FINAL REPORT

An Integrated Field and Laboratory Study of the Bioavailability of Metal Contaminants in Sediments

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Section I: Nicholas S. Fisher—Metal-biota interactions

Relating the sediment phase speciation of As, Cd and Cr with their bioavailability for the deposit-feeding polychaete *Nereis succinea*

Abstract
We studied the influence of sediment geochemistry on bioavailability of arsenic, cadmium and chromium in deposit-feeding polychaetes. Metal phase speciation in sediments was determined with a sequential extraction scheme, and assimilation efficiencies (AEs) of ingested metals were determined by pulse-chase feeding experiments using γ-emitting isotopes. Worms were fed sediments collected from geochemically diverse estuaries that were labeled by sorbing dissolved radiotracers or mixing with radiolabeled algae. Uptake of sediment-bound metals was compared to that from labeled algae or goethite. Metal AEs showed a positive relationship with the exchangeable and carbonate sedimentary fractions, while metals in iron and manganese oxides and acid volatile sulfides, or in pyrite and other refractory material were inversely correlated with AEs. Arsenic was most bioavailable from algae (72%), less from sediments mixed with algae (24 -70%) and least from sediments labeled directly (1 – 12%). Arsenic AEs in sediments labeled directly showed a positive correlation with sedimentary Mn and Al and negative correlation with Fe. Cadmium AEs were positively correlated with salinity and negatively correlated with sedimentary organic carbon. AEs of Cr from sediments or algae were < 5%, but 34% from pure goethite. By quantifying the relationship of metal speciation in sediments with their bioavailability for deposit-feeding polychaetes, the present study provides new insight into understanding metal bioaccumulation in benthic invertebrates.

Introduction

Elevated concentrations of trace metals are presently found associated with sediments on the floors of rivers and their estuaries, often accumulated as historical pulses several centimeters below the surface. Data on total sediment concentrations of specific metals are valuable, but by themselves may be of limited use in evaluating the risks associated with this contamination. Concern over elevated metal concentrations stems primarily from the risks these contaminants can pose for living organisms (including people who might consume contaminated seafood). Because organisms must first accumulate metals before any toxic effect can be manifested, it is necessary to assess the extent to which the metals bound to sediments are accumulated in benthic animals. Contaminated sediments may be a dominant source of metals for benthic animals (Langston and Spence, 1995a) if the contaminants are in a form that can be accumulated into biological tissue. The non bioaccessible contaminants irreversibly bound to sediment (Semple et al., 2004), are not assimilated into biota and should have little ecological impact. The extent to which sediments can serve as sources of contaminants for marine organisms and the mechanisms responsible remain under-studied for most sediment types and organisms. Complicating factors include metal partitioning into various sedimentary mineral and organic phases that may affect the uptake and assimilation of the metals by benthic organisms. Metals associate with these phases through surface adsorption, co-precipitation during diagenetic formation or actual incorporation such as covalent bonding in organic matter or precipitation into insoluble metal sulfides.

The chemical speciation of a metal dissolved in water and its solid phase speciation in sediment both can influence its bioavailability (Campbell, 1995; Tessier and Campbell, 1988). In this respect, the total concentration of a sedimentary metal could have little relevance to its

bioavailability, just as is the case with dissolved metals. The bioavailability of different metals in contaminated sediments is likely to be a function of a metal's characteristics - for example charge, ionic radius, and oxidation state, its phase speciation in the sediment, and the physiological and ecological characteristics of the organism inhabiting the sediment (Griscom and Fisher, 2004; Lee et al., 2000b; Luoma and Bryan, 1982; Tessier et al., 1993). Consistent with another study that related phase speciation with bioavailability, metals bound to more refractory fractions such as pyrite or part of the sediment matrix itself would be expected to be less available for animals than metals loosely bound to sediment particle surfaces in exchangeable and carbonate fractions (Griscom et al., 2000).

Bioavailability of sedimentary metals has been assessed in light of abiotic factors such as ratios of simultaneously extracted metals (SEM) to acid volatile sulfides (AVS) (Ankley et al., 1994; Lee et al., 2000b), metal partition coefficients (Kd) - often related to sediment grain size and organic matter content (Griscom et al., 2000), pore water metal concentrations (Ankley et al., 1994), and geochemical heterogeneity in an animal's immediate microhabitat (Lee et al., 2000a). Biological factors that influence the assimilation efficiency (AE) of ingested metal, a key parameter in kinetic (or biodynamic) bioaccumulation models (Wang et al., 1996), include ingestion rate (Ahrens et al., 2001a; Wang and Fisher, 1999a), gut passage time (Griscom et al., 2000), and the gut environment (Chen and Mayer, 1998; Chen et al., 2000b; Griscom et al., 2002a), which determines the amount of metal that can be freed from particles into the gut fluid. For example, gut surfactants help extract organic matter coating mineral particles in the sediment (Ahrens et al., 2001b) and anoxia can help reduce iron oxides, thereby releasing metals bound to them (Griscom et al., 2002a)

The assimilation of metals from contaminated sediment in deposit-feeding invertebrates such as polychaetes can effectively transfer the metal from abiotic substrate to living tissue, which can subsequently be transferred to their predators. To provide a better mechanistic understanding of metal transfer into benthic food chains from sediments, the present study involved a series of experiments that used gamma-emitting radioisotopes to evaluate the bioavailability to deposit-feeding polychaetes of sedimentary As, Cd and Cr – all of which often display high concentrations in industrialized estuaries, sometimes reaching concentrations that are toxic to resident organisms (Langston and Spence, 1995a). These three metals have contrasting biochemical associations in living organisms, contrasting geochemical associations in sediments, and divergent particle-reactivities and residence times in aquatic systems (Fisher and Reinfelder, 1995a).While each of these metals is an environmental contaminant of concern in its own right, simultaneously studying their behaviors can reveal the behaviors of other metals with similar divergent characteristics. Further, As and Cr exist as oxyanions in solution but also have multiple oxidation states that vary with the oxygen content of the water and sediments. In oxic sediments arsenate is known to bind with Fe and Mn oxides, and with pyrite in reduced sediments (Huerta-Diaz and Morse, 1990). Chromium III and VI both associate with organic matter, although Cr III is far more particle-reactive. In contrast to As and Cr, Cd is a non-redox cation in water that is strongly chloro-complexed (Bruland, 1983). Each of these metals shows distinct patterns of mineral/organic association with particulate matter and distinct biological behavior.

Deposit-feeding polychaetes ingest large amount of sediments (Wang et al., 1999a), whose nutritional value is typically low in comparison to algae or bacteria but which can vary seasonally with productivity in overlying water. Metals associated with sinking biogenic debris

can eventually associate with various geochemical fractions in the sediments. Thus, metals can loosely associate with the surfaces in the exchangeable or carbonate phases, bind to or precipitate with Fe/Mn oxides, bind to less labile organic matter or iron sulfides including AVS and pyrite in anoxic sediments. Typically only a small fraction of sedimentary metals loosely associates with particles (e.g. exchangeable) and metals that are associated with oxygen or pH sensitive phases can be released into the solution when these conditions shift.

The assimilation efficiencies (AEs) of ingested As, Cd, and Cr from surface sediments collected from two sites in the Chesapeake and one in San Francisco Bay in deposit-feeding polychaetes was compared with their AEs from the same sediments amended with organic matter from algal debris, pure algal detritus, and from goethite. These AEs were regressed against the geochemical fraction patterns of these metals in the sediments. The three study sites were chosen based on differences in their sediment composition (organic carbon content, degree of metal contamination, S content, etc.).

Pulse-chase feeding experiments using radiotracers have been used to determine metal AEs and efflux rates out of animals. These parameters are components of a metal bioaccumulation model that can evaluate the relative importance of dietary and aqueous sources of metals for aquatic animals and enable predictions to be made of steady-state metal concentrations in animal tissues in different geographic locations (Wang et al., 1996). Modeling has shown that many metals are found to be accumulated significantly from diet in diverse invertebrates (Wang and Fisher, 1999a). The application of gamma-emitting radioisotopes in bioaccumulation experiments provides the advantage that environmentally realistic metal concentrations can be used and analyses are rapid, accurate and precise. Previous studies have evaluated AEs of metals bound to different types of sediment in diverse marine invertebrates

(Gagnon and Fisher, 1997; Griscom and Fisher, 2004; Griscom et al., 2002a; Griscom et al., 2000) but most earlier studies did not relate AEs with metal fractionation patterns in sediments.

Materials and methods

Choice, collection and handling of the test organism

The present study used the surface deposit-feeding polychaete, *Nereis succinea*, ubiquitous in muddy sediments along the US coastline. This species, whose ecology and physiology are well described, was used previously in experiments that studied contaminant bioaccumulation (Ahrens et al., 2001a). Worms of similar size (~10 cm) were hand collected from a local salt marsh at Flax Pond, from early spring to late fall. Individuals were placed with a small portion of sediment in separate containers to avoid cannibalism, because although *N. succinea* feeds on surface sediment, it is predatory given the opportunity. Animals were transported to the lab, rinsed with Flax Pond water and placed in clean containers with Flax Pond water (salinity 28) and ~0.5 g (wet weight) of Flax Pond sediment. Prior to each feeding experiment ~20 individuals that were regularly producing fecal pellets were selected and further acclimated for 2 to 7 days (depending on water salinity) to the experimental conditions. The final selection of worms (n=5-8 individuals) which were fed radiolabeled sediment was based on the presence of feces in their chambers.

Pulse-chase feeding experiments

Feeding experiments were conducted at 21 °C by placing individual worms in feeding chambers that consisted of two small (φ =5 cm**)** plastic Petri dishes connected by tygon tubing

(length =15 cm; φ = 4 mm). Each chamber was filled with water and ~0.5 g of radiolabeled sediment was placed at the "head" end of the tube; fecal pellets were collected from the other end of the tube (Wang et al., 1999a). After worms were placed in the chambers, water was periodically changed or filled to the top and feces were removed.

Study sites

Sediments and water used for feeding experiments were collected from three locations- two sites in Chesapeake Bay and one in San Francisco Bay. They were Baltimore Harbor (BH; 39°12'.25"N, 76°31'40"W) [Baltimore, MD], Elizabeth River (ER; 76°20' 09" W, 36°52' 32" N) [Norfolk, VA] and Mare Island (MI; 38°04'23" N, 122°14'91" W) [Vallejo, CA in San Francisco Bay]. Sediments from Norfolk were collected in May, BH in June, and MI in October. After collection they were stored at 4°C in plastic containers for up to two years prior to experimentation, where deeper layers of the sediments stored in the bucket were anoxic. Sediment location choices were based on differences in their geochemical properties and extent of metal contamination. For example, BH sediments had the highest content of organic carbon and nitrogen (5 and 0.33%) compared to ER (2 and 0.12%) and MI (1.5 and 0.12%), but lowest salinity (8.5) compared to ER (19.5) and MI (23). The greatest degree of contamination with Cr and As was in BH (Cr: 322.6 and As: 47.19 µg g^{-1} dry wt; ER- Cr: 33.3 and As: 6.37 µg g^{-1} dry wt, and MI: Cr: 47.4 and As: 1.43 µg g^{-1} dry wt), and with Cd in MI (2.39 µg g^{-1} dry wt; BH: 0.96 and ER: 0.46 µg g^{-1} dry wt) (Z. Baumann et al. in preparation).

Chemical analyses

Measurements of organic carbon, organic nitrogen, and sulfur (CNS) were conducted using a Carlo Erba 1500 Elemental Analyzer on sediment samples that were dried (50 °C), ground, and sieved through a 150 μm nylon mesh screen (Cutter and Radford-Knoery, 1993). Such prepared sediments were also used for metal concentration analysis. Sediment subsamples were digested in two steps with trace metal-grade concentrated nitric acid and then concentrated perchloric acid in a boiling bath. All of the sediment manipulations were conducted in a clean lab and under a high efficiency particulate air laminar flow bench. Sediment digest solutions were analyzed using ICP-MS (Finnigan Element 2).

Food types and radiolabeling

Feeding experiments considered 4 different foods, each uniformly radiolabeled with gamma-emitting isotopes, either ^{73}As (V) (half-life = 80.3d) or a combination of ^{109}Cd (half-life = 461.4d) and ^{51}Cr (III) (half-life =27.7d): (1) unamended sediment from the upper 10 cm at each site, mixed thoroughly immediately prior to radioisotope additions, (2) sediment to which radiolabeled diatom debris was added and mixed in, (3) pure diatom debris, and (4) pure goethite purchased from Sigma-Aldrich (goethite ~35% Fe; EC No. 2437464). Radioisotopes were added as arsenate for ^{73}As, cadmium chloride dissolved in 0.1 M HCl and chromic chloride dissolved in 0.5 M HCl.

Unamended sediments were radiolabeled by a direct addition via pipette of radioisotope dissolved in dilute HCl; microliter quantities of dilute NaOH were immediately added to the unamended sediment so that the pH was not affected by isotope addition. Amended sediments received radioisotopes by mixing 22.3 ± 4.5 mg (dry wt) of previously radiolabeled diatoms (*Thalassiosira pseudonana*, clone 3H) with ~2 g (wet wt) of sediment. The ratio of dry to wet

weight for all the sediments ranged between 0.7 and 0.5 as determined by Baumann et al. (in preparation). Both types of sediments were aged at 21 °C prior to the feeding experiment for 2 or 30 days to assess the influence of sediment aging on metal partitioning and bioavailability over this time period. Given the radioactive half-lives of the isotopes, it was not practical to conduct experiments involving bioavaiability of all three isotopes for longer time periods.

Radiolabeled *T. pseudonana* was produced as described previously (Wang et al., 1996). Because the assimilation efficiencies (AEs) of ingested metals in marine invertebrate herbivores from phytoplankton diets reflects the cytoplasmic distribution of metals in the algal cells (Fisher and Reinfelder, 1995a; Reinfelder and Fisher, 1991), the cellular distribution of each metal in aliquots of the diatom cells was determined by differential centrifugation of algal components after cells were broken (Reinfelder and Fisher, 1991). Most radioactive diatom cells were harvested for mixing with the sediments to feeding to the worms. Radioactive diatoms were first harvested by filtration on 1 μm polycarbonate membranes, then resuspended in 10 ml of seawater and centrifuged at 840 *g* for 5 minutes; the resulting radioactive pellet containing the cells was mixed thoroughly with the sediments or fed without sediment to the worms. Goethite (2.5 g dry wt) powder was radiolabeled after suspension in 2 ml of seawater. Radioactive goethite upon thorough mixing was briefly centrifuged to remove excess seawater and then goethite pellets were fed to *N. succinea*. For sediments which received direct addition of dissolved radioisotopes a small amount of dilute sodium hydroxide was added to neutralize the acid associated with radioisotope additions (dissolved in dilute HCl). The radioactivity and the added metal concentration in each food, determined using the specific activity of each added radioisotope, are given in Table 1. The radioisotope additions contributed only a small fraction of the measured background concentrations of these metals in surface sediment (<<1%) (Z. Baumann et al. in

preparation). Carbon additions in the form of added algal debris were 8% of background organic matter in the BH sediment, 19% in ER sediment, and 27% in MI sediment.

Measurement of radioactivity

Radioactivity of worms was measured following 1-6 h of feeding, depending on the presence of feces in the chamber. Worms were removed from their feeding chambers and rinsed 3 times with filtered seawater and twice with an Ethylenediaminetetraacetic acid (EDTA) solution in seawater [10^{-4} M] to remove adsorbed metal and adhering particles. To assay their radioactivity, individual worms were placed into 50 ml plastic containers, filled with a small amount of water to assure their position on the bottom of the container, and inserted into a well-type NaI(Tl) gamma detector. Counting times were typically 5 min, yielding propagated counting errors that were typically < 5%. Radioactivity of ^{73}As was detected at 53 keV, of ^{109}Cd at 88 keV, and of ^{51}Cr 256 keV. This kind of radioactive counting is non-destructive, so individual worms could be counted repeatedly over different time periods. For all radiolabeled worms, after counting, individuals were returned to their feeding chambers which were filled with new water and nonradioactive sediment that they could ingest to purge their guts of unassimilated radioactive material. After determining the radioactivity of worms following their radioactive feeding, the retention of the radioisotopes post feeding on nonradioactive food over time in the individual worms was determined to quantify the AEs of the ingested metals for each treatment, as described in Wang et al. (Wang and Fisher, 1999a).

Sequential extraction procedure

Sedimentary metal phase speciation was evaluated using a modified scheme originally described by Tessier et al. (Tessier et al., 1979) and Huerta-Diaz and Morse (Huerta-Diaz and Morse, 1990). We determined 7 sedimentary fractions, nominally identified as: exchangeable, carbonate, acid volatile sulfides (AVS), Fe/Mn oxides, two organic pools, and pyrite. It is important to note that these fractions were determined chemically and names assigned to each of them are strictly operational; details are given elsewhere (Z. Baumann et al. in preparation). Briefly, metals in the exchangeable pool were extracted for 1 h by 1M $MgCl_2$, in carbonate for 1 h by sodium acetate solution at pH =5, for 0.5 h in AVS by 0.5 M HCl. Sediments were incubated for 6 h in a hot reducing solution of hydroxylamine to dissolve the Fe/Mn oxides and metals associated with them. Metals in the organic fractions were extracted first for 8 h by a hot 1N NaOH solution and later concentrated H_2SO_4 for 6 h. Metals remaining in sediments, thought to associate with pyrite, were extracted for 2 h with 11 M HNO_3. After each extraction step the extractant was separated from the sediment by centrifugation at 834 *g* for 10 min and transferred to a separate container. This extract was then analyzed for the amount of radioactivity due to each of the elements. After the final extraction there was a small “residual” fraction of radioisotopes remaining in sediment.

The geochemical fractionation of As, Cd, and Cr in operationally defined sedimentary fractions (Z. Baumann et al. in preparation) was related to AE values for all metals, sediment locations, label types and ages.

Results

Assimilation efficiencies

Individual worms feeding on radiolabeled sediments ingested at least 1 mg wet wt of sediment, corresponding to 1 - 32 Bq of ^{73}As (or 0.02 - 0.53 fmol of As), 4-169 Bq of ^{109}Cd (or 0.38 – 16.17 fmol of Cd) and 2 - 87 Bq (or 0.01 – 0.50 fmol of Cr). In nearly all cases, there was sufficient radioactivity in each worm to measure the depuration rates of radioisotopes from individual worms over time. Assimilation efficiencies of ingested metals, determined by analyzing the loss patterns of metal during depuration, were determined for all experimental treatments; Figure 1 shows representative results of pulse-chase feeding experiments in which the retention of initially ingested radioactivity in *N. succinea* over time after feeding on radioactive food is given for ^{73}As, ^{109}Cd, and ^{51}Cr. Typically a two-phase loss pattern is observed, a sharp decline within 24 h reflecting loss of unassimilated metal via defecation, followed by a much slower loss, representing physiological turnover of assimilated metal (Wang and Fisher, 1999a). It is evident that *N. succinea* generally retained more As and Cd than Cr, resulting in correspondingly higher AEs for As and Cd (Table 2). Assimilation efficiencies of Cr in most cases were very low (<5%) except when worms were fed pure radiolabeled goethite, yielding AEs of about 34% (Table 2). There were significant differences in AEs for metals that were labeled via direct addition of radioisotopes to the sediment or via addition of radioactive algal debris to the sediment. The AEs of As were much higher for all sites when worms fed on sediments with added algal debris than on unamended sediment (Table 2). These differences were between sediments from BH and MI that were labeled with ^{109}Cd aged for 2 days (one-way ANOVA: $p < 0.01$), and between 2 and 30-day old MI sediments labeled with Cr (one-way ANOVA: $p < 0.05$). Significant differences in metal AEs were noted for As between all sample sites and for Cd and Cr between some sites (one-way ANOVA: $p < 0.01$; Cd: BH vs. ER and BH vs. MI, Cr: BH vs. ER and ER vs. MI). Generally, AEs of As and Cr decreased (one-way

ANOVA: $p < 0.01$) from sediment with aging, regardless of whether or not the sediments were amended with diatom debris.

Sub-cellular distribution of metals in *Thalassiosira pseudonana*

The cellular distribution of As, Cd and Cr in the radiolabeled diatoms indicated that 83% of Cr, 55% of As, and 43% of Cd were bound to cell surfaces (Table 3). AEs of As in *N. succinea* that fed on pure algal debris were much higher than when fed goethite (72 ± 3 *vs.* 2.5% ± 0.7; Table 2). The opposite trend was apparent for Cr (2.8% ± 1.6 *vs.* 34 ± 6; Table 2). For Cd, the method of labeling the sediment had no significant effect on AEs (23% ± 20 *vs.* 24 ± 2).

Distribution of ^{73}As, ^{109}Cd and ^{51}Cr in sedimentary fractions

The geochemical fractionation patterns of the metals in the sediments that were fed to the worms are shown in Table 4. Arsenic was primarily in organic fractions, with smaller amounts in other fractions among which oxides dominated. Cadmium was primarily in the exchangeable fraction, but carbonate, oxide and, to a lesser extent, AVS fractions also contained this metal. In the sediments radiolabeled directly with Cr, this metal was predominantly in the AVS and oxide phases after 2 d incubation, but, unlike the other metals, the distribution of Cr changed significantly over time so that the oxide and AVS fractions were largely replaced by pyrite after 30 d.

Relationship of AEs with metal fractionation

The relationships of all metal AEs with their geochemical fractionation patterns in all sediments are given in Table 5, which presents regression slopes between AEs of all metals and

the per cent of metal in individual or combined geochemical fractions of all sediments. These relationships are also presented for all metals as a function of sediment with or without added algal debris, by each individual metal, or as a function of sediment age (Table 5). Statistical analyses in Table 5, performed on arcsine transformed data to normalize the distribution of the data (AEs and percentages in each geochemical fraction), using PASW 18 statistical software indicate that arsenic's association with the exchangeable pool alone or combined with carbonate had a significant positive slope (Table 5). Relationships between metal AEs and metal association with the exchangeable pool or when combined with the carbonate pool as a whole are positive for sediments from Norfolk and Mare Island but not for Baltimore Harbor, whereas metal AEs show a negative relationship with their association with the Fe/Mn oxide fraction for all sites (Table 5). For both 2 and 30-d aged sediments metal AEs in the exchangeable + carbonate fractions had a positive relationship and metals in oxides (Fe/Mn oxides + AVS) had a negative one (Table 5).

The significant (one-way ANOVA: $p < 0.05$) positive relationship of metal AEs with their fractionation in the exchangeable + carbonate sediment fractions (exchangeable and carbonates) for all metals and sample sites with unamended and amended sediments (both 2 and 30 d) is shown in Fig. 2. The significant (one-way ANOVA: $p < 0.05$) inverse relationship of metal AEs with their fractionation in oxides and AVS fractions in sediment labeled via algal detritus and aged for a month is shown in Fig. 3. Figure 4 summarizes the general pattern of slopes from regressions relating metal AEs and sediment fractionation for all metals, sampling sites, sediment labeling methods, and sediment age. This figure shows significant (linear regression: $p < 0.05$) positive relationships for exchangeable (fraction 1) and carbonate (fraction 2) fractions alone or combined (fraction 9) and significant (linear regression: $p < 0.05$) negative

relationships for Fe/Mn oxides (fraction 4) alone or when combined with AVS (11). Pyrite (fraction 7) and fulvic acid (fraction 6) phases also show a negative slope.

