cl None	**3.86**	**0.0326**	Hi Low Cont	*	*
	EXT	**$F_{4,29}$=15.50 *p* <0.0001**	0.95	0.3985	Zn None Cl	**30.70**	**<0.0001**	Hi Low Cont	*	*
	CDN	**$F_{4,29}$=11.60 *p* <0.0001**	**20.54**	**<0.0001**	Cl Zn None	**4.62**	**0.0181**	Hi Low Cont	*	*
	MRG	$F_{4,29}$=1.67 *p*= 0.1828	2.48	0.1012	Cl Zn None	1.15	0.3302	Hi Low Cont	*	*
	ORG	**$F_{4,29}$=4.17 *p*= 0.0086**	**7.45**	**0.0025**	Zn Cl None	0.55	0.5837	Cont Low Hi	*	*
	HLP	**$F_{4,29}$=7.08 *p*= 0.0004**	**13.73**	**<0.0001**	Cl Zn None	0.48	0.6206	Hi Cont Low	*	*
	HSP	**$F_{4,29}$=11.35 *p* <0.0001**	**20.68**	**<0.0001**	Zn Cl None	**3.70**	**0.0372**	Hi Low Cont	*	*

Table 4 – Results from two way ANOVA and multiple comparison (REGWQ) analysis comparing the 0, 800 and 1600 µg/l aqueous treatment levels for the aqueous-only treatment, the non-contaminated dietary treatment and the Zn-contaminated dietary treatments. REGWQ comparisons read highest (left) to lowest (right). Insignificant interaction terms were dropped from analysis and reanalyzed (*).

Proportion of Zn bound to each Fraction	$F_{model\ d.f.,\ Error\ d.f.}$ =	p =	REGWG and MEAN	
CDN	$F_{1,67}$=5.29	p =0.0245	Ameletus 0.48330	Epeorus 0.43844
MRG	$F_{1,67}$=0.03	p =0.8568	Epeorus 0.0393	Ameletus 0.0383
ORG	$F_{1,67}$=87.04	p <0.0001	Ameletus 0.17814	Epeorus 0.05432
HLP	$F_{1,67}$=33.24	p <0.0001	Epeorus 0.07080	Ameletus 0.03917
HSP	$F_{1,63}$=53.45	p <0.0001	Epeorus 0.39715	Ameletus 0.29467

Table 5 – Analysis of variance and multiple comparisons (REGWQ) between *Epeorus* and *Ameletus* for each subcellular fraction.

APPENDIX A

ACCUMULATION AND DEPURATION OF ZINC IN *SCENEDESMUS* SP. : an effort to create environmentally realistic dietary exposure of metals to grazing insects in toxicity tests.

ABSTRACT OF APPENDIX A

ACCUMULATION AND DEPURATION OF ZINC IN *SCENEDESMUS* SP. : an effort to create environmentally realistic dietary exposure of metals to grazing insects in toxicity tests.

Laboratory testing methodologies used for developing water quality standards rely solely on aqueous exposure. In reality, organisms in the field also experience chronic exposures to metals through both aqueous and dietary pathways. Recent studies (Irving et al. 2003; Conley et al. 2009; Xie et al. 2010) have attempted to create metal laden algae for use as dietary exposure in toxicity tests using grazing insects. These studies have relied on bathing algae in a metal solution to create various exposure levels.

It is unlikely that bathing algae in a metal solution can produce a subcellular distribution of toxicants similar to that observed in algae from contaminated field sites, where algae is cultured in the presence of contaminants. I hypothesize that algae cultured in the presence of contaminants may have a smaller proportion of contaminants bound to the exterior of algal filaments and far more bound within the proteins of inner cell walls, cytosolic proteins and subcellular structures.

I examined the efficacy of using the periphytic algae *Scenedesmus* sp. as a dietary treatment in toxicity tests and compared 6 preparation techniques including culturing and bathing algae in zinc (Zn) solutions. After 24 hour depuration in uncontaminated water, algae that was cultured in growth media contaminated with Zn retained Zn longer than algae bathed in Zn solutions. Cultured algae had subcellular profiles that were more similar to controls than algae simply bathed in Zn solutions. Subcellular partitioning methods were employed to detect differences in compartmentalization of Zn absorbed into and adsorbed to algae tissue.

Introduction

Density and diversity of macroinvertebrates observed at metal-contaminated sites is often significantly lower than the levels predicted by water quality criteria (Warnick 1969; Winner et al. 1980; Clements 1991; Clements et al. 2000). Acute water quality criteria are almost exclusively developed using only aqueous exposure (U.S. Environmental Protection Agency 1985; ASTM 1997). While laboratory toxicity tests have shown that mayflies are highly tolerant of aqueous zinc (Zn) exposure (Warnick 1969; Brinkman and Johnston 2008), field biomonitoring studies have shown significant decreases in mayfly abundance at relatively low metal concentrations (Carlisle and Clements 2003; Schmidt et al. 2010). Dietary exposure through algae consumption may be partially responsible for increased sensitivity of mayfly populations in the field (Neptun 2001). Failure to include dietary exposure is a major shortcoming of toxicity testing methodologies and may result in water quality standards that do not adequately protect organisms in the field (Fisher and Hook 2002).

Given the range of feeding strategies and diets of aquatic macroinvertebrates, the standardization of ecologically relevant food sources for use in dietary experiments presents a challenge. Seasonal variation of metal concentrations in algae (Anishchenko et al. 2010) further complicates our ability to predict dietary exposure in the field setting.

