ABSTRACT

DITTMAN, ELIZABETH KATHERINE. Manganese and Mountaintop Removal Mining: Bioaccumulation and Biological Effects. (Under the direction of David B. Buchwalter.)

Mountain top coal mining and subsequent valley fills have profound effects on water chemistry in Appalachian streams, and result in impaired biological diversity downstream of mining sites. It is well known that manganese is a constituent of mine effluent, and high levels of Mn (up to 4 mg L⁻¹) can be found downstream of valley fills. Little is known, however, about potential bioaccumulation of this metal by aquatic insects living in these systems. These invertebrates represent an important link in aquatic food chains and are widely used as indicators of stream health, but it remains unclear if Mn plays a role in biodiversity reduction. We examined dissolved Mn uptake and depuration kinetics in conjunction with studies of subcellular compartmentalization in aquatic insect species exposed to environmentally relevant Mn concentrations, revealing complex behavior.

First, insect species (n = 9) varied widely in Mn accumulation rates, with a considerable percentage of total Mn accrued associated with the integument in the form of Mn-oxides (mean 74%, range 24 - 95%). Increased ambient calcium concentrations decreased both adsorbed and absorbed Mn accumulation from solution. Though species showed similar Mn efflux rate constants (0.032 – 0.072 d⁻¹), the primary mode of Mn loss was through molting. More Mn was lost during the molt than Mn estimated (via combined ascorbate and EDTA rinses) to be associated with the integument. Subcellular compartmentalization studies revealed an overwhelming tendency for internalized Mn to
associate with the heat stable cytosolic protein fraction. After short dissolved Mn
exposures, intracellular glutathione and cysteine levels were markedly reduced relative to
controls. It was also found that exposure to manganese can hinder Ca\(^{2+}\) uptake from
solution, and there is some evidence that insects’ respiration may be altered after Mn
exposure. These findings suggest that Mn exposure results in transient physiological
stress in aquatic insects which is likely relieved, in part, during the molting process.
BIOGRAPHY

Elizabeth was born and raised in Cincinnati, OH. She attended Miami University in Oxford, OH, where she earned a B.S. in Biochemistry in 2008. The following fall, she began her graduate work at North Carolina State University for Dr. David Buchwalter.
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INTRODUCTION: Manganese and mountaintop mining: Biological effects in aquatic organisms

Mountaintop removal coal mining (MTM) operations in Appalachia dramatically alter the landscape in which they occur. Not only is the regional topography modified, but these mining activities have effects reaching beyond just the mining site. During MTM operations, excess earth that is removed from the tops of mountains is dumped into surrounding valleys. These valley-fills (VFs) result in destruction of habitats and burial of headwater streams [1]. As a consequence, waters downstream of the VF are often impaired both in terms of water chemistry parameters and biological considerations, such as community composition and species richness (see review [2]).

Stream chemistry is profoundly changed in sites downstream from MTM-VF processes compared to undisturbed streams. First, hydrological effects alter stream flow, causing higher volume but shorter lasting storm events due to soil compaction and decreased infiltration [3 - 5]. These changes can impact geochemical cycles, as well as modify habitat structures for stream-dwelling organisms. Second, the pH in water downstream of VFs has been shown to be elevated [1]. Lastly, the chemical and ionic makeup of the water changes. For instance, water downstream of MTM-VF sites has elevated levels of selenium [1, 6], total dissolved solids (TDS) [2], and manganese [1, 7], among other changes in ion levels. Selenium is a known teratogenic agent for fish and birds, and has been shown to have effects on aquatic insect life [6]. TDS and increased
ion concentrations (conductivity) may impair aquatic organisms’ abilities to ionoregulate. Manganese toxicity, however, has often been overlooked or ignored.

These changes in water chemistry have been shown to negatively impact life downstream of MTM-VFs [1, 5, 7, 8]. Aquatic macroinvertebrate surveys are the most commonly used approach to evaluate the ecological conditions of streams and rivers. As insects dominate the invertebrate species pool in most freshwaters, most survey methods focus upon insects. Aquatic insect richness and diversity can be severely decreased as a function of mining impact. Species in the orders Ephemeroptera, Plecoptera, and Tricoptera (EPT taxa) are particularly impaired by mining activities. Insects in these orders can be sensitive to water chemistry changes, and are often used in bioassessments to determine stream health [9, 10]. However, survey methods only assess the occurrence and abundance of these important bioindicators, without considering the factors controlling their presence or absence. Lab studies to determine these causal relationships are lacking, and stream insects are generally underrepresented in toxicity databases [11]. Aquatic organisms that are often included in these databases, such as daphnids, generally do not occur in the same habitat or do not share fundamental physiological traits with aquatic insects. Toxicity values that do exist for insects tend to be acute values that do not represent exposure levels known to cause impairment in nature [12 - 14].

Not only are these insect species important for bioassessment surveys, but they are also essential for healthy stream function [15]. Insects contribute to important
biogeochemical processes, such as nutrient cycling, as they play a vital role in processing organic material. Insects may be primary consumers, detritivores, and secondary consumers, converting relatively poor quality organic carbon into high quality food for higher trophic level organisms such as fish and birds.

Manganese is elevated downstream from VF sites. Pond et al. [1] reported mean concentrations ~4 times higher in mined streams compared to reference sites (range 9 – 904 µg L⁻¹ versus <5 – 83 µg L⁻¹, respectively). Mining effluents can have concentrations of manganese ranging up to 4 mg Mn L⁻¹. In aquatic systems Mn can exist in many forms and oxidation states, but the most common are Mn(II) and Mn(IV). Mn(II) predominantly occurs in the dissolved Mn²⁺ form, but can occasionally be bound onto particulates such as oxides [16]. It is thought that the ionic form of manganese is the most bioavailable in aquatic systems, capable of being accumulated into animal tissues [17]. Mn(IV) can often be found as Mn-oxide precipitates. These oxide particles can be found in the water column and in sediments, but they have also been found as coatings on both animals in streams and on the cobble substrate at the bottom of water channels. These oxide precipitates are capable of scavenging other metal from the water column, such as arsenic and chromium [18]. Mn-oxides can form slowly through abiotic processes, but biotic oxidation of Mn(II) to Mn(IV)-oxides can occur rapidly, performed by certain types of bacteria and fungi [18 - 21]. There is evidence that in many aquatic systems biogenic Mn-oxides are the principal form present [18, 22]. In acidic conditions, Mn-oxides are often reduced, and Mn²⁺ is released into the water column [23]. However, at higher pH (above
8). Mn$^{2+}$ becomes oxidized and precipitates out as Mn-oxides [16]. Dissolved oxygen content of the water also influences Mn redox cycling. In hypoxic conditions, Mn-oxides are reduced, releasing Mn$^{2+}$ into the water. However, in well oxygenated systems, such as streams with steady flow, Mn-oxides are likely to precipitate out of the water column. This geochemical cycling of manganese can be interrupted by mining activities that alter the pH and flow regimes downstream from VFs.

