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# Cadmium exposure route affects antioxidant responses in the mayfly *Centroptilum triangulifer*

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ABSTRACT

Aquatic organisms accumulate metals directly from water and from their diets. Exposure to metals is known to generate oxidative stress in living organisms and this stress may be ameliorated via activation of antioxidant enzymes and non-enzymatic antioxidants. To determine if antioxidant physiology is dependent on Cd exposure route in the mayfly *Centroptilum triangulifer*, we exposed larvae to environmentally relevant concentrations of Cd from isolated dissolved or dietary exposure routes to achieve comparable tissue concentrations. Dissolved Cd had no effect on the antioxidant enzymes examined. However, dietary Cd significantly suppressed catalase and superoxide dismutase activities, and decreased concentrations of the reduced (active) form of glutathione in *C. triangulifer* larvae. These findings suggest that dietary Cd is potentially more toxic than aqueously derived Cd in this mayfly. We further examined the effect of dietary Cd tissue loading rates on antioxidant enzyme suppression and found that absolute tissue load appeared more important than loading rate. These results may help explain why insects are routinely unresponsive to dissolved metal exposures in the laboratory, yet highly responsive to metal pollution in nature.



## 1. Introduction

In industrialized countries worldwide, regulatory entities are challenged to develop regulatory, societally "acceptable" environmental standards such as dissolved concentrations of chemical contaminants (e.g., trace metals) in surface waters (hereafter referred to as criteria). Ideally, these criteria reflect the state of scientific knowledge of contaminant bioaccumulation and toxicity to aquatic organisms. While the scientific community has acknowledged the potential importance of dietary metal toxicity in aquatic ecosystems (e.g. Meyer et al., 2005), regulatory entities have yet to incorporate this understanding into criteria development. Generally, criteria are intended to protect 95% of species in aquatic ecosystems and are derived from standard toxicity assays which do not typically incorporate or consider dietary contributions to toxicity.

In freshwater ecosystems, it is not uncommon for aquatic insects to comprise 90–95% of the invertebrate species pool, particularly in lotic systems (e.g. Arscott et al., 2006). Yet, insects are grossly under-represented in toxicity datasets (Baird and Van den Brink, 2007; Brix et al., 2005), largely because suitable test species have not been developed. As a result, daphnids often serve as the "go to" surrogate for aquatic invertebrates despite the fact that they do not share fundamental physiological traits with insects and are generally not found in lotic environments. One way in which daphnids and insects appear to differ is in the relative importance of dissolved and dietary sources for trace metal bioaccumulation. In insect studies, dietary trace metal bioaccumulation is reported to be more important than aqueous bioaccumulation (Hare et al., 2003; Martin et al., 2007; Munger et al., 1999; Xie et al., 2010; Roy and Hare, 1999), while the opposite is reported for daphnids (Barata et al., 2002).

The lack of representation in toxicity studies is not the only concern with respect to insect responses to trace metals. At this point a substantial number of studies utilizing several different insect taxa have demonstrated that insects are generally unresponsive to dissolved metals in traditional toxicity bioassays at environmentally relevant concentrations (e.g. Brinkman and Johnston, 2008; Brix et al., 2005; Clubb et al., 1975; Mebane et al., 2008; Spehar et al., 1978; Williams et al., 1985). However, in the field, insects are reported to be highly responsive to trace metal pollution (e.g. Cain et al., 2004; Canfield et al., 1994; Carlisle and Clements, 2003; Clements et al., 1992; Clements, 2004; Clements et al., 2000; Gower et al., 1994; Luoma et al., 2009; Winner et al., 1980). Why do laboratory and field studies provide such different information?

We previously commented that inadequate exposure duration in traditional toxicity assays could contribute to the disconnection between laboratory and field data for metal toxicity to aquatic

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insects (Buchwalter et al., 2007). Another possible cause for the discrepancy between lab and field data centers on the dietary route of exposure and the potential for dietary bioaccumulation to elicit physiological stress and toxicity. Based on the way in which criteria are developed, it appears that dissolved exposures are assumed to be more toxic than dietary exposures, despite a few studies that point towards dietary metal toxicity (Hook and Fisher, 2001; Irving et al., 2003; Bielmyer et al., 2006).

Here, we examined the roles of Cd exposure route (diet vs. dissolved) and dietary dosing rates on antioxidant responses in the mayfly *Centroptilum triangulifer*. While the aquatic toxicology literature has generally focused upon ionoregulatory disturbance as a mechanism of metal toxicity, the biomedical community has largely been focused upon metal induced oxidative challenge. This aspect of physiology has received relatively little attention in stream insects to date, but is likely important in understanding organism responses to environmentally relevant metal exposures. Here, we isolated exposure routes (food vs. water) and examined how comparable Cd tissue concentrations, derived from these routes affected *C. triangulifer* antioxidant machinery. We discuss the implications of these findings with respect to the limitations of traditional testing methods with insects and the uncertainties associated with the protectiveness of current water quality criteria.

## 2. Material and methods

Artificial soft water (ASW) was the base water used in all experiments: 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4.2H_2O$, 30 mg/L $MgSO_4$, and 2 mg/L KCl, pH 7.8. A clonal line (WCC-2) of the parthenogenetic mayfly *C. triangulifer* was initially obtained from the Stroud Water Research Center (Avondale, PA, USA) in 2008 and is cultured in the laboratory using periphyton plates (also from Stroud) as described elsewhere (Conley et al., 2009; Xie et al., 2009, 2010). Each group of experiments presented here used clonal offspring from a single mother. Offspring from five different individual mothers and five batches of periphyton were used in the experiments presented below.