Several previous studies have examined effects of contaminated periphyton on aquatic insects (Irving et al. 2003; Conley et al. 2009; Xie et al. 2010) and cladocerans (Sofyan et al. 2006). However, these studies relied on

bathing algae or periphyton mats in a metal solution. Here, we compared the efficacy of preparing these dietary treatments by bathing the algae *Scenedesmus* sp. in Zn solutions and culturing *Scenedesmus* sp. in a Zn contaminated growth media.

Methods

Periphyton Culturing

Periphyton-covered cobble substrate was collected from a reach of Clear Creek, a Colorado stream with high levels of Zn contamination from historical mining activities. Substrate was placed in 1-2 cm water of Guillard's f/2 growth medium (Guillard 1975), fortified with 1.356 millimolar Si, using dissolved sodium meta-silicate 9-hydrate in 11.2 cm x 6 cm PVC troughs. Pumps (Taam Inc., Rio 600) provided a recirculating flow of 200 gallons per hour. Periphyton was cultured on 6.25 cm^2 unglazed porcelain tiles (Cinca Tile Co., Fiães, Portugal). Tiles were arranged close together in culture trays to limit algal growth to the top surface, thereby producing uniform mats of periphyton that remained bound to tiles while transferring to and from artificial streams. The algae covered tiles will be referred to as "feeding tiles" for the duration of this appendix. Cultures received 12-hour cycles of fluorescent light from wide spectrum plant and aquarium bulbs. Zinc (as $ZnSO_4$) was added to growth media of the Zn-contaminated cultures to a concentration of 1600 µg/l to produce algal tissue concentrations similar to those observed in a survey of Colorado mountain streams (µ=1483.5µg/g sd=5381 n=227. Travis Schmidt, unpublished Research, USGS Central Colorado

Assessment Program). Algae was maintained in an exponential growth phase by limiting use of each culture to 3 weeks after which a new culture was inoculated with tiles from a Zn contaminated culture. Three weeks prior to use in experiments cultures were started using Zn-contaminated (1430 µg/l Zn) and non-contaminated (27.8 µg/l Zn) growth media. Periphyton communities were dominated by the diatom *Scenedesmus* sp. (Dr. Sarah Spaulding, USGS, Boulder Colorado).

Periphyton Accumulation and Desorption

Six methods of preparing algae for use in dietary toxicity tests were compared.

- Control Algae or non-contaminated cultured algae: Algae simply cultured in non-contaminated Guillard's f/2 growth media which acted as a control to which the preparation methods below were compared.
- Zn-contaminated cultured algae: Algae Cultured in Guillard's f/2 growth media that was intentionally contaminated with Zinc Sulfate to a target concentration of 1430 µg/l Zn.
- 15 m bathed algae: Algae cultured in non-contaminated Guillard's f/2 growth media then bathed in 1600 µg/l Zn for 15 m.
- 24 h bathed algae: Algae cultured in non-contaminated Guillard's f/2 growth media then bathed in 1600 µg/l Zn for 24 h.
- 50 h bathed algae: Algae cultured in non-contaminated Guillard's f/2 growth media then bathed in 1600 µg/l Zn for 50 h.

- 50 h bathed then depurated algae: Algae cultured in non-contaminated Guillard's f/2 growth media then bathed in 1600 µg/l Zn for 50 h then depurated in 0 µg/l Zn for 24 h.

Ideally, a food source in toxicity tests should have subcellular distributions of Zn similar to algae cultured in non-contaminated growth media (control algae) or algae from contaminated field sites. An ideal algae for use as a dietary exposure will also resist absorption and depuration of Zn when placed in aqueous treatment levels used in our dietary experiments (0, 800 and 1600 µg/l).

Prior to the be beginning of the experiment feeding tiles from the non-contaminated culture were bathed in 1600 µg/l Zn for 15 min, 24 h and 50 h. In addition feeding tiles from the non-contaminated culture were bathed for 50 hours in 1600 µg/l were then allowed to depurate for 24 hours in control treatment cages (0 µg/l) to reproduce the equilibration process used to prepare contaminated periphyton in some toxicity tests (Xie et al. 2010; Conley et al. 2009). Samples (n=4) of these 4 preparation types as well as non-contaminated cultured algae and Zn-contaminated cultured algae were analyzed for total and subcellular concentrations of Zn as described below.

Feedling tiles from each of the 6 preparation methods were randomly assigned to 850 ml circular artificial streams (Brinkman and Johnston 2008) receiving 0, 800, and 1600 µg/l Zn for 24 hours (n=4 for each preparation method) to assess the rate at which algae accumulated and depurated Zn.

After 24 hours of exposure feeding tiles were rinsed in control water, photographed and processed for subcellular determination of Zn concentrations as described below. These concentrations were compared to the concentrations observed before exposure in 0, 800, and 1600 µg/l Zn aqueous treatment levels.

Delivery and removal of these feeding tiles to exposure tanks was carefully scheduled and timed to ensure all replicates experienced the same exposure durations (+/-10 seconds) and appropriate time was allotted for photographing feeding tiles and subcellular partitioning.

Dry mass of algae on tiles was estimated by photoanalysis of feeding tiles. Underwater photographs of feeding tiles were taken before subcellular partitioning using an Olympus Stylus 850 SW (F=4.0. Shutter 1/250) under uniform lighting and shutter speed. Image J version 1.40g (Abramoff 2004) was used to crop, rotate, and convert photos from JPEG to TIFF format. Matlab R2009b on Fedora Core 10 was used to calculate average green saturation of the pixels in a cropped photo based on a 255 RGB color model. This value is referred to as the Mean Greenness. The dry mass of algae on each tile was estimated using the following equation based on an index of mean greenness: Dry Mass (µg) = 0.00005661 X [255-(Mean Greenness)] – 0.00330380; (R^2 = 0.813, n = 13).