Mn is recognized as a micronutrient and an important constituent of certain forms of the antioxidant enzyme superoxide dismutase. However, little is known about the effects of elevated Mn in aquatic systems. Manganese is a known neurotoxin in humans and other mammals [24], and acute and chronic toxicity values have been determined for standard aquatic lab organisms [25 - 30], but toxic mechanisms of manganese in aquatic animals are understudied. Toxicity data for stream-dwelling insects is virtually nonexistent. Most of the work that has been done concerning manganese and aquatic organisms has focused on fish and crustaceans, with a few studies using algae. Even fewer of these studies have focused on the toxicity of manganese, but rather the uptake and tissue distribution of the metal. Manganese uptake has been shown to occur rapidly, with steady state concentrations being reached within a few days of exposure [31 - 33]. In algae, a large portion of accumulated Mn was shown to be oxide formation on the outside of the cells, attributed to the basic microenvironment that is caused by photosynthesis [34]. For other aquatic organisms, however, dissolved Mn$^{2+}$ is thought to be the most bioavailable form, and there is some evidence that transport of the ion occurs through
channels meant for other divalent cations such as Ca\(^{2+}\) [35, 36]. In fact, Mn toxicity in aquatic organisms is decreased as water hardness, particularly ambient calcium concentrations, increase [26, 29, 37]. Presumably, increased ambient Ca competes for transport sites with Mn\(^{2+}\), decreasing Mn uptake into tissues. It is understood that toxicity does not occur if the toxic agent does not reach its target, and if less Mn is taken up by an organism in harder waters, fewer toxic effects would be expected to occur.

It is worth understanding the fate of the metal once it is incorporated into tissues, however. Many Mn tissue distribution studies have focused on fish and crustaceans. In fish, manganese is found in large quantities in liver tissue [38, 39]. Being the liver is the major organ responsible for detoxification, this finding is not surprising. Another site where Mn is relatively abundant in fish is the gills [39, 40]. As ion transport cells occur on gills, they are an expected location to find accumulated manganese. Lastly, there is some evidence that manganese can be incorporated into calcified structures such as bone [38]. In fact, manganese exposure in brown trout resulted in impaired skeletal calcification [28], and skeletal deformations resulted from Mn exposure in sea urchins [41]. In crustaceans, a large amount of manganese (up to 98% in lobsters [42]) could be found in the calcified carapace [42, 43]. Interestingly, crustaceans have shown to have increasing manganese in their exoskeleton and gills over a molting cycle, with the highest concentrations found in premolt animals [42]. However, EPT taxa have chitinous integuments instead of calcified carapaces, so it remains to be seen if the same distribution would apply to those organisms.
The rare studies that do report on manganese in aquatic insects usually focus solely on Mn concentrations in the animals. Generally, these studies are attempting to show the potential uses of insect larvae for biomonitoring purposes, as indicators of stream pollution [44 - 46]. Rarely do these studies focus on EPT taxa, which are composed of species that are generally sensitive to habitat alteration and are often used in bioassessment surveys. The research presented in this thesis attempts to fill some of the data gaps pertaining to manganese effects in aquatic organisms and contributes to the growing body of work concerning the impacts of MTM-VFs in Appalachia. I first explored the uptake and efflux of dissolved Mn into field collected aquatic insects to determine the bioaccumulation potential of Mn and discovered that insects lose a significant portion their accumulated Mn during the molting process. I performed subcellular fractionation experiments to reveal the cellular compartments that were targets for Mn, and subsequently determined the effect of Mn on thiol concentrations. After finding that Mn-oxides form readily of the integuments of some species of insects, I quantified the total amount of Mn that was oxide versus tissue bound. Lastly, I probed possible effects of Mn-oxide formation on the animals, such as ionoregulatory issues and respiratory distress.
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CHAPTER 1: The many facets of manganese: Surface Mn oxide coatings, molting loss, and Mn(II) thiol scavenging in aquatic insects

(Formatted for Environmental Science and Technology)

Abstract

Streams below mountaintop removal-valley fill coal mining operations often have elevated Mn concentrations, but it remains unclear if Mn plays a role in biodiversity reduction. We examined dissolved Mn uptake and depuration kinetics in conjunction with studies of subcellular compartmentalization in aquatic insect species exposed to environmentally relevant Mn concentrations, revealing complex behavior. First, insect species (n = 9) varied widely in Mn accumulation rates, with a considerable percentage of total Mn accrued associated with the integument in the form of Mn-oxides (mean 74%, range 24 - 95%). Increased ambient calcium concentrations decreased both adsorbed and absorbed Mn accumulation from solution. Though species showed similar Mn efflux rate constants (0.032 – 0.072 d⁻¹), the primary mode of Mn loss was through molting. More Mn was lost during the molt than Mn estimated (via combined ascorbate and EDTA rinses) to be associated with the integument. Subcellular compartmentalization studies revealed an overwhelming tendency for internalized Mn to associate with the heat stable cytosolic protein fraction. After short dissolved Mn exposures, intracellular glutathione and cysteine levels were markedly reduced relative to controls. These findings suggest that Mn exposure results in transient physiological stress in aquatic insects which is likely relieved, in part, during the molting process.
Introduction

Manganese is the 12th most common element in the Earth’s crust, yet it is not commonly considered problematic in freshwater ecosystems relative to other transition metals such as zinc, copper, cadmium, and mercury (the 24th, 26th, 72nd, and 73rd most common elements in the earth’s crust, respectively [1]). Much work has focused on Mn in sediments, however Mn in cobble bottomed streams is surprisingly understudied, and perhaps overlooked in situations where it co-occurs with more toxic elements. Manganese is a common constituent of mining and smelting related discharges and other industrial effluents. Our interest in Mn stems from its occurrence at relatively high concentrations below mountaintop removal – valley fill (MTM-VF) coal mining operations in Appalachia [2, 3].

The environmental chemistry of Mn is complex. At neutral to mildly alkaline aerobic conditions, such as those found in Central Appalachian coal mining areas, Mn is expected to occur in both Mn(II) and Mn(IV) oxidation states. Mn(II) is considered to be the most bioavailable form, particularly while occurring as the free ion Mn^{+2}. Mn(IV) occurs as relatively insoluble oxide precipitates under aerobic conditions and can be visible as dark coatings on stream bottoms and on the integuments of stream insects. Importantly, the oxidation of Mn(II) to Mn(IV) can be carried out by several species of bacteria and fungi, which often occurs more rapidly than abiotic oxidation [4 - 6].
It is increasingly recognized that ecological conditions in streams below MTM-VFs are typically poor (see review [7]), and field studies show that aquatic insect diversity is decreased as a function of mining impact, with mayfly genera being particularly affected [2, 3, 8, 9]. Changing hydrology [9 - 11] and degraded water quality [3] are often thought to be potential causes. Water quality concerns include elevated pH [3] and elevated concentrations of dissolved solids [7], selenium [12], and manganese [2]. Pond et al. [3] report that average dissolved Mn concentrations in mined streams were more than 5-fold higher (mean 113.4, range 6.5-853 µg Mn L⁻¹) than those in un-mined streams (mean 20.9, range <5-55 µg Mn L⁻¹). Mine effluent directly discharged into streams can contain Mn concentrations ranging up to 4-5 mg L⁻¹. One field study found a negative correlation between richness of Ephemeroptera, Plecoptera, and Tricoptera (EPT) taxa and Mn concentration [2], though interpretation of these findings is confounded by the presence of other water chemistry parameters that are well outside of typical regional values. It therefore remains unclear whether these elevated Mn concentrations contribute to degraded ecological conditions in MTM-VF affected streams.

Aquatic insects dominate stream ecosystems and play important roles in ecosystem function as a link between the base of the food web (e.g., algae, bacteria) and higher order consumers (e.g., fish, birds). In general, bioassessment (survey) methods that focus on insect communities cannot determine which stressors are most responsible for ecological impairment. Moreover, stream insects are generally not well represented in
toxicity databases [13] and may not be adequately represented by standard invertebrate toxicity models (e.g. daphnids) that either do not occur in streams or do not share fundamental physiological traits. As we are unaware of any laboratory studies focused upon aquatic insects and Mn, this paper represents a first step toward understanding Mn bioaccumulation and physiological responses in stream insects, and is a starting point for future investigations.