Discussion

The positive relationship of metal assimilation efficiencies with exchangeable + carbonate sediment fractions for As(V), Cd, and Cr(III) is consistent with reports that suggested that metals bound to these two sediment fractions can be more bioavailable for benthic invertebrates than other fractions (Tessier et al., 1984). Ligands in the gut fluid of deposit-feeders provide a site for metal ion exchange (Chen et al., 2000b), hence the choice of combining the two metal ion exchanging pools (e.g., exchangeable + carbonate fractions) was made. Generally, oxic sediments have more metal associated with these phases and therefore metals in oxic sediments would be expected to be more bioavailable than metals in anoxic sediment; exceptions have been noted however, such as for Cd, Cr, and Zn in the suspension-feeding mussel *Mytilus edulis* (Griscom et al., 2000). Metals bound to operationally identified phases of iron and manganese oxides, organic phases, and pyrite in sediments showed an inverse relation with assimilation efficiencies in *N. succinea* (Fig. 4), indicating that metals associated with these phases have low bioavailability for *N. succinea*. This polychaete is a surface deposit-feeder and hence feeds primarily on oxic sediment.

In comparing the behavior of metals in sediments from different sample sites, assimilation of As showed a negative relationship with total concentrations of Fe ($r^2 = 0.97$) and Mn ($r^2 = 0.99$) in sediment but a positive relationship with Al ($r^2 = 0.99$) in sediments from all three study sites (Fig. 5). It is widely accepted that in sediments arsenic shows a strong association with iron and manganese rich phases such as Fe/Mn oxides, AVS and pyrite

(Oremland and Stolz, 2003). Lower As AEs occurred for sediments with higher Fe and Mn concentrations (Fig. 5), further supporting the observations that Fe/Mn and AVS (Fig. 4) phases control the bioavailability of ingested As. In sediments, Al can be found in the mineral structure of aluminosilicates. Al's positive correlation to As AEs may be explained by a weak metal binding with the surfaces of aluminosilicates, which can more easily release metal ions into the gut fluid than Fe and Mn oxides (Lin and Puls, 2003). The particle reactivity of Cd is inversely related to chloride concentration (Muller, 1996) and as salinity increases Cd's retention by ingested particles would be expected to decrease, releasing more Cd into the gut where it could be eventually assimilated, consistent with observations shown in Fig. 5. The organic carbon content in sediments from our study sites showed an inverse relation to Cd AEs (Fig. 5). Degraded organic matter can bind metals and thereby limit their bioavailability. The negative relationship of Cd AEs with its association with extracted organic fractions was also significant (Table 5), further supporting this relationship. AE values for Cr were very low and variable, and relationships with sediment characteristics are more tentative.

Generally, these findings suggest that metals must be released from ingested particles into the gut fluid before they can be transported across the gut lining and become assimilated into tissues. As such, these findings support the contention of Mayer and colleagues that metal and organic contaminants must be released into gut fluid before they can be assimilated. Most of these earlier studies focused on release from particulates to the dissolved phase in gut fluid and did not assess their AEs, although some studies determined both the solubilization and absorption of some organic contaminants (Ahrens et al., 2001a; Weston and Mayer, 1998).

Assimilation efficiencies of As in *N. succinea* were highest when diet consisted of fresh algal debris or sediments amended with algal debris, suggesting that As, which is bound to some

labile sugars in algal cells (Andreae and Klumpp, 1979), is the bioavailable form and arsenic bound to sedimentary organic matter such as humic or fulvic acids is not assimilable in worms. Further, arsenobetaine, a common form of As in marine invertebrates (Larsen et al., 1993) is far more assimilable (42%) by crustacean predators, *Crangon crangon*, than inorganic As species – arsenate (1.2%; Hunter et al., 1998), consistent with our observations that inorganic As bound to goethite was much less assimilable (2.5%) by *N. succinea* than organic As in algal debris (72 %). Because sugars that contain As in cells are likely labile and degrade over time, the decreased assimilation of As over 30 days in sediment mixed with algal debris was expected as these sugars decompose over time. There are few other studies that determined AEs of As in marine invertebrates. AEs of As in another nereid worm *Nereis diversicolor* reached 62% (Waring and Maher, 2005), comparable to our results but much higher than AEs reported for the deposit-feeding polychaete *Arenicola marina* (4-11%; Casado-Martinez et al., 2009).

Studies by Griscom et al. (Griscom et al., 2000) and Baumann et al. (in preparation) illustrate the shift of metals from exchangeable + carbonate phases to more refractory phases over time which can possibly result from microbial degradation of labile organic matter or an increase in Fe/Mn oxide-metal associations after a month of sediment incubation (Z. Baumann et al. in preparation) . Labile organic compounds represent the most biologically available source of C, in contrast to more refractory sources, with 55-95% of C assimilated by *N. succinea* feeding on fresh algae compared to only 5-18% of reworked organic matter in sediment (Ahrens et al., 2001b). This difference between labile and refractory organic matter coincides with decreased AEs of As in *N. succinea* (the present study), Cd and Ag in the clam *Macoma balthica* (Griscom et al., 2000), Cd, Cr and Zn in the clam *Ruditapes philippinarum*, and Cd in the mussel *Perna viridis* (Chong and Wang, 2000), although Wang et al. (Wang and Fisher, 1999a) found that AEs

of physiologically regulated Co, Se and Zn were unaffected by metal-sediment exposure time. Other studies that examined bioavailability and toxicity of chemicals for worms in soils similarly showed a decrease in bioavailability with increased exposure time (Lanno et al., 2004).

With further sediment aging, ferric iron associated with iron oxides would be expected to be ultimately reduced and dissolve as ferrous iron. Ferrous iron upon reaction with dissolved sulfide species would precipitate as iron sulfides, contributing to the AVS phase. In anoxic sediments AVS can ultimately be transformed to pyrite. AVS and pyrite, when present in sediments, can bind metals and organic matter that are dissolved in pore water (Lee et al., 2000a). Metals bound to these phases are more tightly bound to particles and can only be released by strong acids (e.g., HCl and HNO_3), which are harsher than the digestive fluids of marine invertebrates.

Unlike As, the AEs of Cd and Cr did not consistently decline with sediment aging (Table 2) suggesting that bioavailability of these metals may not be tied directly to their association with labile organic C in sediments. This is further supported by the observation that AEs of Cd bound to pure goethite were comparable to those bound to pure algal debris. Presumably, Cd is released equally well into gut fluid from goethite and algal debris. Cd showed a wide range of AEs (from < 1% to nearly 69%), whereas others reported a narrower AE range for *N. succinea*, from 29 to 39% (Wang et al., 1999a). Bivalves appeared to assimilate similar amounts of Cd in comparison to worms. Filter-feeding mussels assimilated from 9.5 to 44.5% (Chong and Wang, 2000; Wang and Fisher, 1999a), and clams assimilated 31-51% of Cd (Chong and Wang, 2000; Griscom et al., 2002a). The estuarine crustacean *Palaemonetes pugio* (Wallace and Lopez, 1997) assimilated 57% of Cd from an oligochaete diet. The AE of Cd in mussels from pure mineral phases was lower than from algal cells (Wang et al., 1996).

As seen in many previous studies with diverse invertebrates, Cr displayed lower AEs than the other metals (Wang and Fisher, 1999a). Commonly, its AE from ingested food is < 10%, particularly for deposit-feeding animals (Lee and Luoma, 1998). In San Francisco Bay, the clam *Potamocorbula amurensis* feeding on particles rich in labile organic compounds collected after a spring bloom assimilated > 5% of Cr, compared with AEs of 1.7% during a non-bloom period (Lee and Luoma, 1998). It is noteworthy that mussels feeding on algae assimilate up to 10% of Cr(VI) but <2% of Cr(III), the difference being explainable by Cr(VI)'s greater ability to penetrate into the cytoplasm of the algal cells (Wang et al., 1997).

Analogous to their fractionation in sediments, metal fractionation in algal cells can significantly influence their assimilation in animals. Previous work has shown that AEs of ingested metals in marine herbivores are strongly correlated with the cytological distribution of the metals in algal cells, with AEs showing a nearly 1:1 relationship with cytoplasmic distribution of the metals in the algal cells that comprised the diet (Reinfelder and Fisher, 1991). The low AE of ingested Cr from pure algal debris (2.8%) coincides with its predominant association with diatom cell walls and membranes, in contrast with Cd and As, consistent with earlier findings. The AEs of algal As (69-76%) and Cd (up to 43%) are comparable ($p < 0.05$) to their extraction in the exchangeable fraction (Cd: 43%) alone or in the exchangeable + carbonate pool (As: 65%) within the algae. Nevertheless, although 18% of algal Cr was extracted in the exchangeable pool (Z. Baumann et al. in preparation), its assimilation in *N. succinea* was low (2.8%). Reasons for this discrepancy are not known, but it is possible that trivalent metals can not readily penetrate the gut lining. Cr also displayed low AEs (< 5%) from sediments (Table 2) and its association with exchangeable fractions in these sediments was correspondingly low (Z. Baumann et al. in preparation).

Thus, the bioavailability of As, Cd and Cr to the surface deposit-feeder *N. succinea* is positively related to their exchangeable + carbonate fraction in sediment and negatively related to their fractions in the AVS, Fe/Mn oxides and pyrite and non-extractable phases. We therefore suggest combining the exchangeable and carbonate pools into a "carbonex" pool recognizing that geochemical and physiological processes can positively impact sedimentary metal assimilation in deposit-feeding polychaetes through an ion-exchange process (ion exchange in both $MgCl_2$ and NaOAc extractions; presence of ligands in the gut fluid that can serve as ion exchange sites).

The present study did not accurately evaluate ingestion rates in *N. succinea* in these experiments. Ingestion rates can vary in deposit-feeding polychaetes depending on the particle being ingested (e.g., natural sediment vs. goethite). It is recognized that gut retention times can influence the AEs of ingested metals (Wang and Fisher, 1999a), and while this may possibly explain differences noted in AEs between metals bound to goethite and algal-supplemented sediment, both of these radiolabeled food sources were purged with the identical unlabeled sediment. It is therefore unlikely that the gut passage times of the various radioactive foods differed significantly and accounted for AE differences.

Worms assimilate more As when fed pure algae and less when algae are mixed with sediment. AEs of As from directly labeled sediment were lower than As AEs from ingested sediments mixed with algae, and were positively related to total Mn and Al concentrations in sediments but negatively related to sedimentary Fe. AEs of ingested sedimentary Cd increased with salinity and decreased with sedimentary organic carbon. The present study confirms that Cr has generally low bioavailability for deposit-feeding polychaetes. Further appreciation of metal assimilation in deposit-feeders will result from physiological and biochemical studies that also consider the sediment geochemistry.

Figures

Figure 1. Metals retained in *N. succinea* after feeding on a pulse of 2 and 30 day old radiolabeled sediment from Baltimore Harbor; data points indicate mean values of % metal retained at time; open circles indicate data for sediment mixed with radiolabeled algal detritus and solid circles indicate data for sediments labeled via direct injection of isotope. The asymmetric error bars indicate one standard deviation.

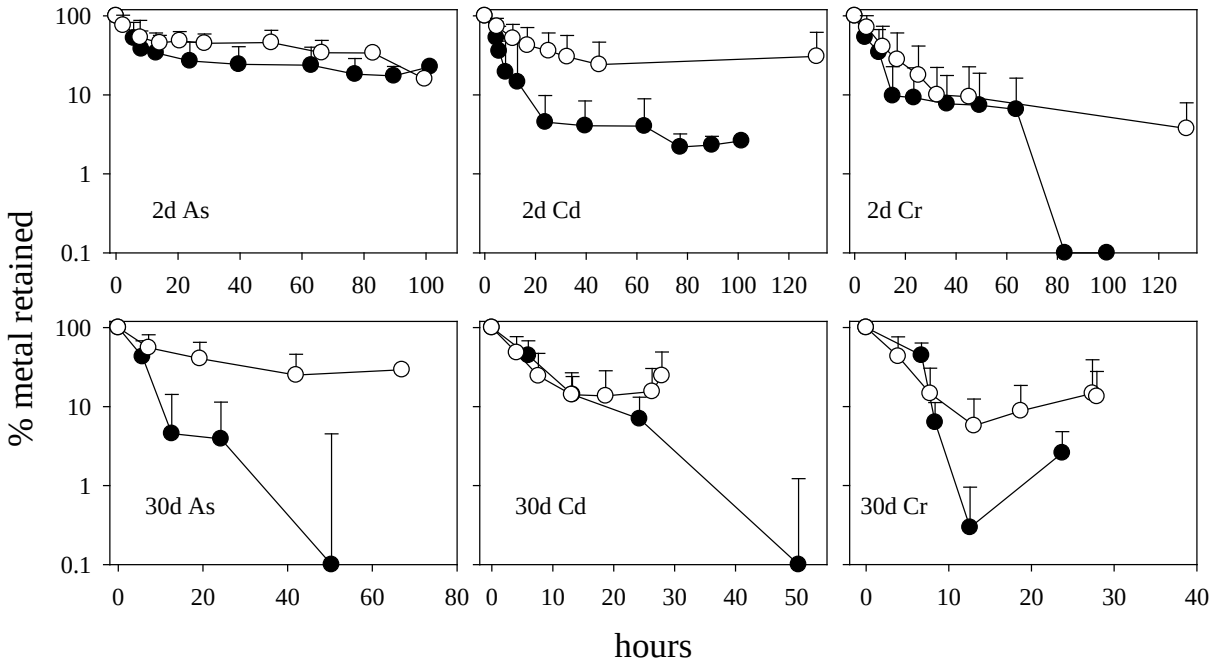


Figure 2. Significant regression ($p < 0.05$) between AE and concentration of metal (As - circle, Cd - triangle, and Cr - square) in exchangeable + carbonate fractions in sediments collected from Baltimore Harbor (BH), Elizabeth River (ER) and Mare Island (MI), which were labeled directly and via algal debris and incubated for 30 days. Dashed line indicates 95% confidence interval around the regression line (solid).

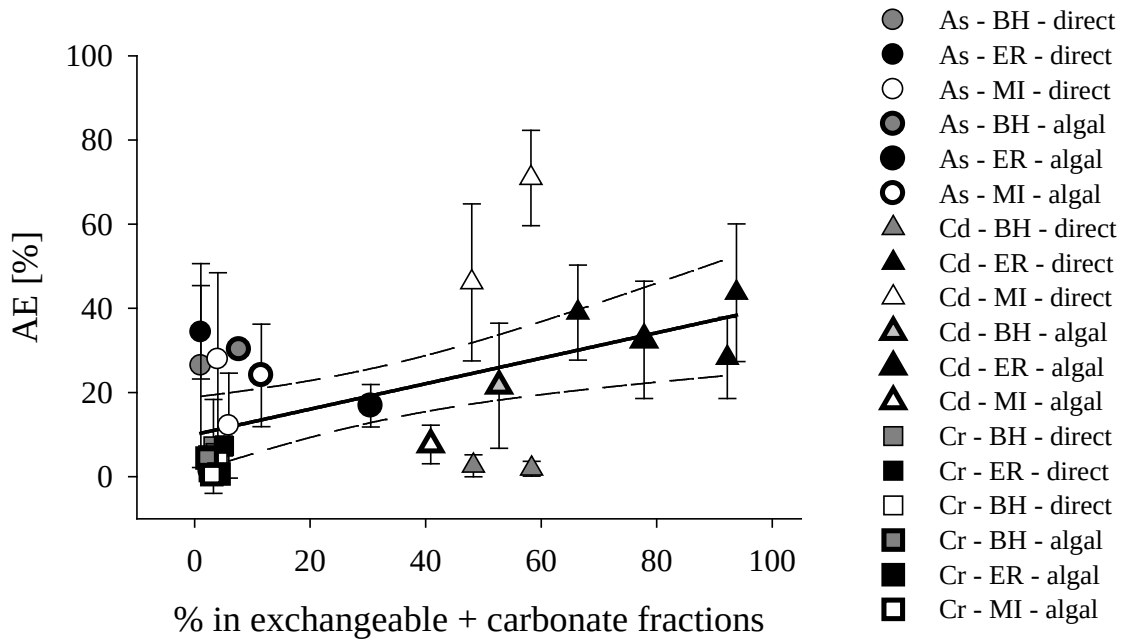


Figure 3. Significant regression ($p < 0.05$) between AE and concentration of metal (As, Cd, and Cr) in AVS + Fe/Mn oxides fractions in sediments collected from BH (gray), NV (black) and MI (white) and labeled via mixing with radiolabeled algal detritus. Dashed line indicates 95% confidence interval around the regression line (solid).

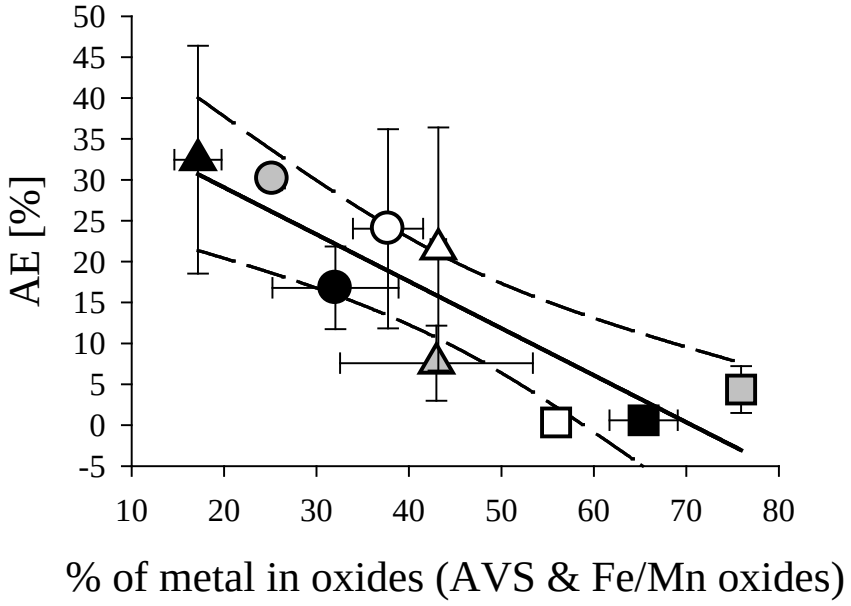


Figure 4. Regression slopes for AEs and % metal in geochemical fractions; regressions were applied to arcsine transformed data. Only slopes of statistically significant ($p < 0.05$) regressions are shown. Single pools: 1-exchangeable, 2-carbonate, 3-AVS, 4-Fe/Mn oxides, 5-humic acids, 6 fulvic acids, 7-pyrite, 8-residue; Pooled fractions: 9- exchangeable + carbonate, 10- exchangeable, carbonate + AVS, 11- AVS + Fe/Mn oxides, 12- humic + fulvic acids, 13- pyrite + residual.

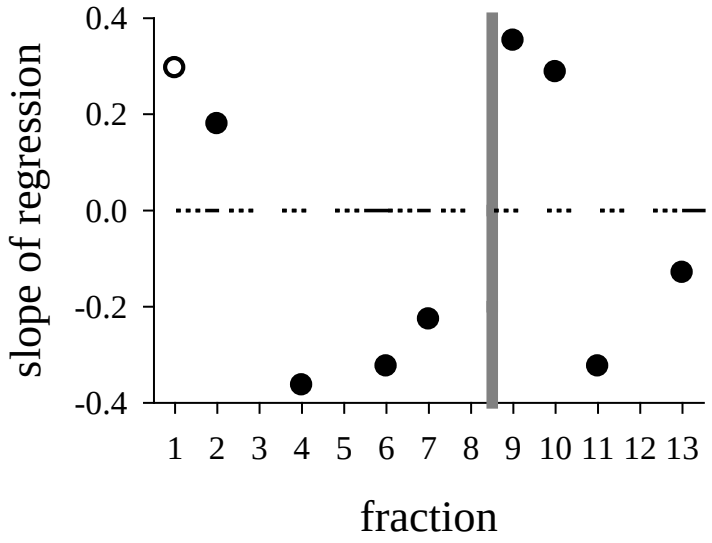
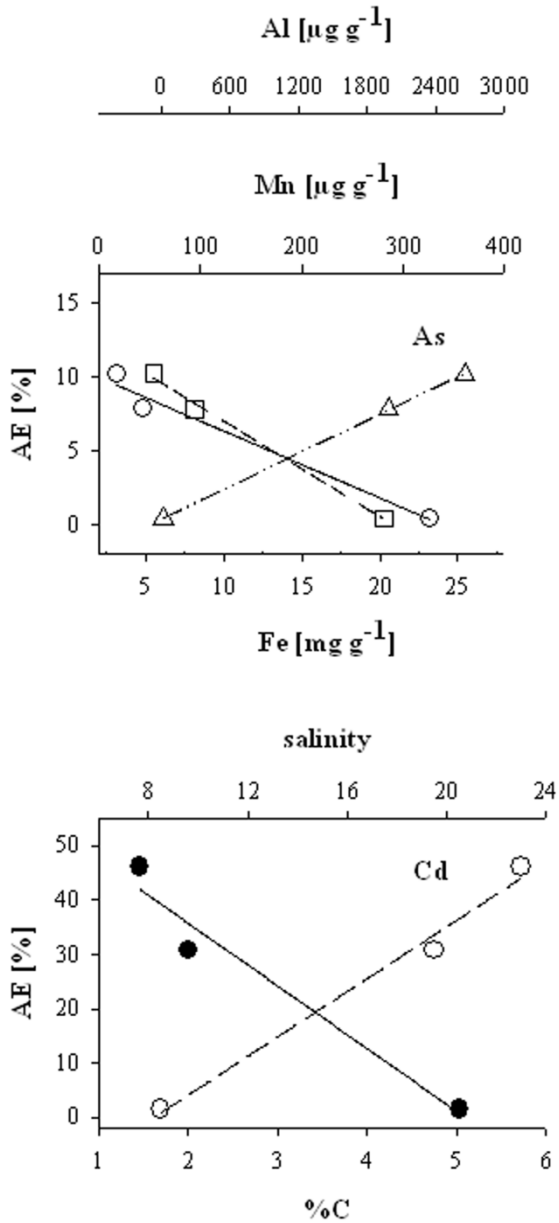


Figure 5. Significant ($p < 0.05$) relationships between As AEs and the total concentrations of aluminum (triangles), manganese (squares), iron (circles) in sediments, and Cd AEs and % C in sediments (black circles) and salinity of overlying water at sediment collection sites (open circles). AEs of As and Cd are for sediments labeled directly and aged for 2 days; AE errors are ignored for clarity of figure and are given in Table 2. *In situ* metal (Al, Mn and Fe) concentrations are from Baumann et al. (in prep). No other significant relationships were found for metal AEs and total elemental concentration in sediment.