Artificial streams received dechlorinated municipal tap water (Fort Collins, Colorado, USA) from continuous-flow serial diluters (Benoit et al. 1982). The diluters delivered a control concentration as well as two concentrations of metal toxicant with a 50% dilution ratio. A flow splitter allocated each concentration

equally among 4 replicate exposure chambers at a rate of 40 ml/min. Food-grade vinyl tubing delivered test solutions to exposure chambers. Metal stock solutions were prepared by dissolving a calculated amount of metal sulfate salts in deionized water. A concentrated stock solution was delivered to the diluter by a peristaltic pump at a rate of 2.0 ml/min.

Water Quality Assessment

Aqueous Zn samples for both field and laboratory studies were filtered using a 0.45 µm disk filter and preserved with ultrex nitric acid in a 15 ml polypropylene centrifuge tube. Analysis was conducted using an Instrumentation Laboratory Video 22 with Smith-Hieftje background correction. Water hardness and alkalinity were determined titrimetrically every other day.

Subcellular Partitioning and Zn Accumulation

To assess subcellular Zn compartmentalization, algae from each treatment were partitioned into subcellular fractions using differential centrifugation (Fig. 1). Fractions included cellular debris and nuclei (800 *g* pellet; Karp 2002), subcellular organelles including mitichondria (15,000 *g* pellet), heat labile cytosolic proteins and heat stable cytosolic proteins. The method also allowed us to differentiate fractions of Zn that were associated with metal-rich granules or loosely bound to the exterior of periphyton and cell fragments.

Each algal feeding tile was rinsed with deionized water, air dried on filter paper for 60 seconds on a Buchner funnel with aspiration, submerged in 3.0 ml

of 0.01M Mg EDTA solution and gently agitated for 60 seconds to remove loosely bound Zn. Two ml of EDTA solution were retained in 2 ml polypropylene centrifuge tubes as a measure of Zn loosely sorbed to the surface of algal tissue (Fraction EXT). This fraction was filtered using a 0.45 µm disk filter due to presence of small amounts of algae dislodged during soaking in EDTA. The EXT fraction is typically not considered when examining the total body burden of organisms because it likely causes little toxicological effect. However, this fraction was included for algae in this experiment because it is bioavailable to primary consumers. Feeding tiles were rinsed with DI water and again dried for 60 seconds on a Buchner funnel. Mass of algae was estimated by photoanalysis.

A modification of the subcellular partitioning methods developed by Bechard (Bechard et al. 2008), Wallace (Wallace et al. 2003), and Brinkman (Brinkman 2008) was used in this study (Fig. 1). Algae were homogenized using a Tissue Terror® (Biospec Products Inc., Dremel, Racine WI, USA) homogenizer in 1.7 ml of helium-saturated 0.05M HEPES Buffer (pH 7.4) with 0.2 millimolar phenylmethylsulfonyl fluoride protease inhibitor. Tris buffer was avoided because of limited buffering capacity at pH 7.4 at 4ºC (Sigma-Aldrich 1996). Homogenate (0.5 ml) was transferred to a 1.5 ml polypropylene centrifuge tube representing the “total” metals (Fraction TOT) and was used to calculate percent recovery.

Homogenate (1.0 ml) was centrifuged at 800 *g* for 10 minutes at 4º C in a 1.5 ml polypropylene centrifuge tube using an Eppendorf 5415 c centrifuge. The

resulting pellet consisted of cellular debris including tissue fragments, membranes, nuclei and metal rich granules. This pellet was held at -20º C until it was resuspended for differentiation of metal rich granules. The supernatant was transferred to a new 1.5 ml polypropylene centrifuge tube and centrifuged at 15,000 *g* for 10 minutes at 4º C. The pellet (Fraction ORG) consisted of subcellular organelles including mitochondria. The supernatant was transferred to a new 1.5 ml polypropylene centrifuge tube, heated to 100 º C for 10 minutes to denature heat-labile proteins, cooled on an ice bath for 10 minutes and centrifuged at 15,000 *g* for 10 minutes. The pellet (Fraction HLP) contains heat-labile cytosolic proteins and the supernatant (Fraction HSP) contained heat-stable cytosolic proteins such as metallothionein, glutathione and phytochelating agents. The pellet representing cell fragments was resuspended in 1M NaOH, repeatedly vortexed while being heated to 80º C for 20 minutes, cooled, and centrifuged at 5,000 *g* for 10 minutes. The supernatant (Fraction CDN) contains resuspended metals not associated with metal rich granules, leaving metal rich granules in the pellet (Fraction MRG).

Throughout the partitioning process all fractions and reagents were chilled on an ice bath to minimize changes in Zn distribution among fractions. Fractions were then dried, digested at 80º C in 0.1ml of concentrated nitric acid for a minimum of 2 hours followed by 0.1 ml 30% hydrogen peroxide and evaporated to dryness. Samples were brought up to 1.5 ml with 10% nitric acid for zinc analysis by flame atomic absorption using an Instrumentation Laboratory Video 22 with Smith-Hieftje background correction.