Here we explore Mn bioaccumulation kinetics from solution in a variety of field collected aquatic insects. We used Michaelis-Menten type kinetic experiments to examine Mn transport in the caddisfly *Hydropsyche betteni*, and compared dissolved uptake and efflux rates in common Southern Appalachian stream insects. We assessed the influence of calcium concentration on Mn accumulation. We further quantitatively discriminate between Mn adsorbed to insect integuments (predominantly as oxides), and Mn incorporated into tissues. The subcellular compartmentalization of Mn is compared among several species, and the effect of Mn exposure on thiol status is examined. Together, these studies provide a framework for understanding the potential effects of Mn on stream insects.
Methods and Materials

*Insect collection and handling*

Insect larvae were field collected using a D-frame kicknet on several dates in 2009 and 2010 from two sites in North Carolina (Basin Creek and Eno River). After each collection, animals were transported back to the lab in a cooler containing river water and substrate (cobbles). Insects were sorted in the lab, and voucher specimens were preserved in ethanol for species verification. Live animals from each collection were allowed to acclimate in artificial soft water (ASW) (48 mg L$^{-1}$ NaHCO$_3$, 30 mg L$^{-1}$ CaSO$_4$$\cdot$2H$_2$O, 30 mg L$^{-1}$ MgSO$_4$, and 2 mg L$^{-1}$ KCl) for at least twenty-four hours with aeration in a walk-in cold room at 11.5 ± 1.0°C and a 12:12 hour light:dark photoperiod.

*Manganese kinetics in multiple species*

We compared dissolved Mn uptake kinetics in several common Appalachian species - *Ephemerella dorothea*, *Acroneuria carolinensis*, *Drunella cornutella*, *Hexatoma sp.*, *Maccaffertium pudicum*, *Epeorus vitreus*, and *Pteronarcys sp.* collected from Basin Creek (Wilkes County, NC) in June, 2009. Five individual larvae from each species (except *Pteronarcys* where n=3, and *D. cornutella* where n=10) were used. A solution with a nominal Mn concentration of 0.4660 μM in ASW was spiked with $^{54}$Mn (total activity = 0.0632 MBq L$^{-1}$) as $^{54}$MnCl$_2$ (0.0249 nM Mn), with the remainder of Mn added
as stable MnCl$_2$. The pH was adjusted to 7.7 using 0.1N NaOH. To ensure that replicates received identical exposure regimes, bulk solutions were prepared and distributed to individual 50 mL HDPE beakers. Each larva was placed into individual beakers with 40 mL of solution with aeration and a piece of Teflon mesh as substrate. At 2, 4, 6, and 8 hours each animal was assayed \textit{in vivo} for radioactivity using a Wallac Wizard gamma counter. After 8 hours, each larva was weighed, and those exhibiting significant Mn accumulation (\textit{E. dorothea}, \textit{A. carolinensis}, \textit{D. cornutella}, \textit{M. pudicum}, \textit{Pteronarcys sp.}) were exposed for an additional 24 - 48 hours to accumulate sufficient radioactivity for subsequent 10 day depuration experiments (see supplemental material). (For molting loss methods and results see supplemental material).

\textit{Dissolved manganese uptake in H. betteni}

To examine dissolved Mn uptake, \textit{Hydropsyche betteni} were field collected from the Eno River (Orange County, NC) on January 30 and February 25, 2009. Animals collected in January were individually exposed to 0.1864, 0.4660, 1.165, 2.912, and 7.281 $\mu$M Mn in 50 mL HDPE beakers containing a total volume of 40 mL ASW with $^{54}$Mn radiotracer (0.0646 MBq L$^{-1}$). February exposures were to a lower range of Mn concentrations: 4.769, 11.923, 29.815, 74.557, and 186.39 nM with $^{54}$Mn radiotracer (0.0608 MBq L$^{-1}$). Solutions were adjusted to pH 7.6-7.7. Animals were exposed as described above. Each concentration was represented by five replicates, with each
replicate consisting of a single larva. After 1, 3, 6, 9, and 24 hours of exposure, the animals were removed, rinsed with clean ASW, assayed *in vivo* for radioactivity, and then returned to their exposure containers. Exposure water was renewed after the 9 hour time point. After 24 hours, the animals were rinsed with clean ASW and weighed. Larvae from the 0.4660, 1.165, and 2.912 μM exposures were frozen and stored at -20°C. Insects from the 0.1864 μM and 7.281 μM treatments were individually placed into 500 mL beakers containing clean ASW for depuration studies (see supplemental materials).

Manganese uptake rates were calculated for each individual at each exposure concentration. The mean of 5 replicate slopes was taken as the uptake rate at a given concentration and Michaelis-Menten kinetic parameters, $V_{\text{max}}$ and $K_m$, were calculated using GraphPad Prism 5.0 software.

*Reducible manganese oxide determination*

Insects were collected from the Eno River, NC on January 13, 2010 (*Cheumatopsyche* spp., *Maccaffertium modestum*, and *Isonychia* spp.) and from Basin Creek on January 28, 2010 (*Cheumatopsyche* spp., *Diplectriona modesta*, *Rhyacophila fuscula*, mixed *Leptophlebiid* species, mixed *Maccaffertium* species, *Acroneuia abnormis*, *Malirekus hastatus*, and *Acroneuria carolinensis*). The insects were exposed to 1.82 μM Mn with $^{54}$Mn as a radiotracer as described above (total activity = 0.0629 MBq
L⁻¹) and assayed daily for radioactivity. On day four, they were counted and rinsed thoroughly with sequential rinses of 0.1M ascorbate followed by 0.05M EDTA, and finally deionized water (henceforth referred to as ascorbate rinses). Animals were assayed again for radioactivity, and percent Mn lost during the rinse was calculated. Selected species from Basin Creek with substantial radioactivity measured after this rinsing procedure were used for subcellular fractionation experiments (see below).

**Calcium competition**

To examine the influence of Ca²⁺ concentrations on dissolved Mn accumulation in insects, we exposed larvae collected from Basin Creek to 0.441 µM Mn with ⁵⁴Mn as a radiotracer (total activity = 0.0637 MBq L⁻¹) under four conditions. ASTM recipes for very soft (VSW), moderately hard (MHW), and very hard (VHW) waters were prepared in addition to the base very soft water recipe with additional Ca (VSW+Ca) added (as CaSO₄) such that total Ca content matched the VHW treatment (Table S1). *Acroneuria spp.*, *Ephemerella dorothea*, *Drunella cornuta*, and *Maccaffertium pudicum* were exposed to Mn under each ambient Ca condition for 24 hours. After 24 hours of exposure, animals were rinsed with DI water, assayed for radioactivity, and then ascorbate rinsed to remove Mn oxide precipitates. Insects were assayed again for radioactivity, weighed, and frozen at -20°C.
Subcellular fractionation

_Cheumatopsyche spp., Diplectrona modesta, Maccaffertium spp., Acroneuria abnormis, Malirekus hastatus_, and _Acroneuria carolinensis_ from Basin Creek were used to determine the subcellular compartmentalization of Mn within insect tissue. Single animals were homogenized in 8mL of phosphate buffer (pH = 7.4). The fractionation scheme was a slightly modified version of Wallace et al. [14] and described elsewhere for insects [15, 16]. Fractions obtained were cell debris, organelles, microsomes, heat-denatured proteins (HDP), and heat-stable proteins (HSP) and are reported on the basis of recovered Mn.

Thiol analysis

_Hydropsyche betteni_ were collected from the Eno River on September 02, 2009. Animals were exposed to Mn at concentrations of 0 (control), 0.091, 0.910, or 9.101 µM Mn for four days. For each treatment there were three replicates consisting of 2-3 insects each. After exposure, animals were rinsed with DI water, weighed, and frozen at -20°C. They were homogenized in 10 mM N-ethylmaleimide (NEM) with 1 µM reserpine in water at a mass (mg) to volume (µL) ratio of 1:19. Samples were centrifuged to remove debris, and the supernatant was analyzed using an LC-MS for concentrations of reduced
and oxidized glutathione and cysteine. Methods were adapted from [17] and described elsewhere [18]. *Cheumatopsyche spp.* and *Maccaffertium modestum* collected from the Eno River on January 13, 2010 were also used for thiol analysis. Animals were exposed to 1.82 µM manganese for four days and then frozen at -20°C. For each species, there were three replicates for both control and Mn exposures, with 2-3 insects per replicate. They were prepared for analysis as described above.