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Modeling metal bioaccumulation in deposit-feeding polychaetes from labile sediment fractions and from pore water

ABSTRACT

Estuarine sediments are often highly enriched in particle-reactive metal contaminants and because aquatic animals have often been shown to acquire metals predominantly from their diet, benthic animals feeding on deposited or resuspended sediments may also accumulate metals through this uptake pathway. Laboratory experiments were performed in which the surface deposit-feeding polychaete, Nereis succinea, was exposed to As(+5), Cd, and Cr(+3) in pore water or in estuarine sediments with and without enrichment with algal debris. These experiments generated metal uptake parameters (assimilation efficiency of ingested metal [AE], uptake rate constant of dissolved metal, efflux rate constants following dietary or aqueous metal exposures) used in a kinetic model of metal bioaccumulation. The model showed that >97% of the body burden of these metals is accumulated through ingested sediment. The kinetic model was further modified to consider the geochemical fractionation of the metals in the sediments because metals bound to some fractions were shown to be unavailable to these polychaetes. The modified model substituted the AE term for each metal by the percentage of metal extracted in neutral and weak acid exchangeable fractions (termed "carbonex" fraction) multiplied by the slope of the regression between the metal AE and its fractionation in carbonex. The modified model generated predictions of As, Cd, and Cr body burdens in polychaetes at three different estuarine sites that matched independent field observations at these sites (r^2 = 0.84 for sediments without organic enrichment, r^2 = 0.87 with organic enrichment). Model predictions that relied on total metal concentrations showed weaker relationships (r^2 =0.11–0.50). This study adds to the evidence for the dominance of dietary uptake of metals in aquatic animals and identifies a key sedimentary fraction of metals that can account for bioavailability of sediment-bound metals.

1. Introduction

It has been widely recognized that coastal sediments can be greatly enriched in particle-reactive contaminants, including many metals and metalloids associated with industrial activities (Kennish, 1997). Considerable attention has been paid toward evaluating the extent to which sediment-bound metals are available for uptake by benthic organisms (Griscom and Fisher, 2004; Lee et al., 2000a; Tessier et al., 1984), but uncertainties remain (Luoma and Fisher, 1997). In particular, it has been shown that not all metals bound to sediment are in a bioavailable form. Thus, metal bioavailability is thought to be related to the composition of the sediment, the geochemical fractionation of each metal in sediments at each site of interest, and the feeding and digestive properties of the organisms of interest (Baumann and Fisher, 2011b; Griscom et al., 2002a; Tessier and Campbell, 1987; Tessier et al., 1984). Sediments are typically complex and metals bound to different sediment components can display very different bioavailability for deposit-feeding animals. Bioavailable components have been shown to include labile organic compounds (e.g., simple sugars or amino acids) (Mayer et al., 1995), and less bioavailable components may include some mineral fractions (Diks and Allen, 1983; Geiszinger et al., 2002).

Benthic animals can acquire metals from both water and diet, although for many animals diet has been shown to be the dominant pathway (Casado-Martinez et al., 2009; Croteau and Luoma, 2005; Wang et al., 1997). To help quantify the relative importance of each uptake pathway, metal bioaccumulation models have been used for both marine and freshwater organisms (Croteau and Luoma, 2005; Luoma and Rainbow, 2005a; Mathews and Fisher, 2009).The so-called biokinetic model or biodynamic model not only delineates the relative importance of each uptake pathway but also can be used to predict metal concentrations in animal tissues on a site-specific basis. Model predictions have generally been shown to closely

match independent field measurements, suggesting that this model accounts for the major processes governing metal concentrations in filter-feeding aquatic animals and that the kinetic parameters used in the model are applicable to field situations (Roditi et al., 2000b; Wang et al., 1996). These kinetic parameters are commonly assessed experimentally using radiotracers using protocols that are well suited for determining uptake and release rates of metals in animals (Fisher et al., 1996a; Wang and Fisher, 1999a).

As described in detail elsewhere (Wang et al., 1996), the model (eq. 1) describes a metal's concentration in an animal at steady state (C_{ss}) as a sum of uptake from the aqueous phase (Eq. 2) and from diet (Eq. 3), where k_u = the uptake rate constant from the aqueous phase, C_w = the concentration of metal in the dissolved phase, AE = the assimilation efficiency of ingested metal from food, IR = the ingestion rate, C_f = the metal concentration in food, $k_{ew\ slow}$ and k_{ef} = the loss rate constants of metal from the animal following uptake from water and food, respectively, and g = the growth rate constant of the animal (generally negligibly small compared to k_e values for most metals). The relative proportion of metal uptake from diet is described in Eq. 4.

$$C_{ss} = \frac{k_u}{k_{ewslow} + g} \times C_w + \frac{AE \times IR}{k_{ef} + g} \times C_f \qquad (1)$$

$$C_{ss,w} = \frac{k_u}{k_{ewslow} + g} \times C_w \qquad (2)$$

$$C_{ss,f} = \frac{AE \times IR}{k_{ef} + g} \times C_f \qquad (3)$$

$$\% \cdot dietary = \frac{C_{ss,f}}{C_{ss}} \quad (4)$$

Currently, there are no published kinetic model evaluations of metal bioaccumulation from contaminated sediments that incorporate metal phase speciation in sediments. This study therefore addresses modeling the bioaccumulation of sediment-bound metals for a deposit-feeding polychaete while considering the geochemical phase speciation of each metal. Three metals of environmental concern were considered: As(V), Cd, and Cr(III), each of which displays distinct chemical and biological behaviors (Greenwood and Earnshaw, 1984). In addition, three estuarine sites (two in the Chesapeake, Baltimore Harbor (BH) and Elizabeth River (ER), Norfolk Virginia, and one in San Francisco Bay, Mare Island (MI)) with distinct sediment characteristics were examined to compare the influence of different geochemical properties on metal bioavailability. The characteristics of the sediments at each site are described in detail in Baumann et al. (in review). Briefly, salinity was lowest at BH (8.5) and higher at the ER (19.5) and MI (23) sites; the dissolved organic carbon concentration in pore water was lowest at ER (10.2 mg C L^{-1}) and highest at MI and BH (29.5 and 33.0 mg C L^{-1}, respectively). Contents of organic carbon (BH-5.0%, ER-2.0%, and MI-1.5%), nitrogen (BH-0.3%, ER-0.1%, and MI-0.1%) and sulfur (BH-0.5%, ER-0.6%, and MI-0.4%) in the surface layer of sediments also varied among the sites. Iron levels varied from 3.2 mg g^{-1} dry wt in MI to 23.2 mg g^{-1} dry wt in ER sediments. Levels of As, Cd and Cr contamination in sediments also varied among study sites such that Cr and As were most enriched in BH sediments (As – 47.2 µg g^{-1} dry wt; Cr - 322.7 µg g^{-1} dry wt) and Cd was most enriched in MI (2.4 µg g^{-1} dry wt) sediments.

The modeling specifically assessed the influence of geochemical fractionation of sediment-bound metals on their bioavailability for benthic animals and quantified which uptake route contributes most to metal body burdens in polychaetes. This study builds on a recent study

that described the assimilation of these metals from these estuarine sites by the polychaete Nereis succinea (Baumann and Fisher, in press). In that study, it was shown that metals in the easily extractable sedimentary phases were also most assimilated in deposit-feeders.

In the present study, we describe experiments which determined metal accumulation in these polychaetes from pore water from each estuarine site and combine the kinetic parameters (k_u, k_{ew}. k_{ef}) from these experiments with AEs, and geochemical fractionation patterns of each metal and site from Baumann and Fisher (in press).

2. Materials and methods

2.1. Experimental organism

Experiments used surface deposit-feeding polychaetes Nereis succinea, commonly present in muddy sediments along eastern US coastline. The ecology and physiology of N. succinea are well described and this species is regarded as suitable for laboratory experiments determining contaminant bioaccumulation (Ahrens et al., 2001a; Wang et al., 1999a). To obtain experimental animals, individuals of similar body length (about 10 cm) were hand collected from a local salt marsh at Flax Pond on Long Island NY, from early spring to late fall and placed with a small portion of sediment in separate containers to avoid cannibalism. After transport to the lab all worms were rinsed with Flax Pond water (salinity 28) and placed in clean containers with Flax Pond water and ~0.5 g (wet wt) of Flax Pond sediment. Prior to each experiment, 20 individuals were selected and further acclimated to the experimental conditions for 2-7 d depending on water salinity, where longer acclimation periods were needed for greater salinity changes (e.g., Flax Pond to Baltimore Harbor water, from salinity 28 to 8.5). Only worms that regularly produced feces were chosen for feeding experiments. Worms designated for the

aqueous metal exposure experiments were further incubated in polycarbonate containers filled with seawater but without sediment so that their guts were emptied prior to exposure to the experimental water containing the metals. This ensured that fecal material would not scavenge the dissolved metals from the water during the metal uptake experiments.

2.2. Kinetic parameters

Parameters used in the kinetic model were generated in two series of experiments using a surface deposit-feeding polychaete, N. succinea, as a model species. Both series used a radiotracer approach that is well-suited for measuring, rapidly and accurately, the assimilation and retention of metals in marine animals while using environmentally realistic metal concentrations (Fisher, 1992). We used ^{73}As, ^{109}Cd and ^{51}Cr, with half-lives of 80.3, 461.4, and 27.7 d, respectively. These isotopes were added as arsenate (As +5), cadmium chloride and chromic chloride (Cr +3). Pulse-chase feeding experiments yielded data used to calculate assimilation efficiencies (AE) of ingested metals and efflux rate constants of the metals following dietary uptake (k_{ef})., The uptake rate constants (k_u) of metals from the aqueous phase and efflux rate constants ($k_{ew\ fast}$ and $k_{ew\ slow}$) following uptake from the aqueous phase were calculated from experiments in which N. succinea were exposed to radiolabeled pore water from each estuarine site. Efflux rate constants (k_{ef}, $k_{ew\ fast}$ and $k_{ew\ slow}$) were calculated as the slopes of % metal retained during the fast and slow turnover phases (Wang et al., 1999a). Aqueous exposure experiments consisted of two parts - uptake and efflux. Uptake rate constants (k_u) equaled the metal concentration accumulated per body mass per day divided by the concentration of metal in solution (Wang et al., 1996). Efflux rate constants from the worms, expressed in units of % d^{-1}, were calculated from regression analysis of % of metal retained in the worms over time.

We used the model described in Eq. 1 to determine steady-state total metal concentrations in N. succinea based on the experimentally determined kinetic parameters for each metal and each estuarine site. As noted above, this model accounts for metal accumulated from diet and water. In this study, the metal bioaccumulation model was modified to also consider the influence of sediment geochemistry on metal bioaccumulation in N. succinea. Using a sequential extraction scheme, we have shown that metal extracted in neutral ("exchangeable" extracted with $MgCl_2$) and weak acid extractable ("carbonate" extracted with sodium acetate) fractions of sediments (termed "carbonex" fraction when pooled together) has the strongest positive relationship with metal AE in this polychaete (Baumann and Fisher, in press). Therefore, this modification took into consideration the % of metal in the carbonex sedimentary fraction ($z_{carbonex}$) and the regression slope between the metal AEs and % of metal in a given sedimentary fraction ($b_{carbonex}$). For example, if 25% of total sediment-bound metal is in the carbonex fraction ($z_{carbonex}$) and the regression of percentages of metal in carbonex and AEs has a slope of 0.4 ($b_{carbonex} = 0.4$), then 10% of total metal in sediment is assumed to be assimilated.

$$C_{ss} = \frac{k_u}{k_{ewslow} + g} \times C_w + \frac{z_i \times b_i \times IR}{k_{ef} + g} \times C_f \qquad (5)$$

$$C_{ss,f} = \frac{z_i \times b_i \times IR}{k_{ef} + g} \times C_f \qquad (6)$$

The metal content in the carbonex pool included the metal associated with the operationally defined exchangeable and carbonate sediment fractions. To determine these fractions, exactly the same types of radiolabeled sediment that were used for the pulse-chase feeding experiments were subjected to sequential chemical extractions, as described in detail elsewhere (Baumann et al., in prep; Baumann and Fisher, 2011b). Briefly, sediment was first

incubated with 1M $MgCl_2$, after which the sediment was centrifuged and the supernatant containing the exchangeable portion of the radiotracer was transferred into another container. The remaining sediment was then incubated with sodium acetate solution at pH 5 to extract metals in the carbonate phase. The radioactivity in the extracted exchangeable and carbonate fractions were assessed by gamma-spectrometry.

2.3. Feeding experiments

For determinations of polychaete ingestion rates, we assumed that ingestion rate equaled defecation rate. Defecation rates, represented by units of dry mass of defecated sediment per dry mass of worm per time ($g\ g^{-1}\ d^{-1}$), were calculated based on the dry mass of feces that were periodically collected. Fecal pellets were filtered onto tared GFF filters, dried, and weighed. The lengths of the experimental worms were measured with electronic calipers. Individual worms and collected feces were dried over >24 h at 68 °C.

Equation 4 was used to determine the % of polychaete metal accumulated from diet. All kinetic parameters used in the modeling were calculated based on experiments presented here, except AEs and k_{ef} values which are taken from Baumann and Fisher (in press). A brief description of the pulse-chase feeding experiments is given here. Feeding experiments were conducted at 21 °C. Individual worms were placed into the feeding chambers constructed of tygon tubing (length =15 cm; φ = 4 mm) connecting two plastic Petri dishes (φ =5 cm), as described in Wang et al. (1999a). The tubing was to simulate the burrow in which these worms live in nature. Chambers were filled with seawater and about 0.5 g of radiolabeled sediment. Field collected sediments were radiolabeled by a direct addition of radiotracer dissolved in dilute acid to the sediment or by mixing the sediment with previously radiolabeled algae (*Thalassiosira pseudonana*) that were separated from water by filtration. Such labeled sediments were incubated

in plastic containers at 21°C for 2 and 30 days prior to the feeding experiment. Exact details of sediment labeling procedure are provided in Baumann and Fisher (in press). Polychaetes spent most of the time inside the tubes. Sediment was placed at the "head" end of the tube; fecal pellets were collected from the other end of the tube. When worms were first presented the radiolabeled sediment, they were given about 4 hours to feed on it and were then removed from their feeding chambers and placed inside the well detector of the gamma counter to determine the radioactivity in their bodies. Immediately after feeding on radiolabeled sediments, the radioactivity of the labeled polychaetes was determined (note this is non-destructive analysis) and the worms were replaced into individual containers and fed unlabeled sediment for up to 5 d. Their radioactivity was periodically determined over time and the retention of each isotope in each individual worm was tracked and k_{ef} values of these isotopes from the worms were determined. Exact procedures describing the feeding experiments are given in Baumann and Fisher (in press).

2.4. Pore water experiments

Pore waters used for experiments that determined metal bioaccumulation from the aqueous phase were extracted from sediments collected from three different field sites- Baltimore Harbor (BH; 39°12'.25"N, 76°31'40"W) [Baltimore, MD] in June, Elizabeth River (ER; 76°20' 09" W, 36°52' 32" N) [Norfolk, VA] in May, and Mare Island (MI; 38°04'23" N, 122°14'91" W) [Vallejo, CA in San Francisco Bay] in October. They were transported to the lab and stored at 4°C in plastic buckets. Field locations were chosen based on differences in sediment and water geochemistry and the extent of metal contamination (Baumann et al. in review). Pore water was separated from the sediment by centrifugation at 7500 *g* for 10 min. Any particles (including microorganisms) remaining in the extracted pore water were removed by filtration through 0.2 µm polycarbonate membranes. The pore waters were then autoclaved to

ensure axenic conditions and the water was cooled prior to injecting the radioisotopes. Water was radiolabeled with γ-emitters either by addition of a single radioisotope ^{73}As or combined ^{109}Cd and ^{51}Cr. The gamma detectors that we used did not allow sufficient resolution to combine all three radioisotopes due to their overlapping energies of emission. The isotopes were taken from stock solutions in dilute HCl, and the acidity from the microliter additions was neutralized by addition of microliter quantities of dilute NaOH. Pore water labeling resulted in the following concentrations of radioisotopes: for ^{73}As 6.8, 11.8, and 11.5 Bq mL^{-1} were added to ER, BH, and MI pore waters, respectively; for ^{109}Cd 80.0, 55.4, and 59.2 Bq mL^{-1} were added to ER, BH, and MI, respectively; and for ^{51}Cr 0.3, 27.7, and 28.3 Bq mL^{-1} were added to ER, BH, and MI, respectively. These additions corresponded to additions of 0.03, 0.06, and 0.06 fmol mL^{-1} of As for ER, BH, and MI, respectively; 8.0, 5.5, and 5.9 fmol mL^{-1} of Cd for ER, BH, and MI, respectively; and 0.14, 13.15, and 13.44 fmol mL^{-1} of Cr for ER, BH, and MI, respectively. Given the background concentrations of these metals in the pore waters from each site (Table 1), the radioisotope additions resulted in additions of <1% of background concentrations of each of the metals examined.

Metal accumulation experiments using radiolabeled pore waters were conducted at 17 °C by placing individual worms in separate polycarbonate containers filled with 50 mL of radiolabeled water for 2-4 h. Worms were then removed from their containers and rinsed three times with filtered seawater to remove adsorbed sediment grains and twice with a 10^{-4} M solution of ethylenediaminetetraacetic acid (EDTA) in the corresponding seawater (ER, BH or MI) to remove adsorbed radioisotopes from body surfaces. After counting the radioactivity of the worms immediately following exposure to radiolabeled pore water, the same individual worms were placed into plastic containers holding unlabeled pore water and their radioactivity was

periodically determined over time. The retention of each isotope in each individual worm was tracked for up to 14 d and the k_{ew} values of these isotopes were determined.

For assaying their radioactivity, individual worms were placed into empty 50 mL plastic counting tubes and inserted into one of the large well gamma detectors. This detector was intercalibrated with an LKB Compugamma well-type NaI(Tl) gamma detector that was used for counting the radioactivity of all samples (water, fecal pellets, sediment) other than worms. Counting times were typically 1 min, yielding propagated counting errors < 5%. Radioactivity of ^{73}As was detected at 53 keV, of ^{109}Cd at 88 keV, and of ^{51}Cr 256 keV.

2.5. Field data

As, Cd and Cr in surface sediments, pore waters and various polychaete species collected from the study sites (BH, ER and MI) and pore waters were measured by ICP-MS. Metal concentrations in surface sediments were determined as in Baumann et al. (in review). We used experimentally determined kinetics parameters combined with metal concentrations in sediments and pore waters from field samples to predict metal concentrations in N. succinea. These model estimates were compared with metal concentrations in field-collected polychaetes at each of the three estuarine sites.

2.6. Statistical analyses

Statistical analyses were performed using PASW 18.0 software. One-way ANOVA combined with ad-hoc Tukey's tests were conducted to detect significant ($p < 0.05$) differences among kinetic parameters such as k_{ef}'s and $k_{ew\ slows}$'s for a specific metal at each location or for a specific location between metals. The number of replicates for each k_{ef} or $k_{ew\ slow}$ typically

ranged from 5 to 8. Data expressed as % were arcsine transformed to normalize their distribution prior to ANOVA.

3. Results

Table 1 shows metal concentrations in surface sediment and pore water and Kd values for metals in sediments from the three estuarine sites. Kd values were highest for Cr. The total average concentrations of As in surface sediment were highest in BH and lowest in MI, Cd was highest in MI and lowest in ER, and Cr was highest in BH and lowest in ER. Polychaetes collected at the different sites generally contained measurable concentrations of As, Cd and Cr (Table 2). Mean concentrations of As were similar for worms from BH and ER, although the range was higher in worms from ER. Arsenic concentration in MI worms was below detection. Mean Cd concentrations were similar in worms from ER and MI, however due to insufficient number of polychaetes it was not possible to establish a concentration range for MI. There was no measurable Cd in BH polychaetes. Worms that were collected at all three sites contained detectable Cr, with highest values but fewest samples noted for MI worms (Table 2). Metal concentrations in field-collected worms did not show any relationship with metal partition coefficients (Kd) in the surface sediments.

The fractionations of ^{73}As, ^{109}Cd, and ^{51}Cr in the combined carbonate and exchangeable (carbonex) pools in sediments from each estuarine site that were radiolabeled by direct injection of radioisotope to the sediments or by addition of radiolabeled algal debris are given in Table 3. Cd was most enriched in the carbonex pool (38-94%), regardless of site or means by which the sediments were labeled, and Cr was least enriched in this pool (2-5%). Aging of the directly labeled sediments from 2 to 30 d had no consistent effect on the association of the metals with the carbonex fraction.

N. succinea exposed to radiolabeled metals in pore water generally showed a linear uptake of metal over time, but Cr uptake from ER pore water and As uptake from MI pore water leveled off after 1 d (Fig. 1). Metal uptake rate constants (k_us, Table 4) were highest for As (ranging from 0.021 L g^{-1} d^{-1} in BH to 0.180 L g^{-1} d^{-1} in MI) and lowest for Cd (ranging from 0.0006 L g^{-1} d^{-1} in BH and MI to 0.005 L g^{-1} d^{-1} in ER); differences among metal k_us were significant (one-way ANOVA, $p < 0.001$). Uptake rate constants of Cd and Cr increased significantly ($p < 0.05$) with salinity (from 8 to 20) and decreased significantly (one-way ANOVA, $p < 0.05$) for Cr as pore water DOC increased from 10 to 30 mg L^{-1} (Fig. 2). Metals that were accumulated by Nereis succinea from water were lost in two phases, most clearly seen for Cr in ER water (Fig. 3). The overall percentage of metal rapidly lost ($k_{ew\ fast}$) was nearly always < 15%, except Cr in ER (74%**;** Fig. 3, Table 4). The rapid metal removal from worms lasted no longer than 24 h and was followed by a slow turnover - $k_{ew\ slow}$. Given the relatively high variability in the data, no systematic differences for k_{ef}'s were evident among metals, sediment labeling schemes, sites or sediment age (Table 5).

Following feeding on radiolabeled sediment, the average ingestion rate (IR) was 0.27 g dry sediment g^{-1} dry worm d^{-1}; ingestion rates were inversely related to worm lengths (Fig. 4). Modeling metal concentrations in polychaetes using whole sediment metal concentrations (C_f) (Eq. 1) used AEs shown in Table 6, an IR of 0.27 g g^{-1} d^{-1}, and $k_{ew\ slow}$ values in Tables 4 and 5; growth rate (g) was assumed to be negligible. The kinetic parameters in the modified metal bioaccumulation model (Eq. 5) also used $z_{carbonex}$ values given in Table 1 and a $b_{carbonex} = 0.353$ (Baumann and Fisher, in press). Using this modified model, it was shown that virtually all ($\geq$97%) of the metal in worms was acquired from ingested sediment, for all metals, sediments, and treatments (Table 6); for As in directly labeled ER sediments after 2 d exposure, 85.5% of

the As was shown to be acquired from diet (Table 6). Model predictions of metal concentrations in polychaetes using Eq. 5, which considered the geochemical fractionation of the metals in the carbonex phases ($z_{carbonex}$ and $b_{carbonex}$), were compared with independent measurements of metal concentrations in field-collected polychaetes (Fig. 5A, B). Regression analyses for all metals and sediment sites showed significant relationships between the two (linear regression, $r^2 = 0.87$ for directly labeled sediments, $r^2 = 0.84$ for sediments labeled via algal debris). The slope for the directly labeled sediment (0.51, Fig. 5A) suggests that the model underpredicts by a factor of 2 the metal concentration in the polychaetes, whereas the slope for the sediments mixed with radiolabeled algal debris (1.88, Fig. 5B) suggests that the model overpredicts by a factor 2 the metal concentrations in polychaetes relative to field-collected worms. However, when using total metal concentrations in sediments (Eq. 1), model predictions showed much weaker relationships with field measurements (linear regression, $r^2 = 0.50$ for directly labeled sediments, $r^2 = 0.11$ for sediments labeled with algal debris) (Fig. 5C, D). Thus, consideration of the metal fractionation in the sediment enables more accurate modeled predictions of metal body burdens in polychaetes than modeling with total metal concentrations in sediments.