Statistical Analysis

Total and subcellular concentrations of Zn were compared by one-way analysis of variance using PROC GLM, LSMEANS and REGWQ for comparison of treatment means using SAS 9.1.3. I also employed confidence intervals and descriptive statistics which were calculated using PROC MEAN. All statistical analyses were conducted using $\alpha=0.05$. Type III sum of square were used if replicates were missing.

Results

Algal Accumulation and Depuration

Characteristics of periphyton that would be useful as dietary exposure to Zn would include a slow loss of Zn when used in aqueous control chambers and a subcellular distribution of Zn that is similar to the control (non-contaminated cultured) algae.

No statistical differences in subcellular proportions of Zn were indicated between algae cultured in the Zn-contaminated and non-contaminated growth media (Fig. 2). Even after 24 h of exposure to 0, 800 and 1600 µg/l aqueous Zn all but one of the subcellular proportions remained similar to the original non-contaminated (Fig. 3) and Zn-contaminated (Fig. 4) algal cultures.

The proportions of Zn in the EXT, MRG, and/or CDN fractions of algae loaded with Zn by bathing often differed significantly from algae cultured in Zn-contaminated and non-contaminated media (Fig. 3). After 24 h depuration tiles

cultured in Zn-contaminated media lost only 14% of total Zn compared to 75% in tiles that were bathed for 50 hours in 1600 µg/l Zn (Table 1).

In an effort to reduce error associated with changes in nutritional, palatability and bioavailability differences, bathed algae was not used for the dietary exposure component of this thesis. Algae cultured in Zn-contaminated and non-contaminated growth media provided more similar profiles of subcellular distribution between dietary controls and treatments and also depurated less Zn when used in aqueous controls.

Discussion

As laboratory toxicity tests are improved to increase environmental realism, dietary exposure should be included. Algae cultured in toxicants were less affected by aqueous exposure than “bathed” algae and had subcellular distributions of Zn more similar to controls (Figure 2).

Although the use of natural periphyton assemblages may have increased ecological realism in our experiments (Xie et al. 2010), we believe that exposing mayflies to a monoculture of *Scenedesmus* sp. reduced variation in nutritional quality and bioavailability. I also believe that exposing organisms to periphyton cultured in Zn has advantages over the traditional approach of rapidly bathing periphyton in a Zn solution. My results showed that Zn was retained in the cultured algae longer than in the bathed algae. The subcellular distribution of Zn in the Zn-contaminated and non-contaminated algae cultures were more alike than any of the bathed options. Unlike the culturing technique, short-term (e.g., 15 min) exposure of periphyton to Zn limits accumulation to superficial adsorption (Irving et al. 2003);(Fig. 2). Contaminants that are bound to certain proteins within natural food sources may be more bioavailable form (Harrison and Curtis 1992; Meyer 2005; Ng and Wood 2008; Reinfelder and Fisher 1991). Even with increased duration of exposure, bathed algal cells may not become fully equilibrated if Zn was not present during the formation of proteins and silica frustules.

Guillard’s f/2 growth media included trace amounts of EDTA and may have reduced the bioaccumulation of Zn in cultured algae especially in the EXT

fraction. To facilitate standardization of dietary exposure in toxicity tests, further development must be made in culturing food sources in a repeatable and ecologically realistic manner.

A primary goal of my research was to compare the relative importance of dietary (via algae) and aqueous exposure to Zn to aquatic insects. However, it proved difficult to produce an uncontaminated dietary treatment because of the high rate at which algae accumulated Zn when these feeding tiles were placed in aqueous zinc treatments. On day 0 of the experiment the concentration of Zn in the non-contaminated treatment was only 14% of the level in the contaminated treatment; however, this difference was negligible after 24 hours of use in the 1600 µg/l Zn treatment chambers. The tendency of metals to rapidly move between water and algae makes dietary studies difficult even if one employees isotopes or tracers.

Examination of subcellular concentrations of heavy metals is a more refined tool that total metal concentration. Since metals are more readily assimilated by consumers when bound to certain subcellular fractions of food (Rainbow et al. 2004; Bechard et al. 2008) subcellular profiles of metal concentrations allow the researcher to ensure that the toxicant of concern is bound to bioavailable fractions similar to those measured *in vivo* food sources.

Although high concentrations of Zn pollution is a primary concern in aquatic ecosystems impacted by historical mining activities in Colorado, Zn is an essential micronutrient for both algae and insects. Non-essential metals, such as cadmium, also occur at high levels in mine-contaminated rivers of the West and

may be regulated differently. Future studies are needed to examine this difference.

Numerous biotic and abiotic factors contribute to the marked disparity between safe metal concentrations predicted in laboratory studies and those observed in the field. Assessing the relative importance of these factors is challenging because of the difficulty reproducing the complexities of natural ecosystems in the laboratory. I suggest that the inclusion of dietary exposure to contaminants represents an obvious improvement in the ecological realism of laboratory toxicity tests and should be utilized in the development and validation of water quality criteria. Creation of environmentally realistic food sources will be challenging because of the wide variation in feeding habits of aquatic insects and the tremendous seasonal and temporal changes that occur in natural populations. However, I believe this is a necessary endeavor if ecotoxicologists are to make any progress addressing the discrepancy between results of laboratory toxicity tests and effects observed in the field.