**Statistics**

Graphs and statistical analysis were completed using GraphPad Prism (Version 5.0) software. Discrepancies from controls were determined using t-tests (α = 0.05). $V_{\text{max}}$ and $K_m$ values were determined by using a best-fit Michaelis-Menton function in Prism. Unless otherwise noted, all values are given as mean ± standard deviation.

**Results**

*Manganese accumulation*

Mn uptake rates ranged over three orders of magnitude across several species exposed to 0.466 µM Mn. The caddisfly *H. betteni* and the ephemerellid mayfly *D. cornutella* had the fastest uptake rates of Mn from solution during short term exposures,
779.7 and 725.0 ng Mn g\(^{-1}\) hr\(^{-1}\), respectively (Figure 1). The tipulid *Hexatoma sp.* and the heptageniid mayfly *E. vitreus* had negligible uptake from solution. The remaining insect species were intermediate in Mn uptake ability: another ephemerellid mayfly *E. dorothea* and the heptageniid mayfly *M. pudicum* had uptake rates of 299.3 and 63.53 ng Mn g\(^{-1}\) hr\(^{-1}\), respectively (Figure 1). Stonefly species *A. carolinensis* and *Pteronarcys sp.* had uptake rates of 70.65 and 18.15 ng Mn g\(^{-1}\) hr\(^{-1}\), respectively (Figure 1).

Manganese accumulation rates across a wide range of concentrations (4.769 nM - 7.28 µM) followed a Michaelis-Menten type kinetics pattern in *H. betteni*. Estimates of maximal transport rate (\(V_{\text{max}} = 156.1 \pm 4.6 \text{ nmol Mn g}^{-1} \text{ hr}^{-1}\), \(r^2 = 0.9955\)) and affinity (\(K_m = 4.855 \pm 0.2728 \mu\text{M Mn}\)) (mean ± standard error) suggest that total Mn accumulation rates in this species are extremely rapid (Figure 2). This finding suggests that Mn transport in *H. betteni* occurs via a low affinity, high capacity transport system. We found no evidence for a complimentary high affinity, low capacity transport system at concentrations as low as 4.769 nM (Figure 2, inset).

As these initial experiments did not discriminate between absorbed (true uptake) and adsorbed Mn accumulation, they should be interpreted as total accumulation and not true transport. Since all larvae used in these experiments (excluding those used in efflux studies as described in supplemental material) were archived (frozen), we were able to use ascorbate rinses to “correct” for surface adsorption of Mn oxides. On average, 41.9%
of total radioactivity was removed with this rinsing process, representing loss of surface adsorbed metal.

We further investigated the relative contribution of surface adsorption (as Mn oxides) and internalized (i.e. absorbed) Mn to total Mn body burdens after aqueous exposures. With exception of *Leptophlebiids*, all species we tested lost the majority (over 50%) of their Mn body burden after the ascorbate rinse (Figure 3). On average, species (n = 11) lost 74 ± 21.7% of their radioactivity after rinsing. *M. hastatus* lost the largest percentage of Mn, 95 ± 1.23%, while mixed *Leptophlebiid* species only lost 24 ± 16.4% of their accumulated Mn. Thus, significant Mn associated with insects seems to be in the form of Mn oxide coatings on the integument.

*Influence of water chemistry on Mn absorption and adsorption*

Because calcium concentrations can be elevated in mine-impacted streams, animals were exposed to Mn under various ionic strengths and Ca concentrations. In general, both Mn absorption and adsorption decreased with increasing ionic strength and Ca concentrations (Figure 4). For *Acroneuria spp.*, Mn oxide accumulation after 24 hours was significantly decreased from controls (VSW) by 84.4 (p = 0.0026) and 80.1% (p = 0.0225) in VHW and VSW+Ca treatments, respectively (Figure 4a). Post ascorbate rinse, internalized Mn accumulation was significantly decreased by 53.2 (p = 0.0127) and
60.3% (p = 0.0346) in VHW and VSW+Ca treatments, respectively (Figure 4e). Though not statistically significant, adsorbed Mn on Drunella cornuta was highest in VSW relative to the other treatments (Figure 4b), but absorbed Mn burdens were significantly decreased in VHW and VSW+Ca treatments by 53.1 (p = 0.0023) and 59.9% (p = 0.0002), respectively (Figure 4f). Mn oxide formation on Maccaffertium pudicum was reduced significantly by exposure in VHW and VSW+Ca treatments, with burden reductions of 77.2 (p = 0.0005) and 76.2% (p = 0.0013), respectively (Figure 4c). The same trend was observed for absorbed Mn in M. pudicum, but the differences were not statistically significant (Figure 4g). Reducible Mn body burdens were significantly reduced by 64.7 (p < 0.0001), 72.4 (p < 0.0001), and 42.7% (p < 0.0001) in Ephemerellid dorothea exposed to MHW, VHW, and VSW+Ca treatments, respectively (Figure 4d). No significant trends were observed for internalized Mn accumulation in this species (Figure 4h).

**Manganese elimination**

Because bioaccumulation of metals from solution is a function of both uptake and efflux, we examined Mn loss kinetics in six stream insect species. Manganese elimination was modest in each species tested, with efflux rate constants ranging from 0.032 day\(^{-1}\) in H. betteni to 0.072 day\(^{-1}\) in E. dorothea (Figure S1).
Interestingly, although the loss of Mn was relatively slow in all species, we observed the apparent loss of nearly all accumulated Mn during the molting process, with the lost Mn recovered in the shed exuvia. Discrepancies between larvae and adult tissue Mn body burdens have also been found in field collected samples. Analysis of mayflies from the Clinch River (CR) and the Little Emory River (LER) in Tennessee show that nymphs had mean body burdens of 975 and 1215 µg Mn g\(^{-1}\) dry weight, respectively. However, adults collected from the same sites contained means of only 3.017 and 2.933 µg Mn g\(^{-1}\) dry weight (Figure S2). Exuvia from the sub-imago to imago molt collected from CR and LER showed mean Mn concentrations of 24.01 and 5.472 µg Mn g\(^{-1}\) dry weight, respectively (Figure S2), but exuvia from the larval to sub-imago molt were unable to be recovered.

Although we found that a significant portion of total accumulated Mn was present in the oxide form that could be rinsed from the integument with a reducing agent, many species still accumulated substantial internalized Mn concentrations (Figure 3). Subcellular fractionation studies revealed that the cytosol was a major sink for Mn bioaccumulation. The mean percentage of Mn associated with the cytosol across all species was 68.6 ± 18.7% and ranged from 39.9 ± 10.1% (in *Cheumatopsyche spp.* to 90.8 ± 2.6% (in *M. modestum*). However, within the cytosol, the distribution of Mn between HDP and HSP fractions was remarkably consistent across species and heavily skewed towards the HSP fractions (Table 1). Manganese associated with HSP averaged 96.2 ± 1.8% of the total cytosolic content in these six species. The HSP protein fraction is
thought to be dominated by small peptides, thiols such as glutathione and cysteine, and metallothionein like proteins (though the latter have never been directly observed in insects) [19].