### 2.1. Antioxidant enzyme assays

For all experiments, frozen mayfly larvae were thawed on ice and homogenized manually in 0.15 mL of 50 mM phosphate buffer (pH 7.4, with 1 mM EDTA) using a Kontes® 2-mL glass homogenizer. The homogenates were centrifuged (10,000 × *g*) at 4 °C for 10 min. All enzyme assays called for the same homogenization buffer therefore aliquots for each assay were taken from individual replicate homogenates and stored at −80 °C until analysis. All enzyme assays were performed in a 96 well format in a Thermo Multiskan FC microplate reader. Catalase activities were determined as in Hansen et al. (2006). Superoxide dismutase (SOD) and glutathione S-transferase (GST) were determined using assay kits from Cayman Chemical Company (Ann Arbor, MI). All enzyme activities were normalized to total protein as determined by the method of Bradford (1976).

#### 2.1.1. Comparing dissolved and dietary Cd effects on antioxidant physiology

To compare the influence of Cd derived from dissolved vs. dietary exposure routes on antioxidant responses, it was first critical to manipulate exposure regimes such that final tissue concentrations were comparable among treatment groups. Previous studies of aqueous and dietary Cd uptake with *C. triangulifer* larvae in our lab facilitated in overcoming this challenge (Xie et al., 2010). We prepared contaminated diets by exposing periphyton to dissolved Cd (see below), and used identical exposure concentrations for dissolved Cd exposures to larvae (see below). Each Cd treatment group (dissolved and dietary) was matched with appropriate fasted and fed controls for each experiment.

*2.1.1.1. Preparing contaminated diets.* We exposed periphyton to 2 μg/L (Exp. 1) or 4 μg/L (Exp. 2) with $^{109}Cd$ (in the form of $CdCl_2$, specific activity: 286.8 Bq $ng^{-1}$ in Exp. 1 and 208.4 Bq $ng^{-1}$ in Exp. 2) as a tracer and stable $CdCl_2$ providing the remainder of the ambient Cd in solution. Cadmium concentrations in the water were monitored for radioactivity daily and reported based on the mean concentration of duplicate 2-mL samples. Based on these measures, appropriate additions of $^{109}Cd$ and $CdCl_2$ were added daily to maintain the designated Cd exposure concentrations. After allowing periphyton to absorb Cd, the plates were transferred to clean water for 1 h to rinse Cd weakly associated with periphyton and then transferred a second time to a bottle containing clean water. Triplicate periphyton samples were collected, dried, weighed, and assayed for radioactivity on a Wallac Wizard 1480 Gamma counter to determine Cd concentrations in the periphyton. Loading of periphyton with Cd lasted 4 days in Exp. 1, and 3 days in Exp 2.

*2.1.1.2. Dietary Cd exposures to C. triangulifer.* Thirty larvae each were added to the bottles with Cd-labeled periphyton and control (Cd free) periphyton. Larvae were close in age in the two experiments – 20–25 days old in Exp. 1 and 23–27 days old Exp. 2. Larvae were allowed to graze on the control and Cd-labeled periphyton for 5 days (Exp. 1) or 3 days (Exp. 2) to achieve comparable tissue concentrations with the fasted, dissolved exposure groups. Duplicate 2 mL water samples were taken daily to monitor for the release of loosely bound Cd from the periphyton to water—which was found to be negligible. Following exposures, larvae were collected, rinsed and randomly divided into 10 composite replicates of 3 larvae each. Each replicate was weighed, assayed for radioactivity, and stored at −80 °C until antioxidant enzyme activities were measured

*2.1.1.3. Dissolved Cd exposures to C. triangulifer.* Thirty larvae per treatment group were exposed to Cd in HDPE beakers containing 800 mL ASW and Cd (stable and radiotracer) as described for periphyton loading. Exposure concentrations were 0 (control) and 2 μg/L Cd for four days in Exp. 1, and 0 (control) and 4 μg/L for three days in Exp. 2. Food was withheld during these exposures. Duplicate 2 mL water samples were taken daily to monitor Cd concentration in solution and appropriate additions of $^{109}Cd$ and $CdCl_2$ were added to maintain the designated Cd concentration. Following exposures, larvae were rinsed with ASW, divided into 10 composite replicates of 3 larvae each, weighed, and assayed for radioactivity. The samples were stored at −80 °C until antioxidant enzyme activities were measured. Experimental conditions and durations are summarized in Table 1.

#### 2.1.2. Dietary Cd effects on reduced glutathione

This experiment was designed to study the effects of dietary Cd on reduced glutathione (GSH)—a major non-enzymatic antioxidant in a variety of organisms. Periphyton was exposed to 0 (control) or 2 μg/L of stable Cd for 6 days and moved to clean water. Forty larvae (15–18 days old) were allowed to graze on clean periphyton (control) or Cd-exposed periphyton for 12 days. Cd concentrations in water ($n = 3$), periphyton ($n = 3$) and larvae ($n = 2$, each composite sample with 3 larvae) were determined by ICP-MS (Analytical Services Laboratory, Department of Soils Science, NC State University). Remaining animals were divided into 5 composite replicates each with three larvae. Larvae were homogenized in 10 mM N-ethylmaleimide (NEM) with 1 μM reserpine as an internal standard in water at a mass (mg) to volume (μL) ratio of 1:19. Samples were centrifuged to remove debris and the supernatant was analyzed for