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FIGURES AND TABLES FROM APPENDIX A

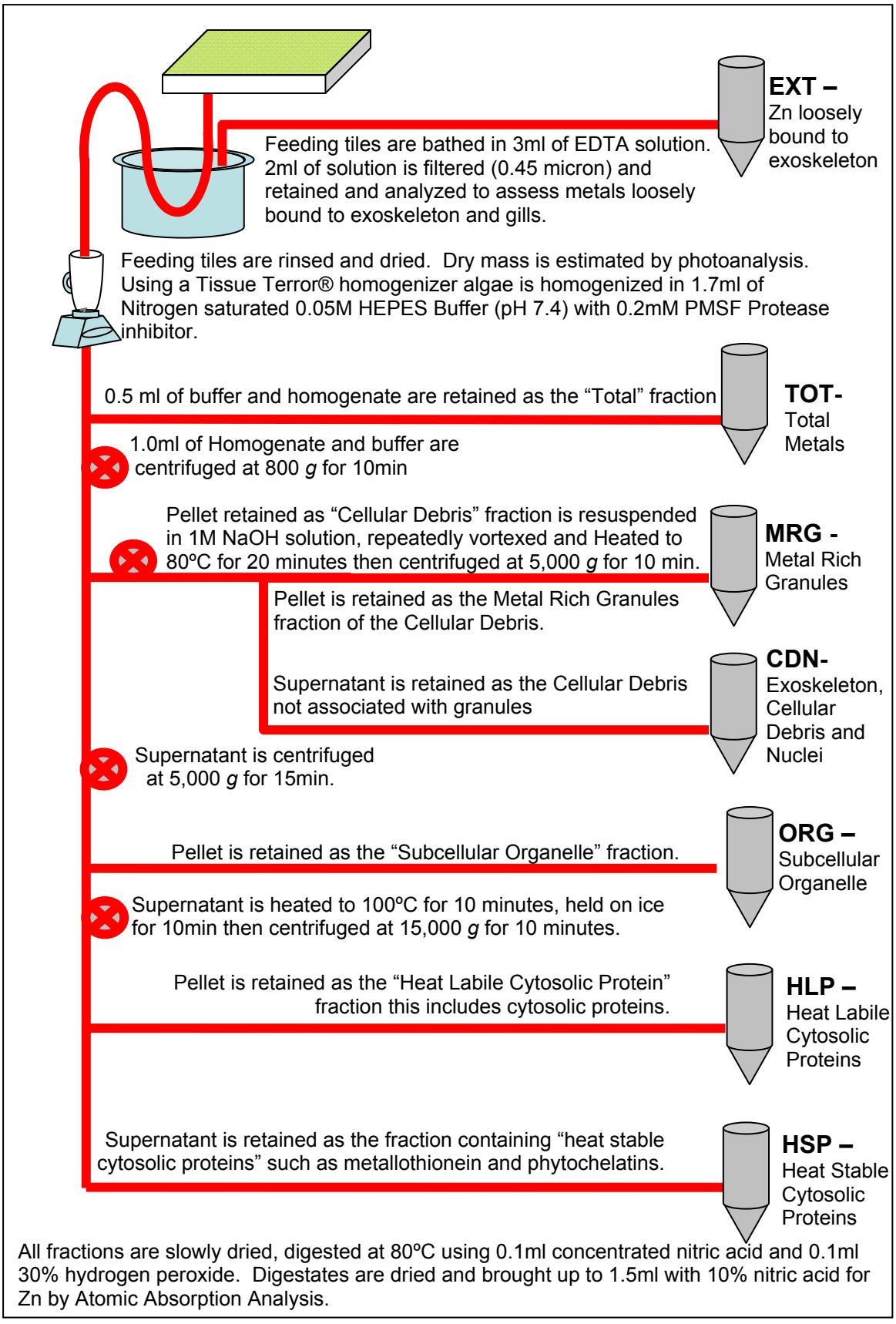

Figure 1 – Flow Chart of Subcellular Partitioning Method

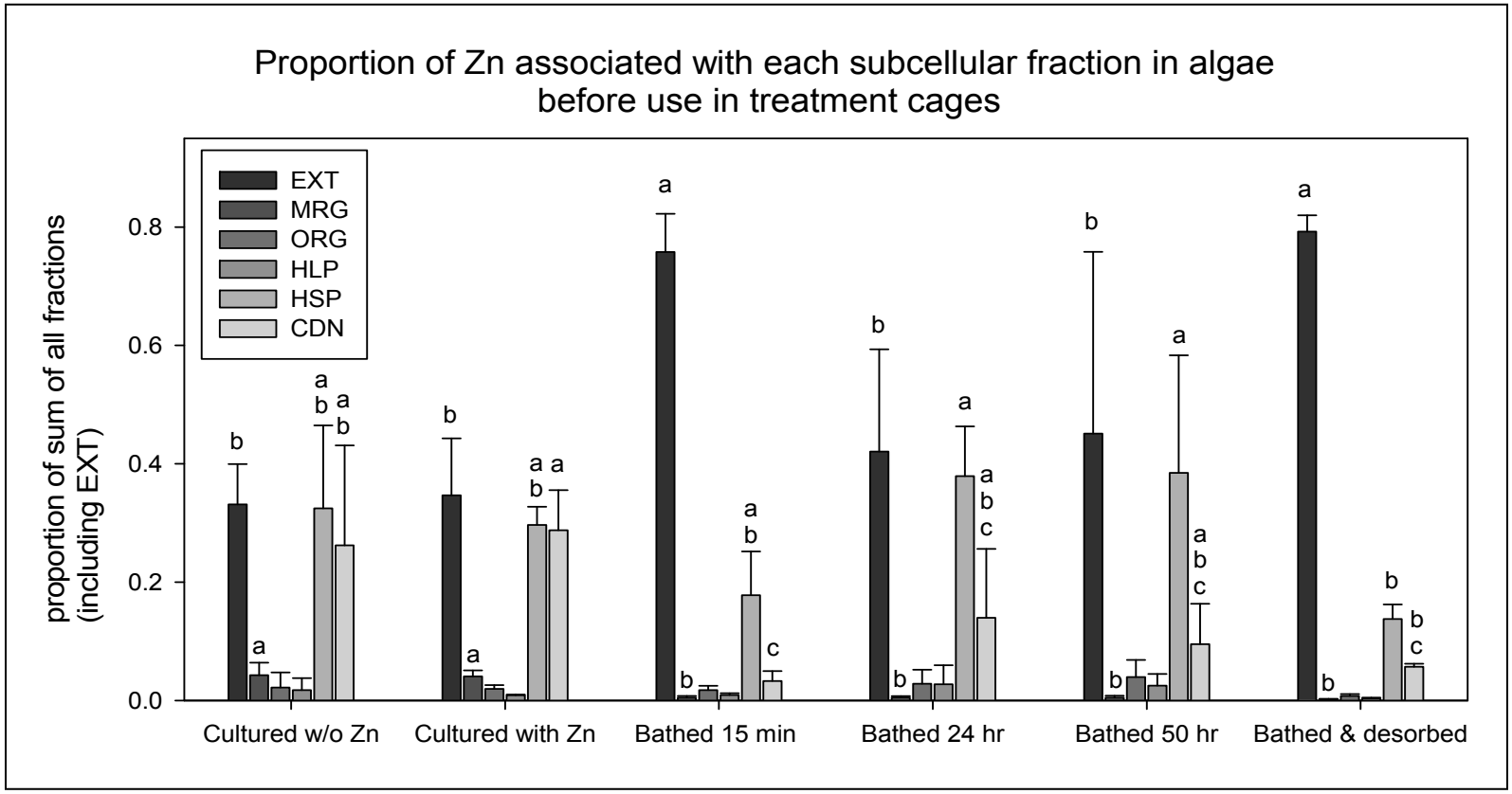

Figure 2 – Subcellular Zn profiles of algae considered for use as dietary treatments. "Cultured w/o Zn" is algae cultured in the non-contaminated growth media. "Cultured with Zn" was algae sutured in a Zn contaminated growth media. "Bathed for 15 min" is "cultured w/o Zn" algae that was bathed in 1600 µg/l Zn for 15 min. "Bathed for 24 h" is "cultured w/o Zn" algae that was bathed in 1600 µg/l Zn for 24 h. "Bathed for 50 h" is algae that was bathed in 1600 µg/l Zn for 24 hours. "Bathed & desorbed" is algae that was bathed in 1600 µg/l Zn for 24 h then depurated for 24 h. Letters for each fraction denote significant differences by REGWQ comparison across preparation methods. Fractions without letters were not significantly different by REGWQ analysis. Error bars denote standard deviation.