To test whether Mn exposure influenced thiol status in insect tissues, we examined reduced glutathione (GSH) concentrations in *H. betteni*. Exposure to dissolved Mn for 4 days resulted in a 55.4% decrease in GSH concentration (*p = 0.0603*) at 0.091 µM Mn, and a 63.2% (*p = 0.0393*) and 66.8% (*p = 0.0288*) decrease in GSH at 0.91 and 9.1 µM Mn, respectively (Figure 5a). Oxidized glutathione (GSSG) concentrations in this experiment were below quantification limits.

Follow up studies revealed a significant effect of Mn exposure on cysteine and cystine concentrations. After four days of exposure to 1.82 µM Mn, cysteine levels were decreased 64.9% (*p = 0.0674*) in *Cheumatopsyche spp.* and 84.4% (*p < 0.0001*) in *M. modestum* (Figure 5b). Similarly, cystine concentrations in *Cheumatopsyche spp.* and *M. modestum* decreased by 75.6% (*p = 0.0083*) and 44.1% (*p = 0.0205*), respectively. Peak areas revealed similar responses to Mn exposure in GSH concentrations, but problems with standards did not allow us to rigorously quantify GSH in this experiment.
Discussion

Manganese is a relatively common, yet poorly studied element in freshwater ecosystems. As we were unable to find any Mn experimental work specifically related to aquatic insects, this paper represents a first step in understanding Mn interactions in this important faunal group. Our studies reveal that Mn interactions with aquatic insects are multifaceted and complex.

Manganese uptake from solution ranged three orders of magnitude across several common insect taxa. This finding is not surprising given similar variability observed for different insect species with other metals such as Cd [15] and Zn [20]. However, it is intriguing that taxa with relatively fast Mn uptake rates (e.g. *Hydropsyche, Ephemerella*) are also those with generally fast transport rates of Cd and Zn. This finding suggests that each of these elements may perhaps be transported by a common transport system – possibly involved in Ca transport.

Detailed kinetic experiments with *H. betteni* revealed a large capacity to accumulate Mn from solution. The large $V_{\text{max}}$ and $K_m$ suggests a high capacity, low affinity transport system. We found no evidence of a second transport system in play at very low ambient Mn concentrations. However, we only performed these detailed kinetics studies under a single ambient Ca concentration (6.98 mg Ca$^{2+}$ L$^{-1}$). Ascorbate rinses (but not EDTA alone) suggest that most Mn associated with the integument was in the form of Mn oxides. Use of the Mn oxide specific dye leucoberbelin blue verified that
Mn oxides were not forming in the water or container walls during these experiments, but were obvious on integuments. This finding leads us to speculate that differences in oxide production may be related to distinctive microbial communities residing on the integument of various insect species. Some species (e.g., *Tipula sp.*) did not seem to cultivate any surface bound oxides, whereas others (e.g., *M. hastatus*) had as much as 94.8% of their total Mn body burdens as surface bound oxides.

Increasing Ca concentrations profoundly decreased both absorbed and adsorbed Mn accumulation, which is consistent with decreased Mn toxicity at increased water hardness levels [21 - 23]. Paired with our finding that Mn is transported via a high capacity but low affinity system, our results support former studies that Mn²⁺ transport can occur through channels meant for other divalent cations, such as Ca²⁺ [24, 25]. Future studies should examine the influence of dissolved Mn concentrations on Ca ion uptake and regulation.

Manganese efflux rate constants were relatively consistent across species, but molting was the primary means by which Mn is eliminated from tissues. During efflux experiments larvae that molted lost the vast majority of their total Mn body burdens – considerably more than could be removed by ascorbate rinses alone. This finding is in agreement with Cid et al. [26], where considerable Mn loss after molting was described in the European mayfly (*Ephoron virgo*) in nature. Our own exuvia analyses from
*Hexegenia sp.* corroborates the finding that many different aquatic insects shed internalized and surface-bound Mn during the molt process.

Manganese sequestration in the exoskeleton has been observed in the calcified body parts of crustaceans, which also lose a substantial amount of accumulated Mn during the molting process [27]. This partitioning may be a detoxification mechanism, or a means to harden or fortify the integument or certain cuticular tools such as mandibles [28 - 30]. Thus there is some precedent for physiological processes in place that allow insects to shuttle Mn to the exoskeleton. Practically, this molting loss makes traditional bioaccumulation modeling approaches (e.g. [31, 32]) insufficient for Mn, and also suggests that field-based values of Mn tissue concentrations in insects should be interpreted with caution.

Despite extensive surface binding, several species still accumulated an appreciable amount of Mn in tissues, with ≤ 90% of internalized Mn found in the HSP fraction of the cytosol. Conventionally, this fraction is thought to contain detoxified metal [14] since molecules such as metallothionein and glutathione are found here. It may be that small peptides or thiols play a role a transporting Mn to the integument during the molting process, though we have no direct evidence of this.

Relatively short and ecologically relevant Mn exposures resulted in marked decreases in thiol concentrations. For example, we found decreased concentrations of GSH in *H. bettenti* exposed to as low as 0.91 µM Mn, consistent with previous studies
finding decreased GSH after Mn exposure [33]. We also found reduced cysteine concentrations in *Cheumatopsyche spp.* and *M. modestum* after Mn exposure. These intracellular thiols play important roles in managing the cell’s redox state. The strong association of Mn with the HSP fraction coupled with the substantial reduction in the free concentrations of thiols following Mn exposure provides some evidence for Mn acting as a direct thiol scavenger in aquatic insects. Brief dissolved Mn exposures did not affect the total antioxidant activity of insect homogenates (as measured by the Cayman Chemical kit which uses the oxidation of 2,2’-Azo-di-[3-ethylbenzthiazoline sulphonate by metmyoglobin). However, previous reports have shown that Mn exposure decreased total antioxidant status in rat brain, the effects of which can be lessened by cysteine addition [34]. As we have yet to examine chronic or dietary Mn exposures, the observation that Mn exposure reduces free thiol concentrations is alarming, especially considering that Mn often co-occurs with other metals and contaminants which may act as pro-oxidants (e.g. [18]).

Together these studies provide a first glimpse of Mn interactions with aquatic insects. We show highly variable transport rates of dissolved Mn among species and highlight the important process of Mn oxide formation on the integuments of different insect species. We provide unequivocal evidence of Mn loss during the molting process and show a strong interaction of Mn with heat stable cytosolic proteins and thiols – the latter of which may constitute a significant physiological stressor. Yet several issues remain unclear and require further study. For example, it remains unknown whether
surface Mn oxide coating affects insects’ ability to exchange gasses or salts with the surrounding water. Moreover, it is unclear whether dietary Mn (as either Mn(II) or Mn-oxides) is bioavailable or associates with thiols. Thus there is much to learn about Mn interactions with this important group of ecological indicators.