4. Discussion

4.1. Dietary exposure

That model predictions showed metal primarily deriving from a dietary source is consistent with findings for other metals in benthic animals. Diet represents a larger relative source of metals for polychaetes than for copepods and bivalves (Wang and Fisher, 1999b). The marine mussel *Mytilus edulis* accumulated between 62 and 87% of Cr(III) and 95-98% of Se from diet but only 24-49% of Cd from diet (Wang et al., 1996). The deposit-feeding clam *Macoma balthica* also

accumulated Se almost exclusively from diet (>98%) (Luoma et al., 1992b). Other metals such as Ag (41-69%) and Zn (48-67%) were appreciably derived from diet (Wang et al., 1996) and most Ag in other benthic invertebrates M. balthica, Neanthes arenaceodentata, and Leptocheirus plumulosus was acquired from diet (Yoo et al.,(2004). Roditi et al. (2000b) found that freshwater zebra mussels accumulated 63-90% of Ag, 75-91% of Cd, 57-82% of Hg, and only 9-45% of Cr from their diet. Griscom et al. (2000) found that 49-93% of Ag and 33-82% of Cd is accumulated from diet in the clam Macoma balthica when deposit feeding and >98% for Ag and 90% for Cd when clams were filter feeding. Williams et al. (2010) showed that >90% of As, Cd, Hg(II), and CH_3Hg was diet derived by the benthic amphipod Leptocheirus plumulosus. Another study showed that > 90% of the body burdens of Cd and Zn and >70% of Ag in lugworms were diet derived (Casado-Martinez et al., 2009). Nereis succinea has been shown to accumulate at least ~60% of Hg(II) from diet and 40-80% of methyl mercury from diet (depending on sediment-CH_3Hg binding properties) (Wang et al., 1998). Croteau and Luoma (2005) demonstrated that the freshwater bivalve Corbicula sp. acquired Cu predominantly from diet. Croteau and Luoma (2008) proposed evaluating dietary uptake by multiplying the dietary uptake rate constant (k_{uf}) by the metal concentration in the food and dividing by the efflux rate constant, which allows for a more direct comparison with the rate of aqueous metal uptake. Croteau and Luoma (2008) demonstrated that despite a three order of magnitude difference between k_{uf} and $k_{u\ aq}$ values ($k_{uf} < k_{u\ aq}$), dietary metals (Cd, Cu and Ni) accounted for most of the overall metal body burden in animals. Similarly, diet has been shown to dominate the uptake for many metals in pelagic vertebrates in marine and freshwater systems (Mathews and Fisher, 2009; Pickhardt et al., 2006).

The finding that acquisition of As, Cd, and Cr is related to their association with labile (easily extracted) sedimentary fractions is consistent with earlier findings for other metals (Tessier et al., 1984). By using the percentage of metal in neutral and weak acid exchangeable pools ($z_{carbonex}$), and $b_{carbonex}$, which is the slope of regression between the AEs and z (Baumann and Fisher, in press) we show here that this geochemical pool of metal in diverse sediments can be used to explain observed metal concentrations in deposit feeding polychaetes in the field. Indeed, a positive regression was found between Cu in exchangeable and carbonate sediment fractions and Cu concentrations in freshwater tubificid oligochaetes (Diks and Allen, 1983), comparable to our findings for As, Cd and Cr (Baumann and Fisher, in press). The positive relationship between measured metal concentrations in field-collected polychaetes and model predictions based on the carbonex associations of these metals (Fig. 5) provides further evidence that metals that are weakly bound to sedimentary particles are more bioavailable. This is presumably due to the fact that weakly-bound metals are more readily released from ingested sediment particles into the gut of the deposit-feeder (Chen and Mayer, 1999; Mayer et al., 1996) and therefore more likely to cross the gut lining and be assimilated (Baumann and Fisher, in press; in prep.). The underprediction of polychaete metal from sediment labeled directly may be attributed to the fact that the sediments had no fresh organic detritus associated with it, and thus less metal bound to labile organic matter than would occur in natural field conditions. The overprediction of polychaete metal from sediment labeled by mixing with algal detritus may be attributable to the fact that there was a higher content of metal in labile organic matter in our experiments because there was more labile organic matter in these experimental sediments than would occur in the field.

The mean ingestion rate measured here for N. succinea (0.27 g $g^{-1}d^{-1}$) was identical to that described for similarly sized N. succinea at the same experimental temperature reported by Cammen (1980) and an order of magnitude lower than that cited by Wang et al. (1999a). We are unaware of published reports of As, Cd, and Cr concentrations in polychaetes in the Chesapeake to compare with our measurements, but Cd concentrations in resident oysters and mussels have been described (Sinex and Wright, 1988; Wright et al., 1985). In San Francisco Bay (but not at Mare Island), As concentrations in several species of polychaetes were 110-130 µg g^{-1}dry wt (Meador et al., 2004) in comparison to polychaetes collected in Mare Island (0 µg g^{-1}dry wt; present study). There is more information available regarding metal concentrations in animals representing other taxonomic groups living in San Francisco Bay (e.g. bivalves, birds). For example Potamocurbula amurensis collected in San Francisco Bay and Lower Suisun Bay had ~4 µg g^{-1}dry wt of Cd and ~6 µg g^{-1}dry wt of Cr (Brown and Luoma, 1995), Corbicula sp. collected all throughout the bay system had As levels in a range of 5.4 to 11.5 µg g^{-1}dry wt, and Macoma balthica contained 9 µg g^{-1}dry wt of As (Johns and Luoma, 1990). Among the waterfowl monitored in San Francisco Bay, the American coot (Fulica americana), collected near Mare Island, had an average 3.7 µg g^{-1}dry wt of As, 105 µg g^{-1}dry wt of Cr, and no measurable Cd in its ingested food. Its liver was also enriched with As and Cr (5.1 µg g^{-1}dry wt of As, 3.3 µg g^{-1}dry wt of Cr, 1.3 µg g^{-1}dry wt of Cd) (Hui, 1998).

4.2. Aqueous exposure

Cd aqueous uptake rate constants from our study were 0.06 to 0.5 times those reported for previously published results for N. succinea (Wang et al., 1999a). Cadmium k_us calculated from data reported by Ng et al. (2008) for the polychaete Perinereis aibuhitensis were approximately 0.003 L^{-1} g^{-1} dry wt d^{-1} (assuming dry wt = 20% wet wt) when worms were

exposed to 4.12 μg Cd L^{-1}. The k_u measured for N. succinea in the present study was approximately 0.005 L^{-1} g^{-1} dry wt d^{-1} in ER pore water, which had a comparable Cd exposure concentration (5.12 μg L^{-1}) and significantly lower ($p<0.05$) k_us (0.0006 L^{-1} g^{-1} dry wt d^{-1}) when worms were exposed to lower Cd concentrations in BH and MI pore waters (0.16 and 0.52 μg Cd L^{-1}, respectively). Yan and Wang (2002) reported a Cd k_u of 0.0018 L^{-1} g^{-1} dry wt d^{-1} for the polychaete Sipunculus nudus and a Cr k_u of 0.019 L^{-1} g^{-1} dry wt d^{-1}. This Cr k_u is within the range of k_us reported here for N. succinea (Table 5). We are unaware of any k_us reported for As in marine polychaetes.

The positive relationship between As k_u and salinity is consistent with recent findings for As accumulation in the killifish Fundulus heteroclitus (J. Dutton - personal communication). Although Cd is a chloro-complexed metal, the lowest salinity water investigated here still has sufficiently high chloride to complex all the Cd in the water, thus the lack of a salinity response over the salinity range 8 to 23 is not surprising. Only the k_u for Cr was inversely related to the DOC concentration in pore water for all sites, suggesting that organic complexation of this metal may reduce its bioavailability for N. succinea.

The efflux rate constants from N. succinea following aqueous and dietary exposures did not significantly differ for any of the metals (one-way ANOVA; $p>0.05$), similar to findings of Norwood et al. (2006) who evaluated loss of As and Cr in Hyalella azteca, and suggesting that the uptake route has no appreciable influence on the biological turnover rates of assimilated metal from the polychaete tissues.

In summary, the present study shows that diet is the dominant source of sediment-bound metals for deposit-feeding polychaetes, regardless of the metal or sediment type. Further it is important to recognize that bulk sediment is a mixture of minerals and organic compounds to

which metals may bind, and that metal bioavailability varies among geochemical fractions. For all metals and estuarine sites considered, modeling metal bioaccumulation in polychaetes most accurately quantifies metal body burdens when considering geochemical fractionation of the metals in the sediments.

Table 1. Total concentrations of As, Cd and Cr in surface sediments (SS; 0-1 cm), pore water (PW), and partition coefficients (Kd) of As, Cd and Cr in these sediments.

Location		unit	As	Cd	Cr
Mare Island	SS	$\mu g\ g^{-1}$	1.43	2.39	47.38
	PW	$\mu g\ kg^{-1}$	1.57	0.52	0.15
	Kd	$L\ kg^{-1}$	9.12×10^{2}	4.61×10^{3}	3.08×10^{5}
Baltimore Harbor	SS	$\mu g\ g^{-1}$	47.19	0.96	322.65
	PW	$\mu g\ kg^{-1}$	1.96	0.16	0.34
	Kd	$L\ kg^{-1}$	2.41×10^{4}	5.94×10^{3}	9.52×10^{5}
Elizabeth River	SS	$\mu g\ g^{-1}$	6.37	0.46	33.30
	PW	$\mu g\ kg^{-1}$	28.44	5.12	0.83
	Kd	$L\ kg^{-1}$	2.24×10^{2}	0.90×10^{2}	4.01×10^{4}

Table 2. Ranges and means (in parentheses) of metal concentrations in field-collected polychaetes. BH n = 4; ER n = 5; MI n = 1 and therefore no range provided for metals in MI. < det indicates below detection limit.

	metal concentrations [µg g^{-1} dry wt]		
	BH	ER	MI
As	6.16 - 21.631 (13.45)	0.70 – 21.10 (13.14)	< det
Cd	< det	0.20 – 3.40 (1.55)	- (0.98)
Cr	2.09 – 31.78 (12.30)	1.2 – 5.8 (3.04)	- (49.34)

Table 3. Percent of ^{73}As, ^{109}Cd and ^{51}Cr in carbonex fraction (exchangeable + carbonate) of sediments from 3 estuarine sites labeled directly with radioisotopes or via mixing with previously radiolabeled algae and aged for 2 (just directly labeled sediment) or 30 days (both)(Baumann and Fisher, in press).

location	label	days	% of radioisotope in carbonex pool ($z_{carbonex}$)		
			^{73}As	^{109}Cd	^{51}Cr
Baltimore Harbor	algae	30	9	48	2
Elizabeth River			30	72	5
Mare Island			12	38	3
Baltimore Harbor	direct	2	1	59	4
Elizabeth River			2	67	5
Mare Island			4	48	4
Baltimore Harbor		30	3	49	3
Elizabeth River			3	94	3
Mare Island			6	58	3

Table 4. Kinetic parameters for uptake (k_u) from pore water and efflux from worms ($k_{ew\ slow}$ and $k_{ew\ fast}$) following metal uptake by *N. succinea*; terms in parentheses represent the % of metal in the slowly exchanging metal pool; values represent means ± 1 SD for n = 5 - 7 individuals; in places where error is not specified individual worms were pooled together to obtain a sufficiently high radioactivity count and hence inter-individual differences were not observed. A fast loss phase was not evident for ^{73}As and ^{109}Cd in ER.

	Baltimore Harbor (BH)	Mare Island (MI)	Elizabeth River (ER)
k_u [L g^{-1} d^{-1}]			
^{73}As	0.021 ± 0.019	0.180 ± 0.166	0.122 ± 0.067
^{109}Cd	0.0006 ± 0.0002	0.0006 ± 0.0003	0.005 ± 0.0027
^{51}Cr	0.0048 ± 0.0045	0.008 ± 0.0043	0.076 ± 0.068
$k_{ew\ slow}$ [% d^{-1}]			
^{73}As	3.4 ± 2.0 (95)	0.2 ± 0.9 (95)	1.6 (100)
^{109}Cd	2.0 ± 0.3 (86)	1.5 ± 0.5 (92)	1.5 ± 1.0 (100)
^{51}Cr	2.3 ± 2.1 (94)	1.7 ± 1.7 (95)	0.8 (26)
$k_{ew\ fast}$ [% d^{-1}]			
^{73}As	16.9 ± 5.6	5.6 ± 4.7	-
^{109}Cd	11.7 ± 11.7	10.1 ± 4.7	-
^{51}Cr	5.4 ± 3.6	6.4 ± 5.5	415.1 ± 179.6

Table 5. Efflux rate constants [k_{ef}; % d^{-1}; mean ± 1 SD, n = 5 - 8] of ^{73}As, ^{109}Cd, and ^{51}Cr in *N. succinea* during depuration after pulse feeding on sediment radiolabeled by direct addition of radioisotopes or by mixing with previously radiolabeled algal detritus. Also shown are k_{ef}s following feeding on pure radiolabeled algal detritus or goethite. nd: not determined

		efflux rate k_{ef} [% d^{-1}]			
diet		directly labeled		labeled algae	
		2 days	30 days	2 days	30 days
Elizabeth River (ER)	^{73}As	nd	nd	7.6 ± 5.3	1.2 ± 0.3
Baltimore Harbor (BH)	^{73}As	3.5 ± 2.5	1.4	nd	nd
Mare Island (MI)	^{73}As	6.3 ± 5.9	nd	3.4 ± 1.2	1.6 ± 0.8
fresh algal detritus	^{73}As			4.29 ± 1.58	
pure goethite	^{73}As	1.0 ± 0.6			
Elizabeth River (ER)	^{109}Cd	5.4 ± 4.3	2.5 ± 1.8	0.3 ± 0.2	5.9 ± 4.4
Baltimore Harbor (BH)	^{109}Cd	0.3 ± 0.3	0.3	5.4 ± 5.5	10.6 ± 9.6
Mare Island (MI)	^{109}Cd	1.8 ± 1.3	6.4 ± 1.5	0.9 ± 1.0	0.6 ± 0.4
fresh algal detritus	^{109}Cd			2.1 ± 2.0	
pure goethite	^{109}Cd	2.5 ± 1.5			
Elizabeth River (ER)	^{51}Cr	2.4 ± 2.7	0.1 ± 0.1	0.05 ± 0.01	0.12 ± 0.17
Baltimore Harbor (BH)	^{51}Cr	1.7 ± 2.0	1.4	nd	1.89 ± 2.01
Mare Island (MI)	^{51}Cr	1.0 ± 0.6	nd	1.7 ± 0.7	0.22 ± 0.26
fresh algal detritus	^{51}Cr			0.6 ± 0.3	
pure goethite	^{51}Cr	3.1 ± 1.7			

Table 6. Model predictions of dry wt based body burden of metals in *N. succinea* following feeding on sediment with or without algal detritus and aged for up to 30 d; AEs from Baumann and Fisher (in press). Percentages showing AEs and dietary source rounded to closest 0.1%.

	location	metal	age	AE	from food	from water	C_{ss}	dietary
			days	%	µg g^{-1}	ng g^{-1}	µg g^{-1}	%
algal detritus	BH	As	2	51.6	112.1	0.123	112.10	100.0
			30	30.1	65.54	0.123	65.54	100.0
		Cd	2	68.7	0.64	0.000	0.64	100.0
			30	21.6	0.10	0.000	0.10	100.0
		Cr	2	1.2	29.85	0.007	29.85	100.0
			30	0.8	85.95	0.007	85.95	100.0
	ER	As	2	69.7	15.32	173.1	15.50	98.9
			30	16.8	6.59	173.1	6.77	97.4
		Cd	2	9.9	2.00	0.948	2.00	100.0
			30	21.5	0.22	0.171	0.22	99.9
		Cr	2	0.9	154.9	0.371	154.92	100.0
			30	3.6	59.58	0.371	59.58	100.0
	MI	As	2	50.7	5.71	1.764	5.71	100.0
			30	24	5.74	1.764	5.74	100.0
		Cd	2	9.4	10.23	0.002	10.23	100.0
			30	7.6	12.32	0.002	12.32	100.0
		Cr	2	5	61.62	0.015	61.62	100.0
			30	0.3	32.44	0.015	32.44	100.0
directly injected radiotracer	BH	As	2	7.8	23.07	0.123	23.07	100.0
			30	6.6	34.65	0.123	34.65	100.0
		Cd	2	1.5	0.25	0.0005	0.25	100.0
			30	2.4	0.40	0.0005	0.40	100.0
		Cr	2	4.2	113.0	0.007	113.00	100.0
			30	4.6	149.6	0.007	149.61	100.0
	ER	As	2	1.2	1.02	173.1	1.19	85.5
		Cd	2	30.8	0.35	0.171	0.35	100.0
			30	43.6	1.06	0.171	1.06	100.0
		Cr	2	4.5	15.89	0.371	15.89	100.0
			30	1	86.82	0.371	86.82	100.0
	MI	As	2	10.2	0.62	1.764	0.62	99.7
			30	12.1	0.73	1.764	0.73	99.8
		Cd	2	46.1	24.98	0.002	24.98	100.0
			30	58.9	8.97	0.002	8.97	100.0
		Cr	2	4	88.59	0.015	88.59	100.0

30	0.7	14.69	0.015	14.69	100.0

Figures

Fig. 1. Accumulation (Bq g^{-1}) of ^{73}As, ^{109}Cd, and ^{51}Cr from pore water by N. succinea over time. Data points denote means (n = 5 – 7) + 1 SD.

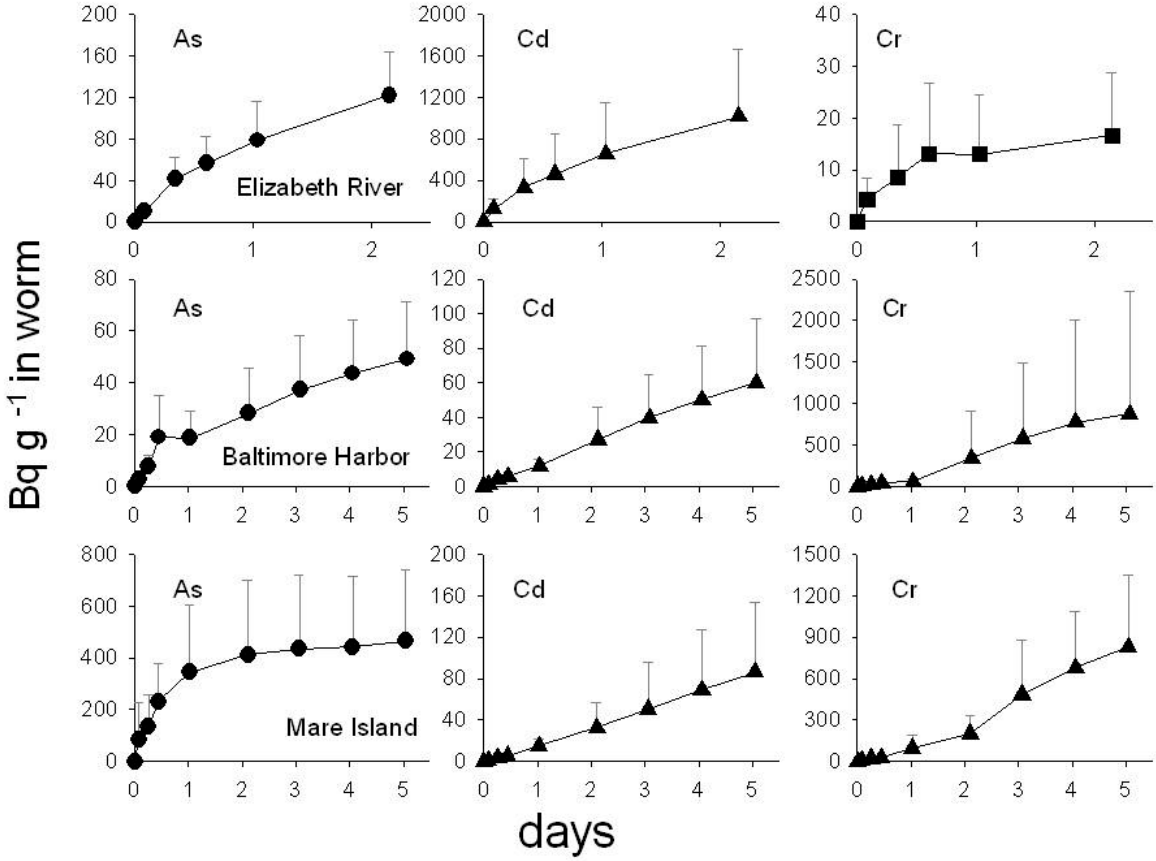

Fig. 2. Relationship between metal uptake rate constants (k_u, L g^{-1} d^{-1}) in Nereis succinea exposed to radiolabeled pore water and the dissolved organic carbon concentration in the pore water or the salinity of the pore water. Pore waters were extracted from field collected sediments from Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI). Data points denote means (n = 5 – 7) + 1 SD.

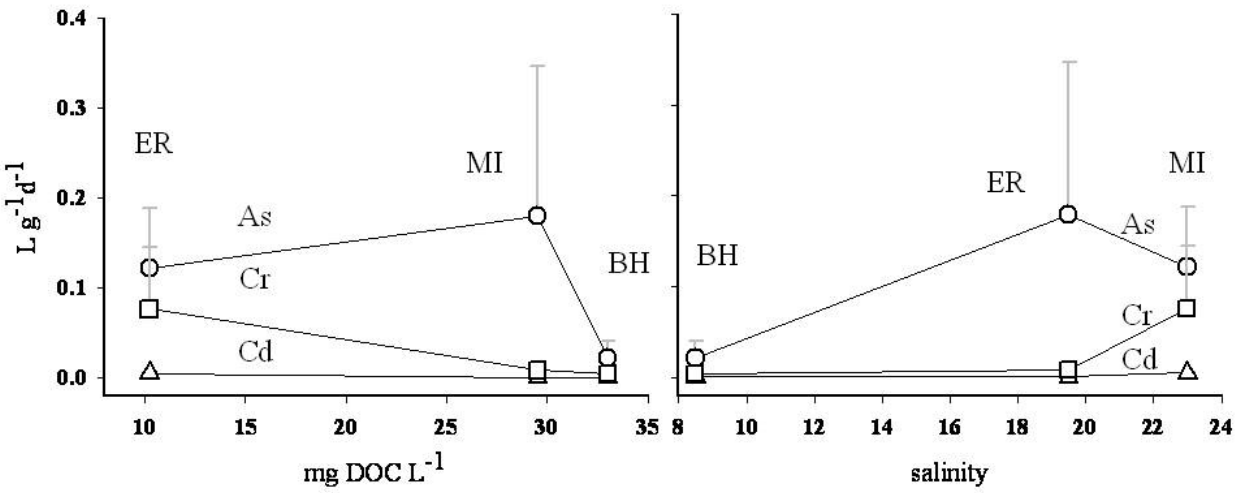

Fig. 3. Percent metal retained by N. succinea during depuration after uptake from pore water. Data points denote means (n = 5 – 7) + 1 SD.