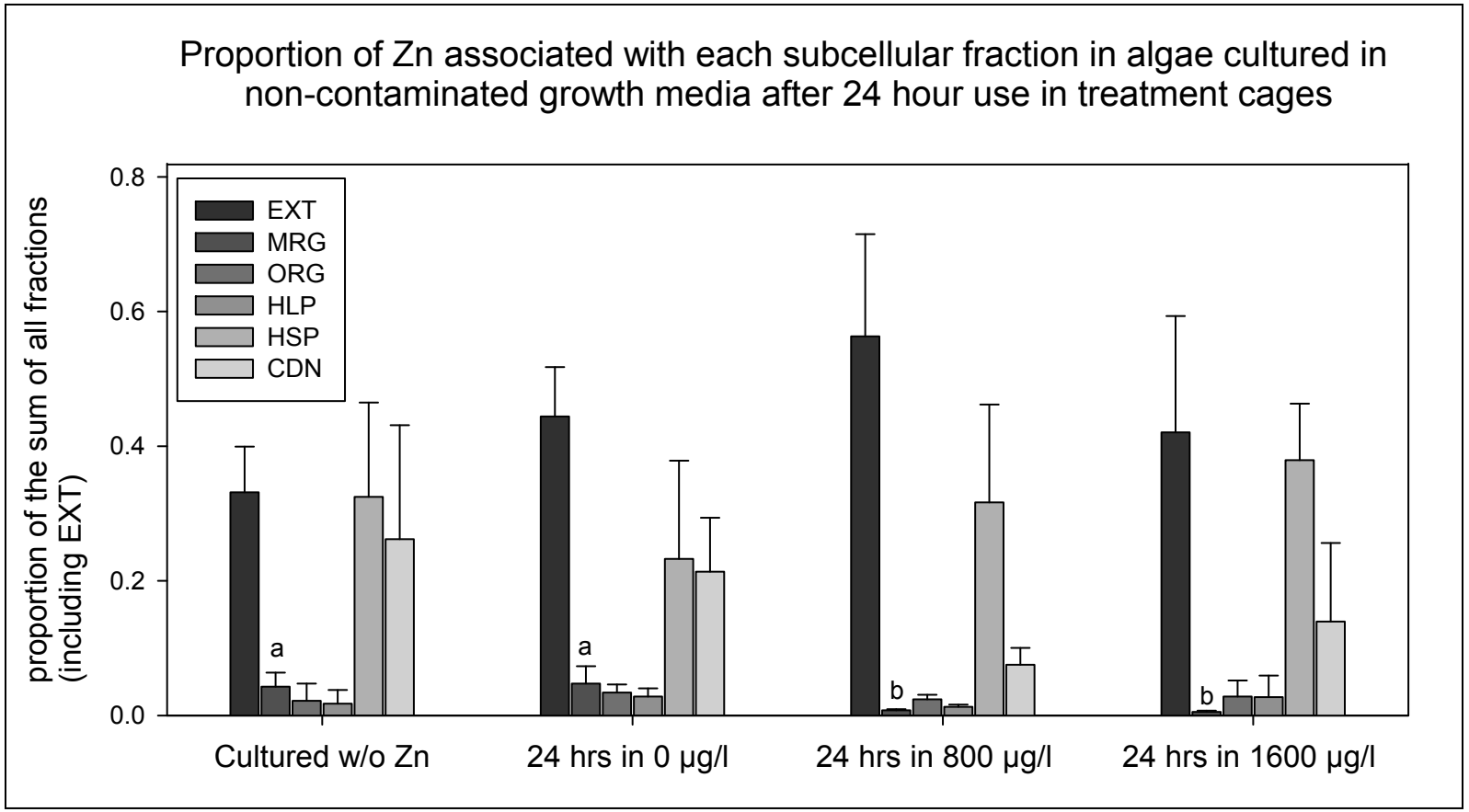

Figure 3 – Proportion of Zn associated with each subcellular fraction of algae cultured in a non-contaminated growth media. Letters for each fraction denote significant differences by REGWQ comparison across algae treatments. Fractions without letters were not significantly different by REGWQ analysis. Error bars denote standard deviation.

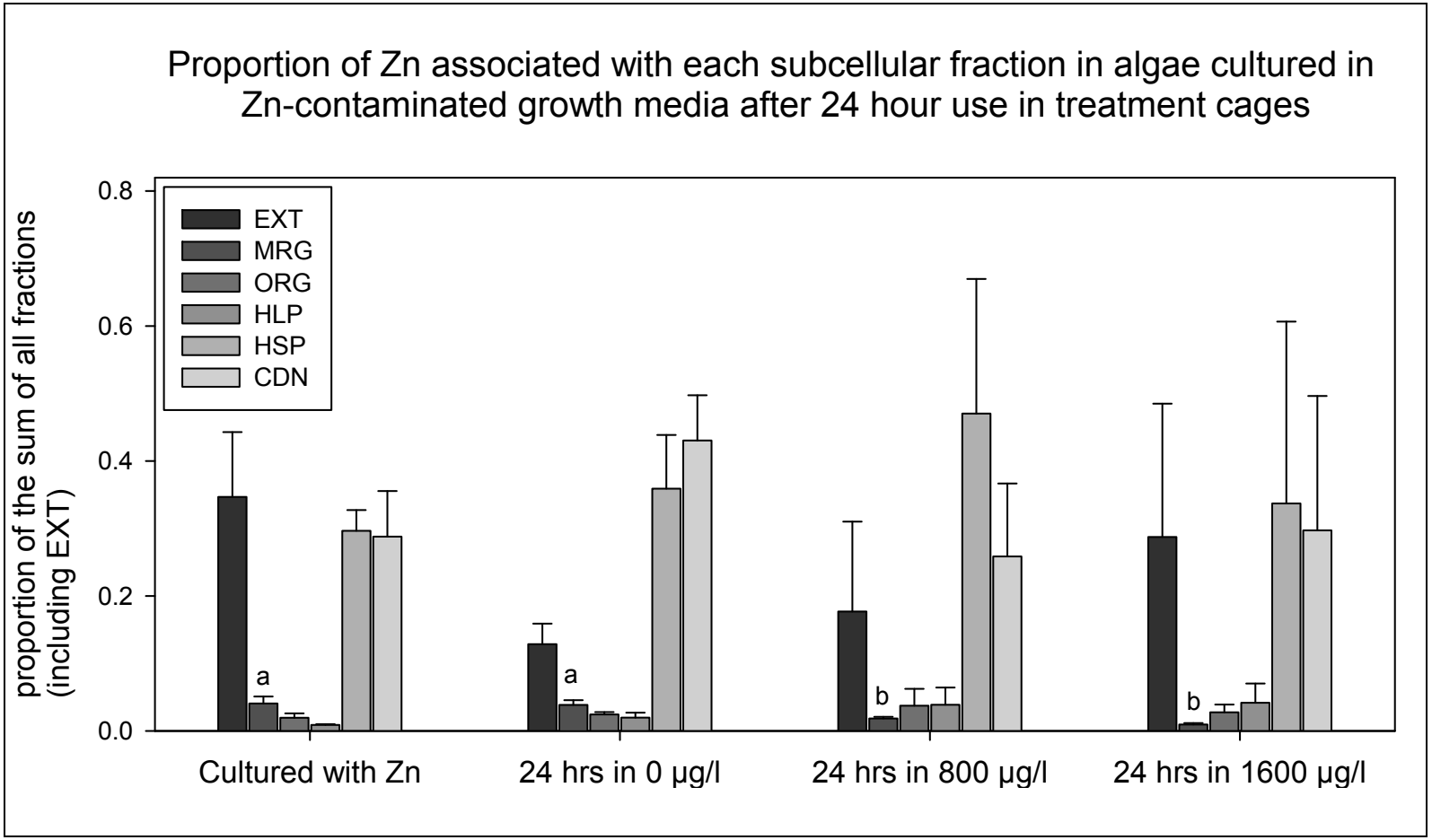

Figure 4 – Proportion of Zn associated with each subcellular fraction of algae cultured in a non-contaminated growth media. Letters for each fraction denote significant differences by REGWQ comparison across algae treatments. Fractions without letters were not significantly different by REGWQ analysis. Error bars denote standard deviation.