**Acknowledgements:** The authors appreciate the taxonomic assistance of William Crouch and Eric Fleek, NC DENR. Norm Glassbrook provided thiol analyses. Lingtian Xie prepared exuvia samples for analysis. Gerald LeBlanc, Justin Conley, Monica Poteat, and Kyoung Sun Kim provided valuable editorial comments. This work was supported by US EPA (83425501-0) and the College of Agriculture and Life Sciences, NCSU.
Table 1. Percent of recovered Mn found in each of five subcellular fractions for six insect species. Fractions represented are cell debris, organelles, microsomes, heat-denatured proteins, and heat-stable proteins. Values given as mean ± standard deviation.

| Species                  | % Cell Debris | % Organelles | % Microsomes | % Heat-stable protein | % Heat-denatured protein | Total body burden (µg g⁻¹) | % Recovered | n  |
|--------------------------|---------------|--------------|--------------|-----------------------|--------------------------|----------------------------|--------------|--|---|
| *Cheumatopsyche* spp.    | 26.6 ± 1.3    | 24.0 ± 6.7   | 9.4 ± 4.7    | 38.4 ± 9.8            | 1.5 ± 0.3                | 59.7 ± 12.6                 | 80.2 ± 6.7  | 2 |
| *Diplecrotuna modesta*   | 26.7 ± 5.1    | 16.5 ± 5.1   | 6.7 ± 5.9    | 48.2 ± 5.6            | 2.0 ± 0.4                | 43.0 ± 26.0                 | 82.1 ± 6.6  | 6 |
| *Maccaffertium* spp.     | 6.1 ± 2.4     | 1.8 ± 0.8    | 1.3 ± 0.6    | 90.1 ± 2.5            | 0.7 ± 0.5                | 10.3 ± 7.9                  | 85.3 ± 4.3  | 6 |
| *Acroneuria abnormis*    | 29.1 ± 18.5   | 3.5 ± 2.2    | 1.9 ± 0.7    | 62.2 ± 20.7           | 3.3 ± 2.3                | 5.6 ± 2.4                   | 90.4 ± 2.0  | 6 |
| *Malirekus hastatus*     | 13.3 ± 1.7    | 3.8 ± 1.1    | 1.9 ± 0.7    | 78.7 ± 1.9            | 2.2 ± 0.4                | 1.4 ± 0.5                   | 85.7 ± 6.4  | 3 |
| *Acroneuria carolinensis*| 23.4 ± 15.1   | 3.6 ± 2.0    | 2.9 ± 1.4    | 65.7 ± 18.4           | 4.3 ± 2.8                | 1.5 ± 0.8                   | 89.8 ± 5.8  | 6 |
Figure 1. Manganese body burdens (ng Mn g⁻¹ wet weight) as a function of exposure time. Insects were exposed to 0.466 µM Mn. Data points (n = 5 except Drunella where n = 10 and Pteronarcys where n = 3) are mean ± standard deviation. The slope of each line represents the uptake rate.
Figure 2. Michaelis-Menten modeling of Mn uptake rates in *Hydropsyche betteni*. $r^2 = 0.9955$. Inset is the linear portion of the curve, showing data from Mn concentrations of 4.769 to 186.4 nM.
Figure 3. Mean body burden of reducible (able to be rinsed off with ascorbate) versus internalized Mn in several species. Bar sets 1-3 represent animals collected from the Eno River, NC on January 13, 2010. Subsequent bars represent insects collected from Basin Creek, NC on January 28, 2010. Error bars represent standard deviation.
Figure 4. Manganese accumulation in four insect species in varying water chemistry conditions. VSW = very soft water; VSW+Ca = very soft water plus calcium; MHW = moderately hard water; VHW = very hard water. Panels a-d represent reducible Mn body burdens of the insects (i.e., Mn oxides). Panels e-h represent internalized Mn body burdens, (i.e., amount of Mn retained after rinsing with ascorbate). Body burdens are given as µg Mn g⁻¹ wet weight, and bars show mean ± standard deviation.
Figure 5. Thiol analysis of Mn exposed animals. a) Concentrations (nmol GSH g\(^{-1}\) wet weight) of reduced glutathione in *H. betteni* exposed to Mn for four days. b) Concentrations (µmol cysteine g\(^{-1}\) wet weight) of cysteine in *Cheumatopsyche spp.* (black bars) and *Maccaffertium modestum* (grey bars) exposed to Mn for four days. Values given as mean ± standard deviation.
Literature Cited


(30) Schofield, R. M. S.; Nesson, M. H.; Richardson, K. A.; Wyeth, P. Zinc is incorporated into cuticular "tools" after ecdysis: The time course of the zinc distribution in "tools" and whole bodies of an ant and a scorpion. *J. Insect Physiol.* **2003**, 49 (1), 31-44.


Supplemental materials

Table S1. Salt additions for solutions of water with varying hardness. Values are given in mg L\(^{-1}\) of DI water.

<table>
<thead>
<tr>
<th>Type</th>
<th>NaCO(_3) (mg/L)</th>
<th>CaSO(_4)·2H(_2)O (mg/L)</th>
<th>MgSO(_4) (mg/L)</th>
<th>KCl (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very soft</td>
<td>12</td>
<td>7.5</td>
<td>7.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Very soft + Ca</td>
<td>12</td>
<td>240</td>
<td>7.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Moderately hard</td>
<td>96</td>
<td>60</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>Very hard</td>
<td>384</td>
<td>240</td>
<td>240</td>
<td>16</td>
</tr>
</tbody>
</table>

Manganese efflux

The acquisition of sufficient radioactivity in larvae exposed to relatively low (0.1864 μM) and high (7.281 μM) Mn concentrations allowed us to test the premise that efflux rate constants are independent of tissue concentrations. Following 24 hours of exposure to these concentrations, *H. betteni* larvae were added to individual beakers containing 500 mL of clean reconstituted soft water. The animals and their surrounding water were assayed daily for radioactivity for 10 days. The water was changed on day four of depuration. The efflux rate constant (k\(_{e}\)) was determined using the equation:
\[ C_t = C_i \times e^{k_e t} \]

Where:

\[ C_i = \text{Mn concentration in the animal at time 0 (}\mu\text{g Mn g}^{-1}\text{ wet weight)}\]

\[ C_t = \text{Mn concentration in the animal at time t (}\mu\text{g Mn g}^{-1}\text{ wet weight)}\]

\[ k_e = \text{efflux rate constant (day}^{-1}\text{)}\]

\[ t = \text{time in days}\]

The same procedure was used to assess efflux in six species used for uptake experiments (\textit{Ephemerella dorothea, Acroneuria carolinensis, Drunella cornutella, Maccaffertium pudicum, Pteronarcys sp.}). Insects were individually placed into approximately 400 mL of uncontaminated ASW for depuration as described above, with water changes at 3 day intervals. Animals were fed periphyton from day 2-7. After ten days, the animals were frozen and stored at -20°C.

\textit{Molting Mn loss from field samples}

We were able to obtain Mn tissue concentrations from US Department of Energy (Oak Ridge) and Tennessee Valley Authority (TVA) biologists from field collected larvae and adult mayflies (\textit{Hexegenia sp.}) from two field sites – the Clinch and Emory Rivers in Tennessee. During the field sampling process, Oak Ridge and TVA scientists
archived shed exuvia (sub-imago to imago molt), which they subsequently sent to us for analysis by ICP-MS.

Figure S1. Mean efflux rate constants, $k_e$ (day$^{-1}$) for six species of aquatic insects. Animals were loaded with Mn and depurated in clean water for ten days. Error bars represent standard deviation.
Figure S2. Mean manganese body burdens of mayfly nymphs, imagos, and exuvia from the sub-imago to imago molt. Samples were collected from two sites in Tennessee. Black bars indicate samples from Clinch River; grey bars represent Little Emory River. Error bars indicate standard deviation.
Chapter 2: Ionoregulatory and respiratory effects of manganese oxide formation on aquatic insects

Introduction

The previous chapter clearly outlined the fact that in addition to aquatic insects taking up manganese into tissues, oxides were forming on their integuments, sometimes in large quantities. The purpose of the research presented in this chapter is to explore the potentially detrimental effects these oxides may cause with regard to insect physiology. In particular, I focus upon ionoregulatory and respiratory issues.