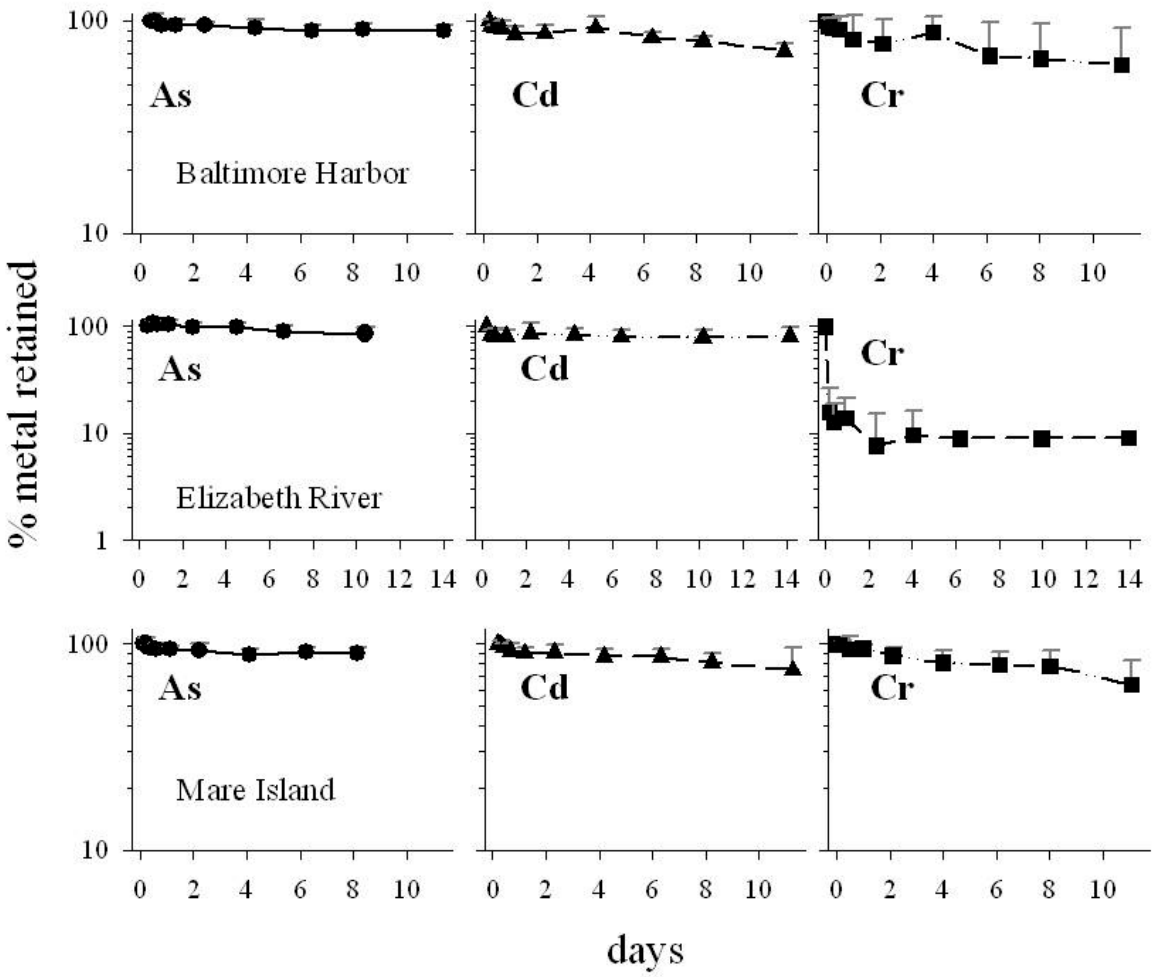

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Fig. 4. Ingestion rates (g dry sediment g^{-1} dry wt worm d^{-1}) of N. succinea as a function of worm length. Mean value is 0.27 g $g^{-1}d^{-1}$; data points denote means of fecal matter collected over three time points (n = 3) $\pm$ 1 SD. When error bars not visible SDs were very small.

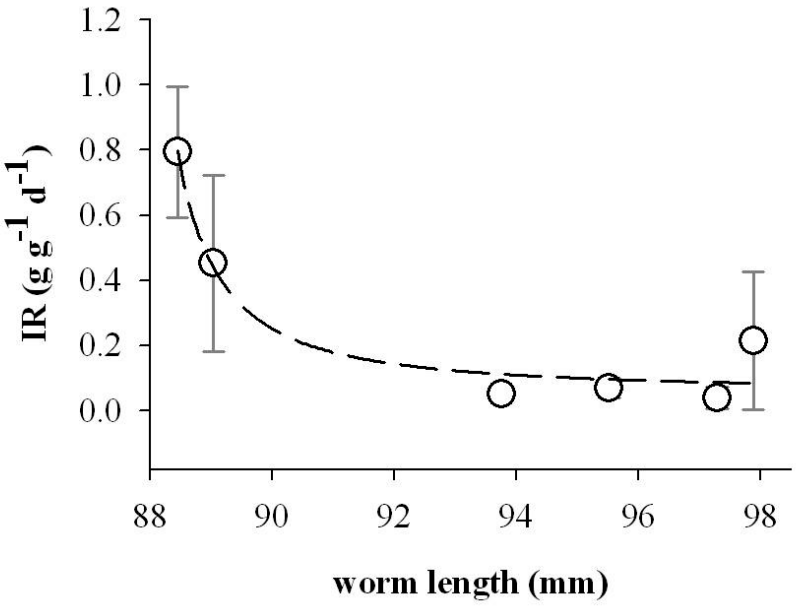
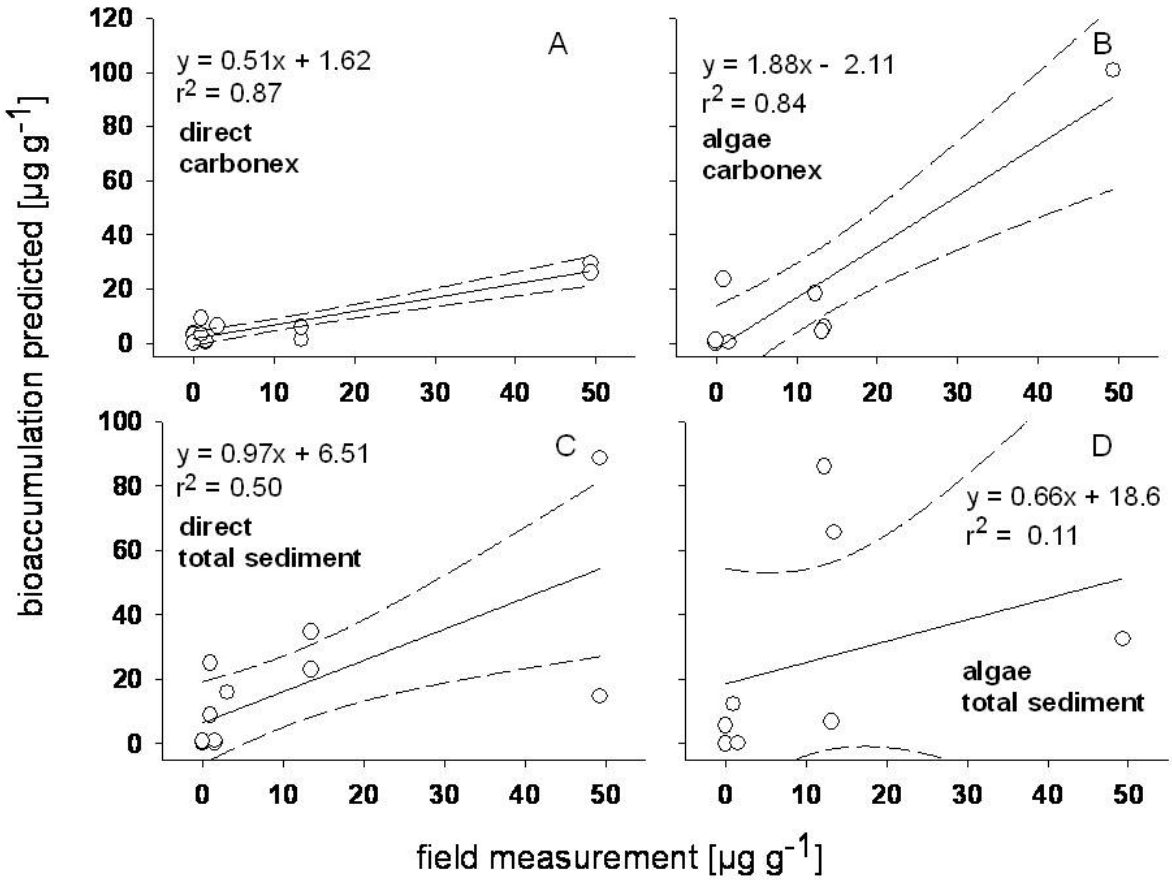


Fig. 5. Regressions (solid line) between field observed metal concentrations in polychaetes and model-predicted metal concentrations in worms for As, Cd, and Cr. Regressions for model predictions using carbonex pool of sedimentary metals (A, B) and model predictions using total metal concentration in sediments (C, D) from ER, BH and MI. Panels A and C show the model predictions for directly labeled sediment and B and D show model predictions using sediments mixed with radiolabeled algae. Dashed lines indicate 95% confidence intervals.



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Factors influencing the assimilation of arsenic in a deposit-feeding polychaete

ABSTRACT

We investigated mechanisms leading to assimilation of particle-bound arsenic (As) ingested by the deposit-feeding polychaete *Alitta succinea* using a radiotracer approach.

Release of As from different particle types into extracted gut fluid or bovine serum albumin (BSA), a gut fluid mimic, was measured. In addition, gut fluid proteins were analyzed by separating proteins via 2D gel electrophoresis, and protein peptide sequences were determined by mass spectrometry. Major ions in the gut fluid were measured by ion chromatography and metals by mass spectrometry. Percentages of particulate As release were related to As assimilation efficiencies (AEs) in polychaetes feeding on different particle types. AEs of As were highest from radiolabeled pure diatoms (72%) and radiolabeled diatoms added to sediment (51%), lower from radiolabeled sediment (10%), and lowest from a radiolabeled iron oxide mineral, goethite (2%).It appears that As release from particles is a necessary but not sufficient requirement of As assimilation. For example, 15% of As was released from goethite into the gut fluid but only 2% was assimilated by *A. succinea*. Our results suggest that the likelihood of As assimilation is higher when it is bound to an organic compound of nutritional value in the ingested particles.

1. INTRODUCTION

Given the importance of sediments serving as a source of metal and metalloid contaminants for benthic invertebrates, we investigated processes that may influence metal assimilation in a deposit-feeding polychaete, *Alitta succinea* (formerly *Nereis succinea*). This study focused on As, which can be enriched in estuarine sediments resulting from natural sources as well as coal combustion and industrial activities (Cullen and Reimer, 1989). Arsenic has been shown to accumulate in high concentrations in estuarine sediments and in resident benthic fauna and has been implicated in risk assessments as a priority pollutant in many coastal sediments (Chen et al., 2000b; Cullen and Reimer, 1989; Fattorini et al., 2005; Fattorini et al., 2006; Jones et al., 1999).

Polychaetes can accumulate arsenic from sediments (Casado-Martinez et al., 2010; Rainbow et al., 2011b) and may serve as conduits of metals from contaminated sediments to benthic predators such as bottom-feeding fish, which in turn can be consumed as seafood. We have recently shown that metals are acquired principally from ingested sediment in *N. succinea* (Baumann and Fisher, 2011a). Previous work concluded that a metal's solubilization from ingested sediment into gut fluid is necessary for that metal to be assimilated across the gut lining (Chen and Mayer, 1998). Building on this earlier work, we conducted a series of experiments with sediment-bound arsenic to relate its release from ingested particles in polychaete guts to its assimilation in polychaete tissues. Baumann and Fisher showed that sedimentary As can be assimilated with efficiencies of ~72% from ingested organic particles in *N. succinea*. In the present study, we consider the form of As in the ingested particle and the chemical composition of *A. succinea* gut fluid in influencing the assimilation of this metalloid (2011b).

It can be argued that metals bound to ingested particles, including sediment particles for deposit-feeding invertebrates, must first be released from that particle into the gut fluid of an animal before it can cross the gut lining and become incorporated into tissues (Chen and Mayer, 1998). Release of metals from sediment particles may be dependent on the characteristics and composition of the sediments and on the chemical composition of the gut fluid. Further, the metal must be released in a form that is able to cross the membranes of the gut lining. A biomimetic approach has been proposed by Mayer et al. (1996) in which a solution of commercially available bovine serum albumin (BSA) is used to mimic the natural gut fluid of polychaetes. This approach is appealing because of the difficulty in obtaining sufficient quantities of gut fluid, particularly for species with small individual sizes. Previous experimental studies involving sediment incubations with gut fluid and BSA led to the conclusion that proteins, amino acids, and other abundant organic ligands in gut fluid can be effective in binding metals and perhaps transporting them across the gut lining, consistent with an animal's "objective" of assimilating nutritious organic compounds (Mayer et al., 1997). This approach has been used to better understand the mechanisms governing dietary assimilation of ingested metal (Voparil and Mayer, 2004; Weston and Maruya, 2002). We have therefore analyzed the composition of the gut fluid of *A. succinea* to provide *in vivo* context for ingested As and for enabling comparison of the gut fluid of this polychaete with previously used gut fluid mimics such as BSA.

Our research evaluated the use of BSA as a substitute for natural gut fluid of *A. succinea* to study the release of As and its eventual assimilation in this polychaete. We used a radiotracer approach that is well-suited for assessing rates of As transfer from particulate to dissolved phases and subsequent assimilation in polychaete tissues. Sediment from an industrial part of San Francisco Bay, Mare Island, was labeled by direct addition of ^{73}As or by mixing the sediments with ^{73}As-labeled diatoms. ^{73}As-labeled goethite was also used to represent the pool of As that is typically associated with iron oxides in sediments (Neff, 1997). Radiolabeled sediments and goethite were incubated in a number of possible extractant solutions, including solutions containing BSA, chloride and BSA, deionized water, and natural *A. succinea* gut fluid to measure the release of particle-bound ^{73}As over a period representative of the gut transit time of ingested particles in this polychaete. The chemical composition of the gut fluid was determined and compared to that of BSA. As noted above, some previous studies considered the release of metals from ingested particles into natural or simulated gut fluid, while others assessed the assimilation of ingested metals bound to sediments. However, studies that have combined these approaches to provide a mechanistic explanation of measured assimilation of metals are generally lacking. Here we consider observations of arsenic release from sediments and its assimilation in a polychaete and identify relevant chemical properties in the gut that may help explain these observations.

2. MATERIALS AND METHODS

2.1. Experimental species

A surface deposit-feeding polychaete *Alitta succinea*, which is found ubiquitously in muddy sediments along the US coastline, was chosen as the experimental species. *A. succinea* has been used in the past for laboratory studies due to its well known ecology and physiology (Ahrens et al., 2001a). This species has also been examined in a series of metal bioaccumulation studies involving contaminated estuarine sediments (Baumann and Fisher, 2011b; Wang et al., 1999a). Polychaetes from a local salt marsh at Flax Pond on Long Island NY were hand-collected in summer, placed in individual containers with a small portion of sediment, and transported to the laboratory. Individual worms were rinsed off and placed in individual containers filled with Flax Pond seawater (salinity ~28) to eliminate any undigested sediment remaining in their guts prior to the experiment.

To obtain the gut fluid samples for pH, protein and amino acid analyses, worms were anaesthetized by immersion in 10% $MgCl_2$ after gut clearance was complete. Individual worms were blotted and placed on a clean glass slide. The body wall was cut open along the body length, and coelomic fluid was removed by a gentle application of a cotton swab. After the coelomic fluid was removed, the gut fluid was pulled towards the foregut by a gentle upward rolling of a glass rod. This resulted in formation of a "bulb" in the frontal region of the gut. The bulb was poked with a sharp needle and ~5-50 µL (depending on the individual worm) of gut fluid was withdrawn by an automatic pipette and immediately transferred into an Eppendorf tube set on ice. Gut fluid from individual worms was pooled together into one tube, later filtered with 0.2 µm PVDF (polyvinylidene fluoride) protein non-binding membranes, and stored at -20°C for further procedures.

2.2. Gut fluid analyses

2.2.1. Bacteria

Three samples of 1 µm Nuclepore filtered gut fluid were fixed with 2% borate-buffered formalin, filtered via 0.2 µm black Nuclepore filter and stained with a fluorescent dye DAPI (4'6-diamidino-2-phenylidole), according to Sherr et al. (2001). DAPI stain was excited by ultraviolet light when bacterial cells were observed and bacterial cells were counted using epifluorescence on an inverted microscope (Leica DM IRB).

2.2.2. Total concentration of gut fluid proteins and amino acids

Freshly extracted gut fluid of *Alitta succinea* was first filtered (0.2 µm PVDF) and then analyzed for dissolved amino acids by high performance liquid chromatography (HPLC) with fluorescence detection (after Lindroth and Mopper (1979)). Total hydrolyzable amino acids (THAA) were determined in the gut fluid samples after acid hydrolysis following the modified methods of Fitznar et al.(1999), Reinthaler et al. (2008) and Mayer et al.(1995).Three 20 µL gut fluid samples were subjected to hydrolysis by 6N HCl and 0.25 wt% phenol for 22-24 h at 110°C. Prior to this incubation samples were promptly subjected to $N_2$and sealed in glass vials with Teflon septa and vortexed. Once hydrolysis was completed, acid was removed from samples by purging them with nitrogen gas N_2. Further, samples were resuspended in distilled water mixed with methanol at a 6:4 ratio. The hydrolysis products of asparagine and glutamine

were included in aspartic acid (ASP) and glutamic acid (GLU) measurements, respectively. For the HPLC analysis o-phthaldialdehyde (OPA) was used to derivatize the gut fluid, ODS HYPERSIL C18 column (Supelco, 150mm, 5μm) with the gradient of 10% methanol in 0.04M sodium acetate as solvent A and 10% 0.04M sodium acetate in methanol as solvent B were used for the separation. During the first 22 min the gradient was ramped up from 10% to 60% solvent B, and within the following 17 min solvent B was increased to and kept at 85%for 2 min, after which it was decreased to 10%. Shimadzu RF-10AXL fluorometer with excitation and emission wavelengths of 330nm and 418nm was used to detect the fluorescently labeled AAs. As a standard we used Amino Acid Standard H (Pierce), combined prior to sample injection, with the standardsfor β-alanine (BALA; Sigma-Aldrich) and γ-aminobutyric acid (GABA; Sigma-Aldrich), which arenon-protein AAs. It was not possible to measure the concentration of cysteine due to the lack of adequate standard. The concentration of total proteins in extracted gut fluid and in solutions of bovine serum albumin (for the protein degradation experiment) was measured according to Bradford(1976) by determining the absorbance of dissolved protein bound to Coomasie Blue stain measured at 595 nm with a Micro Plate Reader (Perkin Elmer, Wallac Victor2 1420 Multilabel Counter).

Analysis of proteins in extracted gut fluid required a prior protein precipitation with 100% Trichloroacetic acid (TCA) with the final TCA v/v concentration of 10%. A solution that included the precipitated proteins was centrifuged at16,000 *g* for 15 min. The supernatant thought to include only the small peptides containing <7-14 amino acids in their chain (Mayer et al., 1995) was removed, and the pellet was resuspended in a solution of protease inhibitor cocktail (Enzo Life Sciences, KI-103) to prevent enzymatic protein degradation. Protein degradation in the gut fluid sample was observed on gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE) generated in the preliminary stage of this research. Mostly, low molecular weight proteins from the gut fluid samples were initially detected on silver stained SDS-PAGE gels. This suggested that the gut fluid proteins had undergone degradation between the gut fluid extraction times and the time of its analyses. Gut fluid **s**amples were stored at 4°C before running the two dimensional electrophoresis (2-DE).

2.2.3. Qualitative protein analysis: Sample preparation, two-dimensional electrophoresis(2-DE), mass spectrometry and data analysis

Salts and other contaminants where removed from the worm gut extract solution using the Bio-Rad ReadyPrep™ 2-D Cleanup Kit (cat# 163-2130). The resultant precipitated protein was dissolved in first dimension rehydration buffer (ST_{50}) consisting of 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% Bio-Rad Biolytes (isoelectric point or pI range = 3-10) and 50 mM dithiothreitol (DTT) and the protein concentration determined using a modification of the Peterson (1977) method that incorporates additional 6% TCA washes to remove DTT, which interferes with the assay.

Two-dimensional electrophoresis (2-DE) was conducted essentially as described in the Bio-Rad ReadyStrip immobilized pH gradient (IPG) strip instruction manual (Bio-Rad catalogue number 163-2099). Briefly, IPG strips (11 cm, pI range 3-10, Bio-Rad catalogue number 163-2014) were passively equilibrated under mineral oil for 18 h at 23 ºC with 47 µg of solubilized protein in 200 µl ST_{50}. The IPG strips were subsequently washed by dipping into deionized H_2O and blotted with filter paper upon which they were transferred to a Protean IEF (Bio-Rad) focusing tray and laid gel side down across wet paper wick covered electrodes and covered with mineral oil. Further, they were focused in a Bio-Rad Protean IEF Cell at 20 ºC, rapid ramp 0-250 V for 1 h; slow ramp 250V-8000 V for 1 h; hold 8000 V to a total of 35000 Vh and subsequently removed from the focusing tray, blotted with moist filter paper and placed gel side up in channels of a clean rehydration/equilibration tray, covered and stored at –80 ºC.

The thawed strips were equilibrated at 23 °C for 10 min in equilibration buffer (EB) composed of 6 M urea, 50 mM Tris-HCl, 2% sodium dodecyl sulfide - SDS, 20% glycerol with 2% (w/v) DTT at pH = 8.8. The equilibration buffer was decanted off and the strip was equilibrated (10 min, 23 °C) in EB with 2.5% (w/v) iodoacetamide. The strips were transferred to IPG wells of pre-cast Criterion 8-16% polyacrylamide gels (Bio-Rad catalogue number 345-0105) and overlaid with agarose. The gels were resolved under constant 200 V for 55 min. The slab gels were stained with 100 ml of SYPRO® Ruby Stain according to the Bio-Rad instruction manual and imaged with a Bio-Rad VersaDoc™ 3000 Imaging System.