	Target aqueous treatment [Zn]	Mean aqueous Zn concentration in algae growing troughs	Total Zn in aglae	REGWQ	Proportion of Zn in the EXT fraction	REGWQ	Proportion of Zn in the MRG fraction	REGWQ	Proportion of Zn in the ORG fraction	REGWQ	Proportion of Zn in the HLP fraction	REGWQ	Proportion of Zn in the HSP fraction	REGWQ	Proportion of Zn in the CDN fraction	REGWQ
Scenedesmus sp.	Zn contaminated algae: cultured algae	2399.3 µg/l (1407.0-4665.0)	722.6 µg/g (502.2-1006.0)	B	0.347 (0.218-0.435)	B C	0.041 (0.028-0.051)	A B	0.019 (0.012-0.026)	A	0.009 (0.008-0.010)	A	0.297 (0.273-0.342)	A	0.288 (0.209-0.369)	A B
	Zn contaminated algae: 0 µg/l	0.2 µg/l (-0.004-1.0)	622.8 µg/g (348.1-837.1)	B	0.129 (0.100-0.172)	C	0.038 (0.030-0.047)	A B	0.025 (0.022-0.029)	A	0.020 (0.014-0.030)	A	0.359 (0.318-0.478)	A	0.430 (0.318-0.488)	A
	Zn contaminated algae: 800 µg/l	820.0 µg/l (808.0-829.0)	3896.1 µg/g (2236.3-5767.7)	A B	0.177 (0.080-0.367)	C	0.018 (0.016-0.021)	B C	0.038 (0.020-0.073)	A	0.039 (0.012-0.064)	A	0.470 (0.284-0.726)	A	0.259 (0.127-0.388)	A B C
	Zn contaminated algae: 1600 µg/l	1624.0 µg/l (1583.0-1647.0)	5957.7 µg/g (2721.3-11012.9)	A B	0.287 (0.122-0.574)	B C	0.009 (0.006-0.011)	C	0.028 (0.018-0.044)	A	0.042 (0.017-0.074)	A	0.337 (0.001-0.593)	A	0.297 (0.133-0.544)	A B
	non-contaminated algae: cultured algae	69.3 µg/l (23.0-123.0)	104.2 µg/g (56.3-164.0)	B	0.331 (0.257-0.422)	B C	0.043 (0.019-0.070)	A B	0.022 (0.000-0.050)	A	0.017 (0.000-0.040)	A	0.325 (0.200-0.478)	A	0.262 (0.156-0.512)	A B C
	non-contaminated algae: 0 µg/l for 24h	0.2 µg/l (-0.004-1.0)	157.4 µg/g (133.7-179.0)	B	0.444 (0.349-0.507)	B C	0.047 (0.020-0.030)	A	0.034 (0.020-0.048)	A	0.028 (0.010-0.038)	A	0.233 (0.082-0.361)	A	0.213 (0.12.7-0.309)	A B C
	non-contaminated algae: 800 µg/l	820.0 µg/l (808.0-829.0)	1951.2 µg/g (978.6-3517.4)	B	0.563 (0.344-0.690)	A B	0.008 (0.006-0.009)	C	0.024 (0.016-0.033)	A	0.013 (0.009-0.016)	A	0.317 (0.226-0.533)	A	0.075 (0.040-0.096)	B C
	non-contaminated algae: 1600 µg/l	1624.0 µg/l (1583.0-1647.0)	5857.2 µg/g (2164.2-13671.4)	A B	0.421 (0.176-0.554)	B C	0.005 (0.004-0.008)	C	0.028 (0.015-0.064)	A	0.027 (0.0100-0.075)	A	0.379 (0.274-0.476)	A	0.140 (0.036-0.273)	B C
	no foodsource: 1600 µg/l for 50h	1624.0 µg/l (1583.0-1647.0)	9780.2 µg/l (5238.1-17494.0)	A	0.451 (0.172-0.780)	B C	0.005 (0.001-0.007)	C	0.040 (0.006-0.059)	A	0.025 (0.008-0.047)	A	0.385 (0.189-0.586)	A	0.095 (0.016-0.139)	B C
	no foodsource: 1600 µg/l for 50h then 0 for 24h	1624.0 µg/l (1583.0-1647.0)	2465.1 µg/g (1666.2-3969.8)	B	0.792 (0.773-0.832)	A	0.002 (0.0006-0.0027)	C	0.008 (0.004-0.012)	A	0.004 (0.0027-0.0054)	A	0.138 (0.103-0.156)	A	0.057 (0.053-0.630)	B C
	no foodsource: 1600 µg/l for 16m	1624.0 µg/l (1583.0-1647.0)	815.7 µg/g (674.8-1016.6)	B	0.758 (0.690-0.844)	A	0.005 (0.003-0.008)	C	0.017 (0.008-0.025)	A	0.009 (0.005-0.012)	A	0.178 (0.104-0.272)	A	0.033 (0.020-0.057)	C

Table 1 – Observed total Zn concentrations and Zn bound to subcellular fractions of *Scenedesmus* sp. both cultured and bathed in Zn solutions. Multiple comparisons by REGWQ compare all preparation types. 95% confidence intervals are provided in parentheses.