Ionoregulatory cells occur on the body surface of most insects – on gills as individual cells or in epithelial patches or protuberances (papillae) [1, 2]. Here, ions such as Ca\(^{2+}\) are moved from the surrounding water into the hemolymph of the insect, often against concentration gradients. Thus, there are 2 ways in which Mn in solution might interfere with ion transport. Increasing water hardness (and calcium concentration) has been shown to decrease Mn toxicity [3 - 5], potentially due to competition for uptake at transport sites. It stands to reason that conversely, Mn\(^{2+}\) could decrease Ca\(^{2+}\) uptake at these sites. Also, Mn-oxide formation on insect integuments may pose a different threat to ionoregulation by physically preventing transport sites on the gills from having contact with the water, hindering Ca uptake. It is worth exploring if these oxides are a factor that could contribute to ionoregulatory distress.
Second, the gills (and sometimes inter-segmental membranes) are also the site of gas exchange for aquatic insects. It is possible that Mn-oxide formation on these surfaces could form a physical barrier obstructing gas exchange and respiration. Stress from metal exposure may also alter respiration rates due to toxic effects. Alone or in conjunction with other harmful chemicals known to be downstream of VF sites, Mn could potentially disrupt respiration in aquatic organisms.

**Materials and Methods**

*Calcium competition*

To explore potential mechanisms for manganese uptake from solution, I examined the influence of ambient Ca concentrations on Mn uptake in *Drunella cornutella*. Each exposure consisted of a solution with reconstituted soft water and 0.4660 μM Mn with $^{54}$Mn as a radiotracer (total activity = 0.0645 MBq L$^{-1}$). Manganese uptake was examined at 6.98, 60, and 120 mg Ca L$^{-1}$, with CaCl$_2$ stock (2 g Ca L$^{-1}$) added to ASW to achieve these Ca concentrations. Five replicates represented each treatment, with each replicate consisting of one larva. Each animal was placed in 40mL of solution in a 50mL HDPE beaker with aeration and Teflon mesh as substrate. Animals were exposed for twenty-four hours, after which they were rinsed with clean ASW and assayed *in vivo* for
radioactivity and weighed. Total accumulated manganese was calculated and compared among treatments using t-tests.

Calcium uptake

To determine the influence of manganese exposure on calcium uptake, I performed a series of experiments using $^{45}\text{Ca}$ as a radiotracer. The first four experiments consisted of three treatments: control, pre-exposed to manganese, and co-exposed to Mn. The last $^{45}\text{Ca}$ experiment had only control and pre-exposed treatments, with three pre-exposure levels.

The first experiment used *Ceratopsyche sparna* collected from Cataloochee Valley (great Smokey Mountains National Park, NC, USA) in September 2009. Fifteen animals were pre-exposed to 500 µg Mn L$^{-1}$ in artificial soft water (ASW) for 24 hours. Pre-exposed animals were then briefly rinsed with ASW and placed into clean ASW containing $^{45}\text{Ca}$ (total activity = 0.0732 MBq L$^{-1}$) as a radiotracer. For each treatment there were three replicates, with five animals per replicate. Co-exposed animals were placed in ASW containing $^{45}\text{Ca}$ and 500 µg Mn L$^{-1}$. After 12 hours, animals were removed, rinsed with DI water, followed by EDTA, and again with DI. Animals were weighed and placed into individual vials. One mL of Soluene® was added to each vial to digest tissues. After animals were digested, 15 mL scintillation cocktail was added to
each vial. Samples were analyzed for radioactivity using a Beckman LS 6500 multi-purpose scintillation counter. Newly acquired calcium body burdens (as measured by $^{45}$Ca) were compared among treatments.

The second $^{45}$Ca experiment was performed using *Hydropsyche betteni* collected from the Eno River in October 2009. Treatments were run both with and without ascorbate present (10 µM) in an attempt to correct for surface- versus tissue-bound Mn effects. Animals were pre-exposed to 500 µg Mn L$^{-1}$ for four days prior to $^{45}$Ca exposure. All exposure solutions were made with ASW and contained $^{45}$Ca (total activity = 0.1067 MBq L$^{-1}$). Co-exposure solutions contained 500 µg Mn L$^{-1}$. Exposures lasted 24 hours, after which the animals were processed as above. For this experiment, 0.2 mL glacial acetic acid was added to each vial with the scintillation cocktail.

The third calcium uptake experiment used *Isonychia spp.* collected from the Eno river in January 2010. Animals for the pre-exposed treatment were put into very soft water (VSW) with 100 µg Mn L$^{-1}$ for five days. During the $^{45}$Ca exposure, the control and pre-exposed animals were exposed to VSW with only $^{45}$Ca added (total activity = 0.1882 MBq L$^{-1}$). Co-exposure animals were exposed in VSW with $^{45}$Ca and 100 µg Mn L$^{-1}$, and there were treatments with and without ascorbate. After 29 hours of exposure, animals were removed, rinsed with EDTA and VSW, weighed, and put into individual vials. One mL of soluene was added to each vial. After digestion, 15 mL scintillation
cocktail was added to each vial and samples were assayed for radioactivity as described above.

The fourth $^{45}$Ca uptake experiment involved *Hydropsyche* collected from the Eno River in July 2010. All exposures took place in VSW with only half the concentration of calcium as VSW (0.873 mg L$^{-1}$). Forty animals were pre-exposed to 500 µg Mn L$^{-1}$ for 24 hours. After pre-exposure, animals were put into solutions with $^{45}$Ca (total activity = 0.1306 MBq L$^{-1}$). Samples ($n = 10$) from control, pre-exposed, and co-exposed treatments were taken at three, six, nine, and twelve hours. Insects from the 3-hour time point were rinsed with VSW, and animals from the 6-, 9-, and 12-hour time points were rinsed with EDTA and VSW. Insects were weighed and placed into individual vials. 1 mL soluene was added to each vial, and after digestion 18 mL of scintillation cocktail was added. Samples were assayed for radioactivity as described above.

Lastly, *Isonychia spp.* collected from Cataloochee Valley in July 2010 were pre-exposed to one of three concentrations of Mn (10, 100, or 1000 µg L$^{-1}$) or held in VSW for controls. After ~21 hours of pre-exposure, insects were rinsed with clean VSW and moved to solutions of VSW containing $^{45}$Ca as a radiotracer (radioactivity). After 4.5 hours of exposure, insects were removed and rinsed with clean VSW and individually placed in 3 mL of 0.5M EDTA. Animals were then removed, rinsed with DI water, and weighed. Insects were placed into clean individual scintillation vials, to which 1 mL soluene was added. After digestion, 18 mL of scintillation cocktail was added to each
vial and animals were assayed for radioactivity. Vial containing the EDTA rinses were also assayed for radioactivity after addition of 15 mL scintillation cocktail in order to determine the amount of calcium that was surface-sorbed to each animal.

**Respirometry**

For a pilot study to examine the effects of manganese exposure on respiration rates, *Hydropsyche betteni* were collected from the Eno River in September 2009. There were four treatments: control, 5, 50, and 500 µg Mn L⁻¹. Animals were put into 200 mL of the respective solutions with ⁵⁴Mn as a radiotracer (total activity = 0.1058 MBq L⁻¹), and volume of oxygen consumed was measured using N-Con Comput-Ox computerized respirometer (model 00-244 SC). After ~21 hours, animals were removed, assayed for radioactivity using a Wallac Wizard gamma counter, and weighed. This procedure was performed twice in order to achieve replication. Volume of oxygen consumed was corrected for total biomass in each treatment, and respiration rates were calculated.

A more refined study of respiration was performed using *Hydropsyche* collected from the Eno River in July 2010. Animals were pre-exposed to 0, 10, 100, or 1000 µg Mn L⁻¹ for 24 hours with ⁵⁴Mn as a radiotracer (total activity = 0.0648 MBq L⁻¹). After pre-exposure, animals were rinsed with VSW and placed in 200 mL of clean VSW. Oxygen consumption was measured for 24 hours as described above. After 24 hours, animals
were removed, assayed for radioactivity, and ascorbate rinsed. Insects were then recounted and weighed. This procedure was repeated three times in order to achieve replication.

Statistics

Graphs and statistical analysis were completed using GraphPad Prism (Version 5.0) software. Discrepancies from controls were determined using t-tests ($\alpha = 0.05$). Unless otherwise noted, all values are given as mean ± standard deviation.