Gel spots were cut out, destained, reduced, alkylated and digested with trypsin (Promega Gold, Mass Spectrometry Grade) essentially as described by Shevchenko et al. (1996) with minor modifications. The resulting concentrated peptide extract was diluted into a solution of 2% acetonitrile (ACN), 0.1% formic acid (FA) (buffer A) for analysis. For solution digest, 10 µl of purified protein was diluted in 40 µl of 50 mM ammonium bicarbonate. The proteins were reduced with 2 mM DTT and alkylated with 4 mM iodoacetamide for 30 min each. Trypsin (0.25 µg) was added to the samples and the digests were incubated for overnight at 37 °C. Protease reactions were stopped with 100% formic acid (final concentration 5%). Ten µl of the peptide mixture was analyzed by automated microcaplillary liquid chromatography-tandem mass spectrometry. Fused-silica capillaries (100 µm inner diameter - i.d.) were pulled using a P-2000 CO_2 laser puller (Sutter Instruments, Novato, CA) to a 5 µm i.d. tip and packed with 10 cm of 5 µm Magic C18 material (Agilent, Santa Clara, CA) using a pressure bomb. Ten µl of the resulting 20 µl concentrate was pressure-loaded onto a 10 cm 100 µm i.d. fused-silica capillary packed with 3 µm C18 reverse phase (RP) particles (Magic, Michrome), which had been pulled to a 5 µmi.d. tip using a P-2000 CO_2 laser puller (Sutter Instruments). This column was then installed in-line with an Eksigent Nano 2D High Performance Liquid Chromatography (HPLC) pump running at 300 $nlmin^{-1}$.

Peptides were loaded with an autosampler directly onto the column and were eluted from the column by applying a 30 min gradient from 5% buffer B to 40% buffer B (98% acetonitrile, 0.1 % formic acid). The gradient was switched from 40 % to 80 % buffer B over 5 min and held constant for 3 min. Finally, the gradient was changed from 80 % buffer B to 100 % buffer A over 0.1 min, and then held constant at 100% buffer A for 15 more minutes. The application of a 1.8 kV distal voltage electrosprayed the eluting peptides directly into an LTQ Orbitrap XL ion trap mass spectrometer equipped with a nano - liquid chromatography electrospray ionization source (Thermo Finnigan). Full mass spectra (MS) were recorded on the peptides over a 400 to 2000 *m*/*z* range at 15,000 resolution, followed by one tandem mass (MS/MS) event triplet on the two most intense ions, each triplet containing of an HCD (higher energy collision dissociation) scan, a CID (collision induced dissociation) scan in the Fourier Transform (FT) at 7,500 resolution and a CID scan in the ion trap of the same ion. Charge state dependent screening was turned on, and peptides with a charge state of +2or higher were analyzed. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the X calibur data system (Thermo Finnigan, San

Jose, CA). MS/MS spectra were extracted from the RAW with ReAdW.exe (http://sourceforge.net/projects/sashimi).

The resulting mzXML file contains all the data for all MS/MS spectra and can be read by the subsequent analysis software. The MS/MS data were searched with Inspect (Tanner et al., 2005) against a database containing peptide sequences for annelids (3932 protein sequences downloaded on December 1, 2009)plus common contaminants and *E. coli* proteins (2777 protein sequences total) with modifications: +16 on methionine, +57 on cysteine. Only peptides with at least a p-value of 0.01 were analyzed further. In addition, MS/MS data were also analyzed by de novo analysis using PepNovo (Frank and Pevzner, 2005). Only hits with a score of >8 were considered for further manual analysis. Possible peptide sequences were used for fasts searches against an NCBI database (downloaded 10/25/2009) (Mackey et al., 2002).

2.2.4. Chemical composition of gut fluid

Worms used for gut fluid extraction and later for ionic composition and trace metal analysis were not anesthetized by 10% $MgCl_2$ recognizing that ionic and metal concentrations in gut fluid could be altered by physiological changes that occur in worm bodies upon exposure to $MgCl_2$. Gut fluid was analyzed for anions (Cl^-, SO_4^{2-}), cations (Mg^{2+}, Ca^{2+}, K^+, Na^+), trace metals (Cr, Ni, Cu, Zn, As, Se, Rb, Cd, Ba and Hg) and pH. The anionic composition was assessed using ion chromatograph Dionex-500with a 4mm AS4A-SC column with 5 mM sodium carbonate/sodium bicarbonate as eluent and the cationic composition using 4 mm CS-12 column with 22mN sulfuric acid as eluent at the flow rate of 1 mL min^{-1}. A sample of gut fluid for the metal analyses was acidified and stored in a trace metal-clean plastic container. Metal analyses were performed using inductively coupled plasma mass spectroscopy (ICP-MS) on three replicate gut fluid aliquots from the larger pooled gut fluid volume. The pH of these gut fluid samples was measured with an optical pH sensor using seawater as a buffer Zhu et al.(2006) .

2.3. Degradation of BSA when mixed with natural sediment

To test if the proteins at a concentration similar to those in gut fluid are rapidly degraded when in contact with sediment an experiment was conducted. Possible protein degradation was monitored for 24 hours. BSA fraction V at a starting concentration of 10% was diluted to a final concentration of 3.8 mg mL^{-1}, as determine by the Bradford assay described above. Three

replicates of 15 mL of BSA solution were mixed with ~1g (wet weight) of sediments from three different locations: Baltimore Harbor, Elizabeth River from Chesapeake Bay and Mare Island from San Francisco Bay. The control consisted of BSA solution without added sediment. Falcon tubes with all treatments were incubated at 21°C on a shaker table to assure mixing of the solution with sediment throughout the incubation. Three liquid samples – 100 µL each, were withdrawn for protein concentration analysis after 1, 2, 4, 6, 8 and 24 hours of incubation and injected into a 96 well plate, previously filled with 200 µL per well of Bradford reagent for protein staining. Well plates were inserted into a Micro Plate Reader (Perkin Elmer, Wallac Victor 2 1420 Multilabel Counter) and absorbance was measured at 595 nm. Protein concentrations were calculated based on a standard curve prepared for a series of BSA concentrations (0, 125, 500 and 1500 $mgmL^{-1}$). A new standard curve for BSA concentration was prepared at every sampling time.

2.4. Application of radiotracer approach

2.4.1. Radiolabeling of sediments and goethite

Sediment collected from Mare Island was uniformly radiolabeled by two methods with the gamma-emitting radioisotope ^{73}As ($t_{1/2}$ =80.3d) as arsenate. One labeling method involved direct addition of 7.7 $kBqg^{-1}$ (dry wt) of ^{73}As (an equivalent of 0.37 µmol g^{-1}; 0.002% of the background As concentration in the sediment) dissolved in 0.1 M HCl to ~2 g wet wtof sediment; the acid from the stock solution of radioisotope was first neutralized by addition of microliter quantities of dilute NaOH prior to radiolabeling so that the pH of sediment was not changed. Portions of sediment (18.3 ± 9.7 mg dry wt) were then transferred into individual tubes for later incubation. The second labeling method involved addition of (32 Bqg^{-1}dry wt) previously radiolabeled diatom biomass to the sediments. To produce the radiolabeled diatoms, a culture of the centric diatom *Thalassiosira pseudonana* (clone 3H) was grown in f/2 medium, except phosphate which was at the level of f/20 (or 3.6 µM) (Guillard and Ryther, 1962) prepared with surface seawater (salinity of 35) collected 8 km offshore of Southampton (Long Island, NY). This water was inoculated with nutrients and 185 kBq of ^{73}As L^{-1} of. Diatoms were grown to stationary phase and were harvested by filtration onto a 3.0 µm Nuclepore filter. Approximately 22 mg (dry wt) of algal biomass was removed from the filter and mixed into 2 g (wet wt) of Mare Island sediment. Small sediment portions (3.9 ± 1.7 mg dry wt) were

transferred from this mixture into individual tubes. Radiolabeled goethite was prepared by suspending dry goethite (from Sigma-Aldrich, goethite ~35% Fe; EC No. 2437464) in seawater (salinity 35) to which ^{73}As was added to produce goethite containing 10.8 kBq^{73}As g^{-1} (dry wt).

2.4.2. Evaluation of assimilation efficiency (AE) of arsenic by *A. succinea*

Assimilation efficiencies of ingested As were experimentally determined in a separate study described in detail by Baumann and Fisher (2011a). In that work, AEs were determined using a radiotracer approach using pulse-chase feeding experiments, an approach that has been used extensively for generating AEs of diverse metals and metalloids in aquatic animals. We used a gamma-emitting radioisotope -^{73}As, with a half-life of 80.3 days, added as arsenate (As +5). AE (%) was determined from the equation for the slow loss over time as the *y*-intercept in this equation (Wang et al., 1999a). The AEs of ingested As from that study are considered here together with As release from particles into gut fluid or other solutions.

2.4.3. Incubation of radiolabeled sediments

Three different solutions were used as sediment extractants. One of the solutions was gut fluid, one was 3.8 mg bovine serum albumin (BSA) per liter of deionized water, and one was 3.8 mg bovine serum albumin (BSA) suspended in 1 liter of 20 g L^{-1} sodium chloride solution; a control consisted of deionized water. In our approach to mimic anoxic conditions in a deposit-feeder's gut, the deionized water (used to prepare the chloride and BSA solutions) was purged with N_2 for 1 h to remove dissolved oxygen. The concentration of BSA was similar to the protein concentration measured in the gut fluid of *Alitta succinea*. Gut fluid used for the experiment was filtered with a 0.2 µm PVDF filter to remove particles such as small sediment grains or microbial cells but not dissolved proteins.

Radiolabeled sediments and goethite were incubated with the solutions for up to 4 h on a shaker table (at 200 rpm) which was placed inside a glove bag that was purged with N_2. A mini centrifuge and all the tools needed for the liquid collection at the end of incubation times were also placed inside the glove bag so that no oxygen would affect the incubated samples. A volume of 100 µL of each of deionized water, BSA dissolved in deioinized water, BSA dissolved in chloride solution, and gut fluid was collected after 20, 60, and 240 min of incubation. Three replicates (200 µL) for each solution were taken. Because of difficulty in obtaining sufficient gut

fluid from *A. succinea*, it was feasible to use three replicates of 200 µL of gut fluid only at the end of the 4 h incubation with sediment labeled directly, sediment mixed with radiolabeled diatoms, and radiolabeled goethite. Sampling times were chosen to observe the rate of ^{73}As release from the sediment into each solution that could occur in the gut of *A. succinea*.

Based on our observation of *A. succinea* in the laboratory under these conditions, the retention time of sediment in the gut was estimated to average 4 h. At each sampling time, three replicates containing substrate + solution were centrifuged at 734 *g* for 5 min to separate liquid from solids. One hundred µL of the liquid was pipetted out of each sample and transferred into separate tubes for determination of radioactivity.

2.4.4. Measuring radioactivity

To assay their radioactivity, samples were inserted into plastic counting tubes and analyzed using a Pharmacia-Wallac LKB gamma spectrometer equipped with a well-type NaI (Tl) detector. Radioactivity of ^{73}As was detected at 53 keV and counted for 5 min per sample, yielding propagated counting errors typically < 5%.

2.5. Statistical analyses

To determine significant differences between treatments in the incubation experiments, one-way ANOVAs were performed using PASW 18.0 software.

3. RESULTS

3.1. Bacteria, proteins and amino acids in gut fluid

Protein analysis using two-dimensional electrophoresis isolated several proteins in the extracted gut fluid (Fig. 1). Eight protein spots were further analyzed by LC/MS/MS to deduce the sequences from these proteins. Due to the limited protein sequences of *A. succinea* in publicly available databases, *de novo* sequencing of peptide fragmentation patters was conducted. Possible peptides for each protein spot were further analyzed by matching the sequences to the NCBI database by using fasts (Mackey et al., 2002). Alignment of the peptide sequences to protein sequences was possible for proteins isolated in two spots (spot 8 and spot 9; Fig. 1). Aligned proteins, alkaline serine protease from *Alteromonas* for spot 8 and fibrinolytic protease

P-III-1 from *Eisenia fetida* (common brandling worm) for spot 9 are proteases, which are normal constituents in gut fluid. However, the peptide sequences alone cannot be used to determine if the analyzed proteins are from *A. succinea* or gut bacteria. To determine these proteins origin further experiments are needed. Bacterial cell counts revealed that the gut fluid of *A. succinea* contains on average 1.15 x 10^8 cells mL^{-1}, therefore, future studies addressing gut fluid proteins originating from bacteria would be worthwhile.

The amino acid composition of the gut fluid of *A. succinea* is given in Table 1. Isoleucine was the most concentrated amino acid in the gut fluid (0.39 mg L^{-1}or 15.9% of total measured amino acids), followed by alanine (14.2%) and glutamic acid (9.8%); γ-aminobutyric acid was least concentrated (0.001 mg L^{-1}, or 0.04% of total amino acids). The total concentration of measured amino acids was 20.6 mmol L^{-1} or 2.46 mg L^{-1} (Table 1).

3.2. Ions, metals and the pH

Measurement of gut fluid pH using the optical pH sensor showed that the gut fluid is neutral (pH =7.15 ± 0.08; data not shown). Ion chromatography analyses of *A. succinea*'s gut fluid showed that its ionic composition was not similar to seawater except for chloride (471.8 ± 0.7 vs. 546 $mmolkg^{-1}$) (Table 2). Concentrations of other ions (Ca^{2+}, Na^{+}, Mg^{2+}, SO_4^{2-}) were 4.5 - 5.7-fold higher (10-foldfor K^{+}) in gut fluid [Table 2] than in seawater (Bruland and Lohan, 2004). Metal concentrations measured for total body and gut fluid alone indicated that only Cr had similar concentrations in whole worms and gut fluid. Other elements such as Ni, Ba, As, Hg and Se were up to 7.7 times more concentrated in the whole worms, and Cu, Rb, Cd and Zn were much higher (up to 66.2-fold) in the whole worms compared to gut fluid (Table 3).

3.3. Sediment incubation experiments

Mare Island sediments, labeled by direct injection of ^{73}As or by addition of radiolabeled algal debris, and radiolabeled goethite, released different amounts of ^{73}As into the different extractants over 4 h period (Fig. 2). At the 4 h time point, the fraction of As released into solution was significantly different between gut fluid and control for sediment labeled directly (one-way ANOVA, $p < 0.05$), and between gut fluid and all other solutions for goethite (one-way ANOVA, $p \leq 0.02$).No significant difference (one-way ANOVA, $p > 0.05$) was found between either of BSA solutions and the control solution. The percentage of As released into the gut fluid

from sediment labeled by mixing with radiolabeled algal debris was highest (33.7 ± 11.7%), lower for directly labeled sediment (17.4 ± 5.7%) and for goethite (14.7 ± 3.0%). Mean AEs for As in A. *succinea* from ingested sediments (Baumann and Fisher, 2011b), were higher than the release of As into any solution except for natural gut fluid.

Percentages of As assimilated by *A. succinea* and that released from goethite into water (control), BSA, or chloride + BSA solutions were not significantly different from one another, however the amount of As released into gut fluid was 18.4, 18.4 and 15.5-fold higher than the other three extractions, respectively (Fig. 2). AEs for As from sediment mixed with diatoms were highest (50.7 ± 9.0%), lower for sediment labeled directly (10.2 ± 6.8%) and lowest from goethite (2.4 ± 0.7%). The AE of As in *A. succinea* feeding on sediment mixed with radiolabeled diatoms was significantly different from As released from this substrate into water, BSA in distilled water, and BSA + chloride solutions (one-way ANOVA, $p < 0.0001$) but not different from the percentage released into gut fluid (one-way ANOVA , $p > 0.05$). Sediments mixed with radiolabeled diatoms released 5.2, 2.1 and 4.2 times more ^{73}As into gut fluid than into control, BSA solution in distilled water, or BSA chloride solution, respectively.

By incubating BSA with sediment for up to 24 hours we have determine d that there was no notable protein (BSA) degradation to peptides consisting of less than 5-10 amino acid units (data not shown).

4. DISCUSSION

This research aimed to identify key factors affecting As assimilation in the gut of the deposit-feeding polychaete *A. succinea*. First, it was evident that As assimilation can vary depending on the composition of the diet with which As is associated. Assimilation efficiencies of ingested As are much lower from diets in which As is attached to goethite and to particles within the largely inorganic sediment than from sediment enriched with fresh algal organic matter with which As is associated (Fig. 5; Baumann and Fisher, 2011b). Second, As release from particles into gut fluid varies among particle types in parallel with AEs. Comparison of AEs and the percentages of As released from unamended sediment and from goethite shows that AEs are either similar to or lower than its release into gut fluid, suggesting that some of the As released from goethite and unamended sediment into gut fluid is not assimilable. This is especially evident for goethite,

where As AE was < 3% compared to 15% of As released into the gut fluid. The assimilability of As clearly depends on its association with the particle types that are ingested, such that inorganic species of As bound to inorganic particles (e.g., goethite or minerals in unamended sediment) are much less assimilable by *A. succinea* than As bound to fresh algal organic matter (e.g. arsenosugars in algal cells mixed with sediment, where As is covalently bound to organic molecules) (Andreae and Klumpp, 1979; Edmonds et al., 1997). This is further supported by findings that As AEs from pure algal cells (not mixed with sediments) reach 72% for *N. succinea* (Baumann and Fisher, 2011b). It would appear that As bound to assimilable organic compounds that are released into gut fluid can be efficiently assimilated. It is also possible that As undergoes biotransformation into organic compounds like arsenobetaine and arsenocholine when taken up by phytoplankton or sediment associated bacteria (Andreae and Klumpp, 1979; Bolan et al., 2006; Riedel, 1993). Biotransformation of inorganic As to organic As can determine its bioavailability in animals ingesting food containing these compounds (Edmonds et al., 1997).Thus, dissolution of As from ingested particles into gut fluid is insufficient on its own to explain assimilation. This conclusion is consistent with the idea that metals must be released into gut fluid before they can be assimilated into animal tissues. It is also consistent with the idea that animals eat to acquire nutritious organic compounds; if metals are bound to those compounds they are transported across the gut lining into the tissues of the animal and become assimilated. The exact mechanism by which As-containing compounds are transported across the gut lining is not known for deposit-feeding polychaetes.

Mayer et al. (1997) argued that gut matrices of deposit-feeding animals containing surfactants and organic ligands are “designed” to solubilize essential nutrients, including compounds to which inorganic and organic contaminants may be bound. This solubilization can lead to elevated contaminant concentrations in the gut fluid of deposit-feeders (Chen et al., 2000b). Consistent with this concept, Roditi et al. (2000b) reported that metals bound to dissolved organic compounds were more highly assimilated by the zebra mussel, *Dreissena polymorpha*, than the metals that were not complexed by organic matter. Previous studies also showed that metals like Cu and Zn that are biologically incorporated into fish prey lead to higher body burdens and higher toxicity in these fish than metals in inorganic forms (Clearwater et al., 2002). More recent work has identified how the compartmentalization of metals in prey can affect the

assimilation in a predator, although only a few studies have determined compartmentalization of As in marine invertebrates (Freitas et al., 2012; Rainbow et al., 2011a).

The higher fraction of ^{73}As released into *A. succinea* gut fluid than in BSA solutions suggests that not just any (e.g., BSA) dissolved proteins can explain As release from particles. Factors that may influence the release of metals from ingested particles into a soluble form within the gut include enzyme activity, surfactancy of gut fluid, and possibly anoxic or reducing conditions within the gut. The latter, for example, may lead to release of As from Mn and Fe oxides. Arsenic that is associated with particles could potentially be taken up by endocytosis as shown by García-Alonso et al.(2011) for Ag-nanoparticles in the gut epithelium of *Nereis diversicolor*, although there are no reports documenting this uptake mechanism for sediment-associated metals**.** For the future studies, it would perhaps be more useful to create a more complex milieu containing enzymes and other proteins that could complex metals, surfactants, reducing chemical agents *etc* that would altogether mimic the natural gut fluid better than BSA, shown here as quite ineffective in extracting As from sediment, diatoms and goethite.

In Mare Island sediment 40% of the radiolabeled As was extracted within the operationally defined fractions such as the *organic matter*, 25% in *acid volatile sulfides - AVS*, and 11% in *Fe/Mn oxides* (Baumann and Fisher, 2011b). Though these fractions are operational we might expect that among other potentially extracted minerals and compounds As was extracted with refractory organic matter – in the *organic* extractions, and iron oxides that were likely present in the *AVS* and *Fe/Mn oxides* fractions. It is plausible that solubilization from this sediment into the gut fluid would occur for As by complexation with organic matter in the gut fluid, which is supported by Redman et al. (2002) according to whom As – natural organic matter interaction aids As release from particles in nature. For oxide-bound As (such as in goethite) As is mobilized by oxides partial dissolution in a reducing environment. The reducing environment of *A. succinea*'s gut fluid is likely due to organic matter decomposition (Griscom et al., 2002a; Mayer et al., 1997; Plante and Jumars, 1992). A study by Griscom et al. (2002a) conducted with deposit-feeding clams (*Macoma baltica*) demonstrated Cd release in conjunction with iron reduction in its gut. In that study ~25% of ingested Cd was released from oxic estuarine sediments into *M. baltica*'s gut fluid in parallel with release of Fe(II) during passage of ingested sediment in the anoxic gut (Griscom et al., 2002a); the acidity (pH 5.5) of the clam gut was

shown not to impact this Cd release. Such mechanism of Cd release from oxide particles in the anoxic gut is likely analogous to As release.

A study (Chen and Mayer, 1998) examining the mechanisms of Cu release from contaminated sediment into the holothuroid *Parastichopus californicus* gut fluid found that gut fluid proteins (as well as proteins in a BSA solution) influenced dissolution of sediment-bound Cu by complexing with this metal. Another study showed that the presence of the enzyme proteinase K influenced the dissolution of Cu, Al, Fe, Pb and Zn (but not As and Mn) from sediment *in vitro* (Turner, 2006). Fan and Wang (2003) studied the effects of gut fluid (in this case, of a sipunculan polychaete) as a Cd extractant relative to seawater at pH 8 and pH 5, and seawater plus surfactant (1% sodium dodecyl sulfonate), finding that natural gut fluid was more effective in mobilizing sediment-bound Cd. In contrast to Cd, all solutions were inefficient in extracting sedimentary Cr (Fan and Wang, 2003). The minimal dissolution of sediment-bound Cr into gut fluid is comparable to this metal's low assimilation efficiency (usually < 5%) in *A. succinea* and other benthic invertebrates (Lee and Luoma, 1998; Wang et al., 1997).

In some cases, seawater has been used as a control to compare the metal extraction capability with gut fluid (Fan and Wang, 2003; Lawrence et al., 1999). However, the ionic composition of gut fluid can differ from that of ambient seawater, as shown for *A. succinea* in Table 2. For example, concentration of K^+ is an order of magnitude higher, and Ca^{2+}, Mg^{2+}, Na^+ and SO_4^{2-} are 4.5 and 5.7-fold higher in the gut fluid than in seawater. Moreover, there is an imbalance of charges measured in gut fluid, with cation concentrations being much higher than those of anions. It is therefore plausible that the negative charge of some organic compounds in the gut fluid – e.g., acidic polysaccharides or other weak organic acid anions could balance these inorganic cations if present in high enough concentration (100's of mM; L. Mayer personal communication). Alternatively, it is also possible that the excessive positive charge in gut fluid could be balanced by the negative charges on the clay particles that worms ingest. The concentration of acidic polysaccharides in the gut fluid of A. *succinea* remains unknown. Concentrations of Ca and Mg in the gut fluid of a sipunculan polychaete *Sipunculus nudus* were 13 and 7 times higher, respectively, than in *A. succinea*, and 56 and 37 times higher than concentrations in seawater (Fan and Wang, 2003). Compositional differences in gut fluid among species may be due to differences in ionic regulation, as described for sulfate and sodium in the

marine gastropod *Aplysia californica* (Gerencser and Levin, 2000). Unlike the variability in ionic composition of gut fluids among marine invertebrates, the pH of deposit-feeder gut fluids tends to reflect the pH of the ingested sediment and pore water (Ahrens et al., 2001a; Lopez, 2005; Plante and Jumars, 1992). Most aquatic invertebrates have a gut fluid pH of 6-8, although the guts of bivalves tend to be more acidic (pH =5-6) (Lopez, 2005). Small pH variations in deposit-feeding polychaetes have been observed due to body length, with greater acidity of gut fluid in juvenile worms (Ahrens et al., 2001a).