Results

Calcium competition

Increased calcium concentration reduced total manganese accumulation in *Drunella cornutella*. Manganese body burdens decreased significantly from a mean (± standard deviation) of 25.98 ± 2.786 µg Mn g\(^{-1}\) in the low calcium treatment to 19.26 ± 1.804 and 17.49 ± 2.062 µg Mn g\(^{-1}\) in the medium and high calcium treatments, respectively (Figure 1). The difference in Mn accumulation between the medium and high calcium exposures was not significant, however.
Calcium uptake was significantly decreased in *C. sparna* pre- and co-exposed to 500 µg Mn L⁻¹. Newly acquired calcium, as measured by $^{45}$Ca uptake, decreased from 5.055 ± 1.778 µg Ca g⁻¹ in control animals to 3.344 ± 1.281 and 2.624 ± 0.512 µg Ca g⁻¹ in insects pre- and co-exposed to manganese, respectively (Figure 2). Although calcium uptake seemed to decrease from pre- to co-exposed treatments, the difference was not significant.

No significant trends were observed in calcium uptake in *Hydropsyche* with regard to manganese exposure. Presence and absence of ascorbate did not significantly change calcium uptake in any treatment (Figure 3).

In *Isonychia spp.*, there appears to be a decreasing trend in calcium accumulation from control animals to animals pre- and co-exposed to Mn (Figure 4). This trend, however, is not significant. Presence of ascorbate in co-exposure solutions did not significantly alter calcium body burdens after exposure.

Calcium uptake rates were calculated for *Hydropsyche* exposed to $^{45}$Ca without, after, or during manganese exposure. Uptake was not significantly different among treatments (Figure 5). Rates were 0.680, 0.745, and 1.815 µg Ca g⁻¹ day⁻¹ for control, pre-exposed, and co-exposed, respectively. Body burdens of newly acquired calcium were also not significantly different at any time point (Figure 6). Note that the body burdens at
the three hour time point are significantly higher than the other time points; this is because the animals at the three hour time point were not rinsed with EDTA to remove surface-bound calcium (Figure 6a).

The amount of calcium that was taken up during the final $^{45}$Ca experiment was not significantly different among treatment levels and ranged from (mean ± standard deviation) 1.734 ± 1.47 µg Ca g$^{-1}$ in the highest Mn treatment to 3.353 ± 2.579 µg Ca g$^{-1}$ in the control group (Figure 7a). While the data may suggest a slight downward trend as Mn concentration increases, the error is too large. It also seems that the amount of Ca adsorbed to the insects’ body surfaces decreased with increased Mn concentration, but this trend was not significant (Figure 7b).

Respirometry

No significant trends were observed in respiration rates of $H. betteni$ exposed to various concentrations of manganese. Mean respiration rates for the pilot study ranged from 0.1218 mL O$_2$ g$^{-1}$ hr$^{-1}$ in the 50 µg Mn L$^{-1}$ treatment to 0.1669 mL O$_2$ g$^{-1}$ hr$^{-1}$ in the control treatments. When comparing rates as proportion of control, it seems that there may be a downward trend which is reversed at high concentrations, but this trend is not significant (Figure 8). It should be noted that rates were calculated based on oxygen consumption between 6 and 21 hours of measurement.
In the subsequent experiment, *Hydropsyche* exposed to 10 and 100 µg Mn L\(^{-1}\) had significantly reduced respiration rates compared to control animals (Figure 8). However, differences in respiration rates between control insects and those exposed to 1000 µg Mn L\(^{-1}\) were not significant (Figure 9). Mean respiration rates during this experiment ranged from 0.1176 mL O\(_2\) g\(^{-1}\) hr\(^{-1}\) in the 10 µg Mn L\(^{-1}\) treatment to 0.1695 mL O\(_2\) g\(^{-1}\) hr\(^{-1}\) in the control treatment.

**Discussion**

Our data show that increased ambient calcium concentration decreased total manganese accumulation in exposed insects. These results were not unexpected, as it has been hypothesized that manganese ion transport may occur through channels meant for other divalent cations such as Ca\(^{2+}\) [6, 7]. Also, these results make sense in the context of previous studies that determined that acute dissolved Mn toxicity decreases as water hardness increases [3 - 5]. If animals accumulate less Mn when higher calcium concentrations are present, they may be somewhat protected from Mn toxicity.

I then examined the possibility that the presence of manganese may interfere with ionoregulation by measuring calcium uptake after and during manganese exposure. The first experiment showed a significant decrease in Ca uptake, however the results were not able to be reproduced in subsequent experiments. The data hints that manganese exposure
may decrease calcium uptake in two ways. First, dissolved Mn\textsuperscript{2+} may compete for uptake at calcium channels so animals may not absorb calcium as easily, as observed by the decrease in Ca uptake in the Mn co-exposure treatments. Second, manganese oxide coatings on the outside of the insects may form a physical barrier over the calcium transport sites, decreasing their contact with ions in the water, evidenced by the decrease in Ca uptake after Mn pre-exposure. These concepts warrant further investigation.

These manganese oxide coatings may also inhibit gas exchange at the gills of aquatic insects, affecting respiration. I measured oxygen consumption in insects during and after manganese exposure to determine if there was an effect. While results were varied and not always significant, the data suggest that exposure to manganese may decrease respiration rates. However, there seems to be a slight recovery at high concentration of manganese exposure. This increase may be due to intensified stress because of higher Mn body burdens. Stressed insects have been shown to have higher oxygen demands than healthy animals would normally require [8]. Again, this is a topic that deserves further study.
Figure 1. Mean Mn accumulation in *D. cornutella* under three calcium concentrations. Body burdens are given as µg Mn g⁻¹ wet weight ± standard deviation.
Figure 2. Newly acquired calcium body burdens in *C. sparna* unexposed, pre-exposed, and co-exposed to Mn. Values given are mean ± standard deviation.
Figure 3. Newly acquired calcium body burdens in *Hydropsyche* unexposed, pre-exposed, and co-exposed to Mn with and without ascorbate. Each bar represents the mean of three sets of replicates and error bars indicate standard deviation.
Figure 4. Newly acquired calcium in *Isonychia spp.* unexposed, pre-exposed, and co-exposed to Mn. Black bars represent treatments without ascorbate, and the gray bar represents the presence of ascorbate. Values given as mean ± standard deviation.
Figure 5. Calcium uptake over time in *Hydropsyche* unexposed, pre-exposed, and co-exposed to Mn. The solid line represents controls ($r^2 = 0.01075$), dashed line indicates pre-exposed ($r^2 = 0.06429$), and dotted line indicated co-exposed ($r^2 = 0.1275$). Each data point represents the mean ± standard deviation of 10 animals at each timepoint for each treatment. Slopes of each line represent uptake rate.
Figure 6. Newly acquired calcium body burdens of *Hydropsyche* unexposed, pre-exposed, and co-exposed to Mn. a) Body burdens at the three hour time point; b) body burdens at the six hour time point; c) body burdens at the nine hour time point; d) body burdens at the twelve hour time point. Values given as mean ± standard deviation.
Figure 7. Newly acquired calcium body burdens of *Isonychia* spp. pre-exposed to various concentrations of Mn. a) Ca body burdens of insects after EDTA rinse. b) Ca burdens able to be rinsed with EDTA (adsorbed).
Figure 8. Mean respiration rates, as proportion of control rates, of manganese exposed *H. betteni*. Bars represent mean ± standard deviation of two replicates for each treatment.
Figure 9. Mean respiration rates, as proportion of control rates, of manganese exposed *Hydropsyche*. Bars represent mean ± standard deviation of three replicates for each treatment.
Literature Cited


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