Total metal body burdens and concentrations in gut fluid measured in sipunculan *S. nudus*, *A. marina,* and *A. succinea* differed by up to three orders of magnitude (Chen et al., 2000b; Fan and Wang, 2003). The most striking differences are among the gut fluid to tissue concentration ratios in *A. marina* and *A. succinea*. For example, in the study by Chen et al. (2000b), *A. marina* gut fluid was much more enriched with metals in relation to its tissues, whereas the opposite was evident for *A. succinea,* where tissues had higher metal concentrations (present study). Reasons for differences between these two polychaetes are not immediately apparent. *A. marina* generally ingests sandy sediments whereas *A. succinea*, ingests finer sediment grains (personal observation; Longbottom, 1970). Because sandy sediments are generally less reactive for metals than fine-grained sediment, metals could be expected to desorb more readily from sandy sediments and be found in the gut fluid of *A. marina*. Furthermore *A. marina* individuals are larger than *A. succinea* individuals, and the ingestion rate of deposit-feeding polychaetes is a function of their body volume (Penry and Jumars, 1990). Hence the absolute rate of sediment ingestion should be higher in *A. marina.* This higher ingestion rate translates into larger volume of sediment being subjected to gut conditions. It is also noteworthy that sand ingesting deposit-feeders have higher ingestion rates than those feeding on finer particles (G. Lopez, personal communication). Therefore, in worms ingesting more sediment, potentially greater amount of metal can be released from particles into the gut fluid. This scenario is plausible if the kinetics of metal release from the particles into the gut fluid are faster than the gut transit time. Also, the surface-to-volume relationship in *A. marina* is lower than in *A. succinea.* Therefore absorption of released metal from gut fluid across the gut lining into the tissues of *A. succinea* may therefore be expected as greater than for *A. marina*. It is also striking that the concentration of As in whole *A. succinea* individuals is 3-fold higher than in the gut fluid alone. Our experimental results, in which less As is assimilated than released from food into the gut fluid, would suggest the

opposite body: gut fluid ratio. It is possible that longer-term feeding studies would have revealed different ratios from the approach used here, based on short term experimentation.

The abundance of proteinaceous compounds in the gut fluid sampled from a variety of deposit-feeding polychaetes (but not *A. succinea*) has been described by Chen et al. (2000b). Proteinaceous compounds have been suggested to complex metals in the gut fluid once they are released from ingested sediment particles. BSA, substituting for natural gut fluid proteins, suggested the importance of proteins for binding a variety of metals and organic contaminants deposit-feeding polychaete guts (Chen and Mayer, 1998; Kalman and Turner, 2007; Voparil and Mayer, 2004). BSA has been shown to have a large capacity to dissolve substantial amounts of sediment-bound metals, but as shown in the present study, BSA can dissolve only a small fraction of sedimentary As, much less than the amount of As released into the gut fluid of *A. succinea*. Concentrations of proteins in the gut fluid of *A. marina* are an order of magnitude higher than protein concentrations measured in the gut fluid of *A. succinea* (Voparil and Mayer, 2004). The concentrations of BSA used in incubation experiments described here and elsewhere were adjusted to be comparable to the total protein concentrations in gut fluids of *A. succinea* and *A. marina* (present study; Chen and Mayer, 1998; Voparil and Mayer, 2004). Inter-experimental differences in metal solubilization capacity in BSA solutions may be attributable in part to differences in protein concentrations used in each experiment and also due to differences in BSA affinities towards different metals and metalloids.

Tables

Table 1. Amino acid gut fluid composition determined by HPLC; values represent the mean ± one standard deviation (n = 3 samples; gut fluid pooled from 16 individuals).

Amino Acid (AA)	gut fluid (GF)			BSA[1]		
	mean ± SD [mmol L^{-1}]	mean ± SD [mg L^{-1}]	% of total AA in gut fluid	[g g^{-1}]	% of total AA in BSA	BSA : GF
Alanine	3.891 ± 0.231	0.35 ± 0.02	14.2	0.082	6.6	0.5
Arginine	1.244 ± 0.017	0.22 ± 0.00	8.9	0.069	5.6	0.6
Aspartic Acid	1.466 ± 0.385	0.20 ± 0.05	8.1	0.102	8.2	1.0
α-Aminobutyric Acid	0.010 ± 0.017	0.001 ± 0.002	0.04	-	-	-
Glutamic Acid	1.641 ± 0.199	0.24 ± 0.03	9.8	0.024	2.0	0.2
Glycine	2.984 ± 0.239	0.22 ± 0.02	8.9	0.029	2.3	0.3
Histidine	0.714 ± 0.151	0.11 ± 0.02	4.5	0.135	10.9	2.4
Isoleucine	2.955 ± 0.649	0.39 ± 0.09	15.9	0.0356	2.9	0.2
Leucine	1.161 ± 0.055	0.15 ± 0.01	6.1	0.179	14.4	2.4
Lysine	0.758 ± 0.538	0.11 ± 0.08	4.5	0.195	15.7	3.5
Methionine	0.119 ± 0.206	0.02 ± 0.03	0.8	0.000	0.0	0.0
Phenylalanine	0.488 ± 0.042	0.08 ± 0.01	3.3	0.103	8.3	2.6
Serine	1.281 ± 0.018	0.13 ± 0.00	5.3	0.052	4.2	0.8
Threonine	0.941 ± 0.102	0.11 ± 0.01	4.5	0.067	5.4	1.2
Tyrosine	0.257 ± 0.022	0.05 ± 0.00	2.0	0.089	7.2	3.5
Valine	0.693 ± 0.011	0.08 ± 0.00	3.3	0.078	3.3	1.0
ΣAA	*20.6* mmol L^{-1}	*2.46* mg L^{-1}		1.24[2]		

1-Bovine Serum Albumin (BSA) data is derived from (Shi et al., 2006); 2-Total mass of amino acids is >1.0 g due to recovery efficiency error

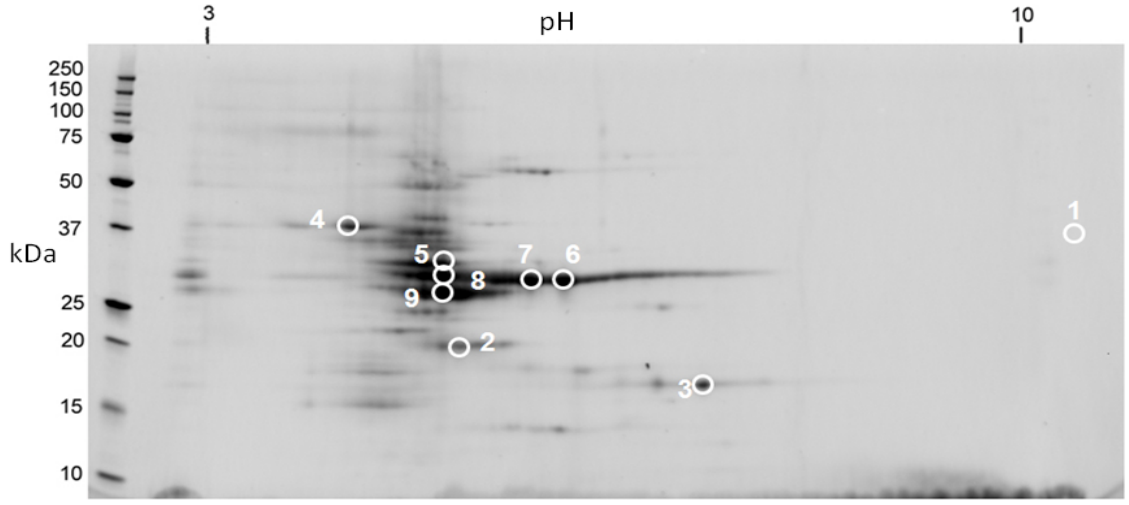
Table 2. Ionic composition of *A. succinea*'s gut fluid (present study), open ocean seawater (Bruland and Lohan, 2004) and their ratio; error indicates one standard deviation (n =3 replicates of gut fluid).

ions	mmol kg^{-1}		gut fluid to seawater ratio
	gut fluid	Seawater	
Cl^-	471.8 ± 0.7	546	0.9
SO_4^{2-}	159.0 ± 1.0	28	5.7
K^+	102.8 ± 0.9	10.2	10.1
Na^+	2271.8 ± 3.5	468	4.9
Ca^{2+}	45.9 ± 0.7	10.3	4.5
Mg^{2+}	266.4 ± 3.9	53	5.0

Table 3. Concentrations (dry wt) of metals in whole worms (μg g^{-1}) and in gut fluid (μg g^{-1}) pooled from several 16 individuals of *Alitta succinea*.

metal	whole worm	gut fluid
Cr	1.72 ± 0.92	1.54
Ni	2.87 ± 1.00	1.62
Cu	75.3 ± 12.5	2.60
Zn	394 ± 47	5.95
As	11.4 ± 0.64	3.55
Se	2.01 ± 0.19	0.26
Rb	3.18 ± 0.17	0.10
Cd	1.56 ± 0.79	0.03
Ba	0.44 ± 0.27	0.19
Hg	0.06 ± 0.01	0.01

FIGURES



Possible sequences for separated proteins:
SPOT 1 (blank): AGALCNN, AGEHSSG, AGEYLAMK, ATGGDLNAALER, ATVSLPR, DGLNAALER, ETVVLGHVDSEER, FASEMSR, GSAGENR, HLQLALR, KALTLFY, LAQGVQLVDGFTK, LASYLDK, LATVSLPR, LDGLFKT, LGSEEGR, QALDVFY, QLGQALR, QLGQALR, SGGSLNR, TLLDLDNTR, TSGLELEG, VVTVSLPR, YTVSLPR; SPOT 2: AGGEEVFVGR, DQAFGLK; SPOT 3: VGGDGAVYEGR, TLTLVTR, YSFDDLPK; SPOT 4: LEGEESR, YYLTGNAR, YSFDDLPK; SPOT 5: STLWGLSR, YLHDTSLR; SPOT 6: ENAGEDPGLAR (**dermicidin – sample contamination**), YLHDTSLR, GSLGGGFSSSK, SYDTTDGAGVR, SYDTTDGAGVR; SPOT 7: TTHQDFGGR, YLHDTSLR (**serine protease II**); SPOT 8: R(-17)AATLVAVK, GSQDTGSR, GTVGGGEFGLAK, LELSELNR, LGT(-18)SDTATSTK, LKEWYEK, LTHDDFGGR, RLSFSLGGSR, STLWGLSR, TSDTATSTK, VLGVGSALLNSLR, VYVLDTGVR, YLHDTSLR (**serine protease II**); SPOT 9: **ASGYAGVYAR**, LSLSTSGGS, **TDACQGDSGGPLVVK**, VYLHDTSLR, YEDLAQK (**fibrinolytic protease**).

Fig. 1. Molecular mass distribution of gut fluid proteins on two dimensional SDS-PAGE (gel electrophoresis); mark spots of gel that were sampled, digested and analyzed by HPLC. Each spot yielded several amino acid sequences (specified below the photo).

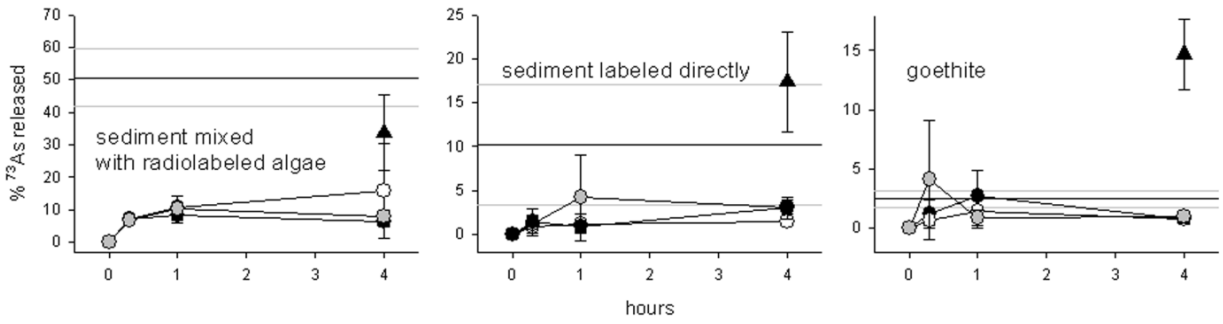


Fig.2. Release of ^{73}As from sediments (n=3 for each incubation treatment) that were radiolabeled directly and mixed with radiolabeled algal debris or from radiolabeled goethite in control treatment (distilled water; black circle), BSA solution in distilled water (open circle), BSA solution in 20 g l^{-1} chloride solution (grey circle) or gut fluid extracted from *Alitta succinea* (black triangle); and ^{73}As assimilation efficiency from Baumann and Fisher (2011; mean indicated by a black horizontal solid line ± 1 standard deviation for n=5 individuals indicated by grey solid horizontal lines above and below the mean).

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Determining the phase speciation of sedimentary metals with radioisotopes to support metal bioavailability studies

ABSTRACT:

Solid-phase speciation of ^{73}As(+5), ^{109}Cd and ^{51}Cr(+3) in radiolabeled fresh algal debris and in sediment alone or mixed with algal debris was determined upon their incubation for up to 90 days. This speciation of added radiotracers was determined by conducting a modified sequential extraction procedure. Operationally-defined geochemical fractions were extracted in following seven steps: *exchangeable*, *carbonate*, *acid volatile sulfides*, *iron and manganese oxides*, *organic I* and *II*, and p*yrite*. Non-extractable *residue* was examined for any remaining radioactivity. Fractionation of ^{73}As, ^{109}Cd and ^{51}Cr was determined to later draw relationships between particular associations (e.g. in *exchangeable*) of radiotracer - their extracted fractions and their bioavailability in deposit-feeding polychaetes. This chapter serves to describe the geochemical aspect of the overall dissertation. Specifically, data sets presented here are used to characterize the geochemical properties of sediment used for further feeding experiments, aimed to determine radiotracer bioavailability from ingested sediments to deposit-feeding worms. Primary results show that fractionation patterns for any of the radioisotopes were similar between sediments collected from three different locations – two in Chesapeake Bay and one in San Francisco Bay, which themselves showed geochemical differences (e.g., organic C content, salinity etc.). As expected fractionation patterns varied between the radioisotopes. Sediment aging with the radioisotopes had no effect on fractionation, except for Cr, which shifted from more easily extractable fractions (*AVS* and *Fe/Mn oxides*) to *pyrite* that was more difficult to extract. Results presented in this chapter were further used in chapter III and IV to help explain the bioavailability of ingested by *Nereis succinea* As, Cd and Cr.

INTRODUCTION

This study was conducted to explore how radioisotopes can be used to gain a better understanding of the geochemical fractionation of arsenic, cadmium and chromium initially added in dissolved form to sediments or solid biogenic debris, and how these fractionation patterns change over time under specific conditions. These patterns can then be used to infer metal bioavailability for benthic animals, further complementing radiotracer methods that have been widely used in metal bioaccumulation studies.

Metal contaminants come from a variety of anthropogenic sources, including diverse industrial activities, and can be delivered in dissolved or particulate form to estuaries by rivers, direct discharge, or via atmospheric precipitation (Luoma, 1996). Mineral particles, can be composed of clays , which range in size from 4 µm (kaolinite) to 2000 µm (intertidal sand) as summarized by Burdige (2006), iron and manganese oxides, carbonates etc. Organic particles can be of different origin, including allochthonous terrestrial material and autochthonous algal debris. Degrading terrestrial and other - non living organic matter can bind with metals in the water column, and algae can passively acquire (through adsorption) or actively incorporate metals into their cells. Once these metals arrive in surface waters, they can be deposited in sediment by being scavenged onto various types of particles that settle out from the water column (Fowler and Knauer, 1986; Turner and Millward, 2002) and become available to the benthos.

Particle-reactive metals and other types of contaminants that are stored in sediments are in much higher concentrations than metals in the water column, which can be reflected by partition coefficients – Kd indicating several orders of magnitude metal enrichment in particles in relation to overlying water. For example metals that are measured in µmol L^{-1} in the water column can be present in sediments in mmol kg^{-1}. Metals and metalloids in sediments undergo diagenetic processes and cycling, which determine their partitioning into different geochemical pools. Metal(loid)s in these pools have different mobilities. Mobility of particle-bound metals varies due to the properties of metals, due to the properties of these geochemical fractions, and diagenetic conditions (Tessier et al., 1979).

Metal contaminant-enriched sediments are ingested by different deposit-feeding animals (clams, polychaetes, fish etc.) for whom sediment serves as the primary food source. Deposit-feeding animals can assimilate these ingested metals if the metals are present in a bioavailable form (Wang et al., 1999a). Metals that are assimilated and internalized into animal cells can be toxic (Clearwater et al., 2002). Assimilated metals can also be passed through the demersal food chain, if predators consume the metal enriched prey. This trophic transfer poses concern due to harmful effects these contaminants may have for local fauna (Luoma, 1996). There is therefore an interest in developing better management strategies for contaminated coastal waters.

Sequential extraction procedures have been used as a tool to approximate the geochemical distribution of metals in operationally-defined (based on the physico-chemical extractability)

pools . These types of extraction procedures intend to selectively target metals in inorganic and organic fractions based on specific reaction kinetics and incubation conditions determined for model fractions. Therefore fractions extracted are, for simplicity, assumed similar to these well characterized model fractions (e.g., specific iron oxide minerals, irons sulfides - AVS, pyrite, etc.) for which the kinetics of solubilization and the reactivity with specific chemical extractants under specific conditions have been determined. Results of such extractions have been used to determine the pools of metals in the bulk sediment that could be potentially bioavailable to animals ingesting these sediments. Bioavailability predictions determined by the sequential extraction procedure could be incorporated into ecological risk assessment protocols.

Sequential extraction methodology has been recognized as problematic due to variable recovery of specific phases or due to non-specificity of chemical extractants that are used in sequential extraction protocols (e.g., specific leaching steps can dissolve more than one targeted geochemical phase) (Martin et al., 1987; Nirel and Morel, 1990; Rapin et al., 1986). Despite these problems, sequential extraction remains a widely used approach to study metal distribution in the geochemical fractions that are operationally-defined. Sequential extraction methodology also provides an option to study the geochemical influences on metal bioavailability in deposit-feeding animals (Diks and Allen, 1983; Fan et al., 2002; Tessier et al., 1984).

Radiotracer approaches have been used in bioaccumulation studies for many metals and metalloids in aquatic organisms, including deposit-feeding polychaetes . The use of radiotracers allows determinations of metal assimilation efficiencies – a parameter directly related to a fraction of metal that is bioavailable upon ingestion (Croteau and Luoma, 2005; Wang and Fisher, 1999b).

In this study I hypothesized that addition of the radiotracers via fresh algal debris, representing metals associated with fresh algal organic matter, to the sediment would show different fractionation patterns of these radiotracers in comparison to sediment labeled directly by metal sorption from solution. Second, I hypothesized that fractionation pattern of radiotracers added directly to otherwise natural sediments would change over time in these wet sediments that were kept sealed in plastic containers. Both of these metal transport scenarios from the water column to seabed will here be mimicked. To address these hypotheses I conducted experiments employing radiotracers of As, Cd and Cr that were introduced to sediment via radiolabeled algal

debris and via direct addition of dissolved isotopes. In addition I also conducted sequential extractions of pure radiolabeled algal debris which could represent settling material following algal blooms, particularly in relatively shallow waters where debris could settle to underlying sediments. Radiolabeled wet sediments that were incubated at room temperature in sealed plastic containers for up to 90 days were extracted using sequential extraction procedures. Radiotracers are a useful tool for determining metal fractionation patterns which may control bioavailability of metals to deposit-feeding invertebrates.

MATERIALS AND METHODS

Sediment collection in the field

Sediment samples were collected using a box corer at 3 sites - two sites in Chesapeake Bay: one in Elizabeth River ("ER"; Norfolk, VA; 36°52'32"N, 76°20'09"W), and second in Baltimore Harbor ("BH"; Baltimore, MD 39°12'25"N, 76°31'40"W) both sampled from the RV Fay Slover, and third site located in San Francisco Bay: Mare Island ("MI"; Vallejo, CA; 38°05'15" N, 122°15'15"W) sampled from RV Questuary. Acrylic sub-core tubes (5.7 cm diameter and 30 cm length) were inserted into the sediment in each of the box cores. These tubes were capped and withdrawn from the box core. On board the ship, sediments in these subcores were sectioned at 1 cm intervals from the surface to 10 cm in depth inside a nitrogen-purged glove bag to limit sediment contact with oxygen in the air. A portion of surface (0-1 cm) sediment from each section was used to determine sediment porosity via wet/dry and other chemical analyses described in the following section. A mixture of sediment from the top layer (~20 cm) remaining in the box core was collected into plastic buckets, transported and stored (4°C, for up to 2 years) for subsequent radiotracer experiments. Sediments used for the experiments were oxidized by leaving them open to air.

Chemical analyses

Portions of the surface (0-1 cm) sediments were dried at 50°C and later ground with an agate mortar and pestle. Samples were then sieved through a 150 μm nylon mesh screen and stored in polyethylene bottles for further analyses. These samples were used to determine organic carbon, organic nitrogen, and sulfur using a Carlo Erba 1500 Elemental Analyzer (Cutter and Radford-Knoery, 1993). To evaluate metal concentrations, sediment samples from the top 1 cm were also

digested for 8 hours first by treatment with nitric acid in a boiling water bath. After cooling, concentrated perchloric acid was added to sediment samples to complete the digestion. All metals but aluminum (Al), were recovered with an efficiency of 98 ± 3% (n = 6). A large fraction of Al bound to silicates remained unextracted and only its extraction with hydrofluoric acid (HF) yielded full recovery. The sediment digest solutions were analyzed for metal concentrations using ICP-MS (Finnigan Element 2), with the standard additions method of calibration to assure accuracy.

Sequential extraction procedure

The extraction scheme used in the present study is illustrated in Fig. 1. The objective of the sequential extraction procedure is to selectively dissolve specific geochemical phases. Although these fractions do not represent pure mineral and/or organic phases and likely include other components, we maintained the original names for these fractions to be consistent with previous geochemical studies (Harvey and Luoma, 1985; Lee et al., 2000c; Tessier et al., 1979). The seven distinguished fractions were (in order of their recovery): *exchangeable, carbonate, acid-volatile sulfides (AVS), iron and manganese oxides* (*Fe/Mn oxide*), *organic I, organic II*, and *pyrite*. Methods were modified from Tessier et al. (1979) for oxic sediments and Huerta-Diaz and Morse (1990) for anoxic sediments. The *AVS* fraction was extracted according to Cutter and Oatts (1987).

We thus employed the following extraction procedure: First, 2 g (wet wt) of sediment was subjected to a 1-h incubation with 8 mL of 1 M $MgCl_2$ to desorb weakly-bound or adsorbed ions. Chloride ions could also complex Cd present in the $MgCl_2$ solution (Ruttenberg, 1992; Stumm and Morgan, 1996). After this first step extracting the *exchangeable* fraction, the remaining sediment was incubated for 1 h with 8 mL of sodium acetate (NaOAC) solution buffered at pH 5, targeting the *carbonates*. Some iron sulfides that are typically defined as the AVS and other pH sensitive minerals remaining in the residue of the sediment could be removed at this lower pH. During method testing nearly 20% of AVS incubated with the sodium acetate solution was dissolved. Further, in this incubation anionic metal groups such as arsenate could exchange with the acetate functional group. This extraction step was designed to target carbonates and will be referred to as *carbonates*, despite the recognized possibility that other fractions (e.g., some weak acid soluble and anion exchangeable materials) could be also removed during this extraction.

The third extraction step was designed to remove *AVS*. It involved 30 minutes incubation at 21°C with 10 mL of 0.5 M HCl. Some iron oxides may have also been extracted in this step (Schwertmann, 1991) and metals in clays could be released as a result of lower pH (Tessier et al., 1979). *Fe/Mn oxides* were the next targeted fraction and the remaining sediment was incubated for 6 h with 15 mL hot (96 ± 3°C) 0.04M hydroxylamine dissolved in 25% (v/v) acetate - a standard solution used in quantitative solubilization of iron and manganese oxides. The extraction of *Fe/Mn oxides* fraction was followed by a sequence of a basic (20 mL of 1 N NaOH, at 80°C) 8-h leach followed by a 6-h hydrolysis in sulfuric acid (10 mL of 5 M H_2SO_4), and it targeted two organic fractions referred herein as fractions *organic I* and *organic II*. According to Huerta-Diaz and Morse (Huerta-Diaz and Morse, 1990) the incubation of sediment with H_2SO_4 would decrease the organic carbon content if the previous step (i.e., 1 N NaOH) was insufficient. It is also important to note that these two steps address the extraction of the organic matter, which may represent refractory compounds such as humic and fulvic acids - extractable by NaOH, and other remaining organic compounds - extractable by H_2SO_4. Previous steps of the sequential extraction procedure – for example the hydrochloric acid extraction, could potentially remove some of the labile organic compounds (e.g., proteins) if these labile compounds were present in analyzed sediment. Hence NaOH extraction was assumed to target detrital organic matter as modified from Harvey and Luoma (1985) and H_2SO_4 was used to extract the remaining organic carbon as described by Huerta-Diaz and Morse (Huerta-Diaz and Morse, 1990). Importantly, incubation of sediment with H_2SO_4, was assumed not to solubilize pyrite. The last step of the sequential extraction procedure targeted a fraction that presumably included primarily *pyrite*. This fraction was targeted with 3 mL of cold 11 N HNO_3 by incubating the remaining sediment for 2 h. It is presumed that if any unextracted refractory organics were left over from the NaOH and H_2SO_4 incubations the last step (nitric acid) of the sequential extraction would fully recover the remaining organic matter in the sediment. At the end of each extraction, sediment was separated from the extract by centrifugation at 834 *g* for 15 minutes. The remaining sediment was twice rinsed with 8 mL of deionized water, and the liquid was again separated from the sediment by centrifugation, and added to the previously collected extract for further analyses, described below.

Radiolabeling of the sediment

Addition of dissolved radiotracers

In one series of experiments, the binding of As (arsenate), Cd (cadmium chloride) and Cr (chromic chloride) to different geochemical phases in sediments was determined using gamma-emitting radioisotopes (^{73}As, ^{109}Cd and ^{51}Cr) added directly to the sediments. Briefly, three replicate aliquots (2 g wet weight) of sediment representing a mixture from 0-20 cm from each site were placed in separate containers (50 ml polypropylene conical tubes) and radiolabeled by addition of ^{73}As ($t_{1/2}$ = 80.3 d) or ^{109}Cd ($t_{1/2}$ = 461.4 d) combined with ^{51}Cr ($t_{1/2}$ = 27.7 d). Depending on the activity of the radioisotope stock solution, up to 20 µl was withdrawn from 0.1 M (^{73}As and ^{109}Cd) or 0.5 M HCl (^{51}Cr) stock solutions with a pipette, mixed with dilute NaOH to neutralize the acid, and pipetted into the sediment. In most cases the entire surface of the sediment was covered by the liquid containing the radiotracers. Following isotope additions, sediments were aged for 2, 30 and 90 d (except sediments labeled with ^{51}Cr, which were only aged for 2 and 30 d due to its short half-life) in closed 50 mL Falcon tubes at 21°C. The 2 d time point allowed evaluation of the rapid metal association with the particles while the 30 and 90 day time points provided material to study any further time-dependent changes in fractionation of the added radiotracers. During the incubation some black spots developed in the sediment indicating anoxia. Activities per mass and molar concentrations of added radiotracers are specified in Table 1. Molar concentrations were small and did not increase the overall metal concentrations in sediments by more than 0.01%.

Addition of radiotracer to the sediment via phytoplankton

In another series of experiments, ^{73}As, ^{109}Cd and ^{51}Cr were added to sediments via mixing with radiolabeled diatom cells. Cultures of *Thalassiosira pseudonana* were grown in f/2 medium (Guillard and Ryther, 1962) prepared with surface seawater (salinity of 35) collected 8 km offshore of Southampton (Long Island, NY) in the presence of ^{109}Cd and ^{51}Cr or in the presence of ^{73}As (at f/20 levels of phosphate, or 3.6 µM). Algae were grown to stationary phase and were harvested by filtration onto 3.0 µm Nuclepore polycarbonate filters. Filters were then immersed into plastic conical vials filled with 10 ml of seawater so that the diatom biomass could be resuspended. Containers with such concentrated algae were centrifuged at 834 *g* for 5 minutes to

separate the liquid from the biomass. Microscopic observations of cells after centrifugation led me to believe the cells stayed intact. Wet algal biomass, whose dry mass equivalent was 22.3 ± 4.5 mg (Fisher and Schwarzenbach, 1978) of these radiolabeled algae were mixed with 2 g wet wt of oxidized sediments collected with a box corer. This increased the total C content of the original sediment from 5.0% to 5.4% in BH, from 2.0% to 2.4% in ER and from 1.5% to 1.9% in MI based on an assumption that the C content in a cell of *Thalassiosira pseudonana* is 8 pg (Pechenik and Fisher, 1979), and equal to 35% of cell dry wt. Labeled sediment was held at 21 °C for 30 days and development of anoxia was visible at the surface of incubating sediments. Activities per mass and molar concentrations of radiotracers added via algae are specified in Table 1.

To evaluate the influence of the sequential extraction procedures on As, Cd, and Cr extractability from pure phytoplankton, aliquots of the radiolabeled algae (not mixed with sediments) were also extracted directly using the first three extraction steps, without mixing with sediments. By doing so, I evaluated the influence of these extraction steps on the removal of metals that are largely bound to organic compounds in cells, at least for As which is known to primarily associated with arsenosugars (Cullen and Reimer, 1989). The biochemical association of Cd and Cr in algal cells is less well-characterized, but an appreciable amount of the Cd is found in the cytoplasm of diatoms (Ettajani et al., 2001; Reinfelder and Fisher, 1991).

Measurement of radioactivity

Initial radioactivity of sediment samples and the radioactivity of samples containing the extracted radioisotopes was measured using a well-type NaI(Tl) gamma detector (Canberra). Radiolabeled sediments and liquids collected at the end of each sequential extraction step were kept in plastic conical Falcon tubes that could hold up to 50 mL of liquid. The final volume of liquid in extracts varied. This required correcting for a variability due to counting geometry changes (i.e. different sample volumes). A function correcting for the counting geometry error was developed based on data generated in a test experiment. Two sets of three conical Falcon tubes (max volume = 50 mL) were inoculated with ^{109}Cd alone (first set), and with a combination of ^{73}As and ^{51}Cr (second set). ^{73}As and ^{51}Cr were combined because their emission energies did not interfere with each other. Initial radioactive counts were recorded for each tube containing no liquid in addition to the µL amount of the radiotracers. Further, volumes of deionized water were added to reach

24, 26, 31 and 36 ml, which were matching the volumes of extracts mixed with rinse water from each of the extractions. Radioactive counts of ^{73}As, ^{109}Cd and ^{51}Cr were recorded for individual tubes for each of the volumes with the instrument counting error of << 1%. Based on the results of the test, the counting geometry correction was applied to each radioactive reading by using the following functions:

$$^{73}\mathrm{As} \quad \%\ cpm\ detected = -0.0252\mathrm{vol}^2 + 0.7768\mathrm{vol} + 96.56;\ R^2 = 0.999$$
$$^{109}\mathrm{Cd} \quad \%\ cpm\ detected = -0.071\mathrm{vol}^2 + 3.4518\mathrm{vol} + 50.446;\ R^2 = 0.991$$
$$^{51}\mathrm{Cr} \quad \%\ cpm\ detected = -0.0375\mathrm{vol}^2 + 1.3248\mathrm{vol} + 68.975;\ R^2 = 0.972$$

Liquids that were extracted during the sequential extraction procedure that were mixed with the rinse solution were counted for periods of time assuring errors <5%, usually < 5 minutes.

Reproducibility of extractions and statistical analyses

Reproducibility of the overall extraction procedure was evaluated with coefficients of variation (CV) for different treatments. CVs for all treatments were found typically below 20% for the dominant phases in Baltimore Harbor and Elizabeth River sediments labeled directly with radioisotopes, and 33% for Mare Island sediments. No systematic differences were noted in reproducibility between metals, sediment phases, or ages in sediments labeled directly. For sediments radiolabeled via addition of algal debris, CVs were 7% for Baltimore Harbor sediments (for dominant phases in all treatments), 13% for Elizabeth River sediments, and 17% for Mare Island sediments. CVs for at all sites were 12% for As, 17% for Cd, and 5% for Cr.

Statistical analyses of replicate (n = 3) samples used PASW 18.0 software. All the data expressed as % metal in a given sedimentary fraction were arcsine transformed to normalize their distributions. One-way ANOVAs were used to detect significant differences in metal partitioning between treatments, including sites, specific sedimentary fractions, and ages.

Assimilation efficiencies

Assimilation efficiency or AE is a parameter directly related to the bioavailability of ingested metal. Assimilation efficiency data of ^{73}As, ^{109}Cd and ^{51}Cr for a deposit-feeding polychaete *Nereis succinea* that were feeding on radiolabeled sediments are from Baumann and Fisher

(2011). Briefly, a series of pulse-chase feeding experiments was conducted to estimate AE values for sediments from all three locations. Sediments labeled directly or by addition of radiolabeled algae to sediments and incubated for 2 and 30 days were placed in feeding chambers and worms were given ~ 4 hours to feed. Polychaetes used in these experiments were previously acclimated to laboratory conditions and specific diet type. Feeding on the pulse of the radioactive sediment was followed by measurement of radioactivity for individual worms in a gamma-counter. After radioanalysis, individuals were placed back into their feeding chambers filled with nonradioactive sediment and refreshed seawater. At this stage worms were allowed to clear their guts off of sediment containing unassimilated radioisotope. The amount of radioactivity in worms was monitored every few hours during the first 24 hours and then daily. AEs were calculated based on radioisotope percentages retained in worms plotted against time, as y-intercepts of the trend line describing the slowly turning over radioisotope pool in worm tissues. Details of AE calculations are provided in Wang et al. (1996).

RESULTS

Sediment characterization

The organic carbon content differed considerably between sediments from MI (1.5%) and ER (2%) and BH (5%). The lower atomic C: N ratios in MI sediments (14) than in Chesapeake Bay sediments (ER: 19 and BH: 18), indicating marine vs. terrestrial inputs of organic matter because the C: N ratio of marine algae is 6.6, while the terrestrial organic matter C: N ratios are higher (~15-30; Hedges et al., 1997). The sulfur fraction in sediments from Chesapeake Bay was slightly higher than in MI sediments (0.5 and 0.6% vs. 0.4%), resulting in higher atomic C: S ratios in BH (10.0) than in MI (3.8) sediments (Table 2).

Metal concentrations in ER sediment were 5-8 fold higher for Fe, ranging between 3.2 and 23.2 mg g^{-1}, and 3-5 fold higher for Mn than in BH and MI sediments ranging between 53.9 and 94.7 µg g^{-1} (Table 2). Concentrations of Ca were 2 orders of magnitude lower in ER sediments (8.6 µg g^{-1}) than in BH and MI (134 and 718 µg g^{-1}, respectively). Both As and Cr in BH were an order of magnitude higher (47.2 and 322.7 µg g^{-1}, respectively) than in ER (6.4 and 33.3 µg g^{-1}, respectively) and MI (1.4 and 47.4 µg g^{-1}, respectively), and Cd showed the least variation among the metals, being lowest in ER and highest in MI (0.5 – 2.4 µg g^{-1}; Table 2). Ratios of

metals to Al in sediments were higher than in Earth crust for Fe (1.2 – 987.2 vs. 0.5), Mn (0.02 – 12.0 vs. 0.01), As (only for BH and ER: 0.02 – 0.3 vs. 0.01), and for Cr (0.018 – 1.4 vs. 0.001) indicating anthropogenic contamination (Table 2). In contrast, Cd did not appear to be a contaminant in the sediments I investigated as the ratios of Cd/Al were lower than in Earth crust.

Metals extracted from pure algae

The recovery of the radioisotopes from the triplicate samples of pure (i.e., not mixed with sediment) radiolabeled algal debris that were subjected to the first three steps (1 M $MgCl_2$ targeting the *exchangeable* fraction, NaOAc at pH 5 targeting the *carbonate* fraction, and 0.5 M HCl targeting the *AVS* fraction) of the sequential extraction procedure was less than 100% for ^{73}As and ^{51}Cr. For As, 80 ± 9% (mean ± 1 SD) was extracted, and 80 ± 7% of ^{51}Cr was extracted, whereas all of ^{109}Cd was recovered during these extraction steps (Fig. 2). The percentage of recovered^{109}Cd from algal debris was > than 100% due to measurement uncertainty. Much of the originally added radioisotope - ^{73}As (38 ± 1%), ^{109}Cd (69 ± 1%) and ^{51}Cr (54 ± 0.4%) was extracted with the acidic (pH =5) solution of sodium acetate in the second step of the procedure. The first extraction step, initially designed to strip off the metals loosely bound to sedimentary particles in the so called *exchangeable* fraction, removed less ^{73}As and ^{51}Cr (14 ± 1%; 9 ± 10%, respectively) than in the two remaining steps. This was likely due to osmotic stress that cells could experience due to high ionic strength of used extractants. A quarter of the ^{109}Cd was already extracted from the algal biomass during the first step. After 65% of ^{73}As was extracted in the first two steps, HCl (third step) leached out an additional 28 ± 8% of this isotope, leaving some of the ^{73}As unextracted. In this third step, only 13 ±1% of ^{109}Cd and 17 ± 2% of ^{51}Cr was extracted from the remaining algal debris.

Radiotracers extracted from sediments mixed with algae

After 30 d incubation of radiolabeled algal debris with sediments, ^{73}As, ^{109}Cd and ^{51}Cr partitioned to different sedimentary fractions (Fig. 3). At the end of the sequential extractions 100% of ^{109}Cd, 98-99% of ^{51}Cr and 82 – 86% of ^{73}As were recovered (Fig. 3). Two main sedimentary fractions accounting for 38 – 58% of ^{73}As were the fourth and fifth in the sequence i.e., Fe/Mn oxides and organic I, which were extracted with a reducing solution of hydroxylamine and solution of sodium hydroxide. Most of the ^{109}Cd was extracted from

sediments mixed with algae by the neutral solution of $MgCl_2$ and the acidic solution of NaOAc in the two most reactive fractions i.e., *exchangeable* and *carbonate*, accounting for 41-78% of this radioisotope. Much (13-39%) of the ^{109}Cd was also in the fraction extracted by HCl (step 3), thought to represent the AVS. Similarly to ^{109}Cd, much of the ^{51}Cr (41-66%) was also released during the hydroxylamine extraction. This was the primary pool of ^{51}Cr in all of the sediments. Between 10 and 13% of ^{51}Cr was released during the HCl incubation, and 12-20% of the ^{51}Cr was extracted from sediment by oxidizing it with sulfuric acid.

Time dependent changes in ^{73}As, ^{109}Cd and ^{51}Cr fractionation in sediments labeled directly

Isotopes introduced to sediments via direct injection partitioned to various operationally defined fractions. Arsenic was the only radioisotope not completely recovered during the sequential extraction procedure. For sediments from all three sites the average percentage of ^{73}As in the final *residual* fraction was <17% at 2 days of exposure and increased, although not significantly, up to 25% at 30 and 90 days (Fig. 3). Only 2% or less of the ^{51}Cr remained in the final residue, whereas ^{109}Cd was fully recovered by the sequential extraction procedure for all of the sediment treatments.

For all sediment types exposed to ^{73}As for 2, 30 and 90 days, 30 - 43% was extracted by incubating the sediment with the oxidizing solution of sodium hydroxide (i.e., the organic I fraction). Sulfuric acid extracted (*organic II* fraction) 11-16% of ^{73}As from BH sediments, hot hydroxylamine (*Fe/Mn oxides* fraction) extracted 14-29% and 8-27% of ^{73}As for ER and MI, respectively. In Chesapeake Bay sediments (BH and ER) the concentration of ^{73}As in the AVS fraction decreased from the initial 9 or 29% (BH, ER) to less than 3% at the end of the three month incubation (Fig. 3). This decrease was the only significant temporal change (one-way ANOVA, $p < 0.05$) for ^{73}As fractionation during the three month incubation experiment.

Overall, in both of the Chesapeake Bay sediments ^{109}Cd was predominantly extracted by $MgCl_2$ (*exchangeable* fraction). There was a significant increase (one-way ANOVA, $p < 0.05$) of ^{109}Cd in the *exchangeable* fraction between 2 and 30 days in ER sediments and between 30 and 90 days for BH sediments, and a parallel significant decrease (one-way ANOVA, $p < 0.05$) by up to 7-fold, in ^{109}Cd fractions extracted as *AVS* and *Fe/Mn oxides*.^{109}Cd from BH and MI sediments exposed for 30 d was extracted in similar proportions in the first four extraction steps (Fig. 2).

Distribution of ^{51}Cr showed the greatest level of agreement among the sediment locations. In all of the sediments at 2 days of exposure, ^{51}Cr was primarily extracted by HCl (40-59%) and hydroxylamine (29-35%) representing fractions identified as *AVS* and *Fe/Mn oxides*. During the 30 d incubation ^{51}Cr, decreased by 56-63% in *AVS* and *Fe/Mn oxides* fractions and significantly increased by 41-56%in pyrite fraction for all sediments (one-way ANOVA, $p < 0.05$).

^{73}As assimilation efficiencies and concentration in exchangeable + carbonate fractions from sediment labeled with algae and via direct addition

In *N. succinea* the assimilation efficiencies of algal ^{73}As incubated with sediments from BH, ER and MI for 30 days were higher than from sediments labeled directly - and incubated for 30 days (Fig. 4). While values for As AEs in algae labeled sediments were available for all sediments (BH, ER and MI), AEs for As labeled directly for 30 days were not available for ER sediment. Arsenic assimilation patterns for sediments labeled by these two methods are in parallel with ^{73}As concentrations as extracted in the pooled operationally defined *exchangeable* and *carbonate* fractions, which are also higher for sediments labeled by algae vs. sediments labeled by direct addition (Fig. 4).

DISCUSSION

Utility of the sequential extraction procedure for algae enriched sediments

Sequential extraction procedures have been designed to extract sedimentary phases from primarily inorganic sediments with small organic matter fractions (Tessier et al., 1979). Traditionally, the refractory organic matter pool represented by the operationally defined *fulvic* and *humic acids* have been extracted by sodium hydroxide and sulfuric acid but extraction of fresh algal organic matter has not previously been addressed in the sequential extraction procedures.

Our results indicate differences in radioisotope extractability from substrates containing fresher and more labile organic matter derived from phytoplankton cells and older, more diagenetically reworked organic matter that is buried in sediment, where the radioisotopes associated initially with algae were more easily extracted. In algal cells organic arsenic, such as arsenosugars (Cullen and Reimer, 1989), was extracted by chemical solutions designed to target inorganic

sediment phases. This is not surprising because solutions used in the first three steps of the procedure could extract some arsenosugars and other organic As compounds. There are however specific protocols designed to extract specific compounds such as arsenosugars, which were not part of the sequential extraction procedure used here (Raber et al., 2000). In the case of elements that are taken up by living organisms such as algae and converted into organic molecules, these molecules could be extracted by solutions other than those targeting the humic and fulvic acids used in the sequential extraction procedures. Therefore, one could mistakenly assume that if these metals are extracted by solutions designed for inorganic fractions these metals are inorganic, while in fact this may not be the case. This realization is important when interpreting extraction results of estuarine sediments, particularly when collected following algal blooms. Sediments at these times could be enriched with relatively fresh algal organic matter containing organometals or metals that are weakly and not covalently associated with organic compounds in decomposing cells (Kowalski et al., 2009). Perhaps these metals could be extracted by $MgCl_2$ solutions but this remains to be tested. Metals associated with microflora living in sediment could also serve as a source of "organic – associated" metals, although this fraction is likely very small due to the small microbial biomass relative to the mass of the sediment.

An assumption that adding to the sediment algae-associated radiotracer would result in its increased concentration within the operationally-termed *organic* fractions is supported by results for ^{109}Cd and for ^{51}Cr in sediments from BH and MI, which show an increased concentration of these radiotracers in the algae mixed sediments in comparison to sediments labeled directly. Specifically, ^{109}Cd in sediments labeled directly that were aged for 2 and 30 days was lower in the two *organic* fractions combined than in sediments radiolabeled by algae, and ^{51}Cr was higher in these *organic* fractions in sediments from BH and MI labeled by algae than in sediments labeled directly and aged for 2 days. This assumption is however not supported by ^{73}As results, although addition to sediment algae associated ^{73}As results in its higher concentration in the most easily extractable fractions i.e., exchangeable and *carbonate* pool in comparison to sediments labeled directly. This finding is important due to previously reported linkage between easily extractible sedimentary metals and their higher assimilation in benthic animals (Baumann and Fisher, 2011b; Lee and Luoma, 1998).

Application of the sequential extraction procedure for investigating ^{73}As, ^{109}Cd and ^{51}Cr bioavailability in a deposit-feeder *N. succinea*

The natural organic matter from field-collected sediments had 2-3 times higher C: N ratios (14-19) than fresh algae (C: N ~6.6), and 1.4 -1.9 higher than algae degraded by bacteria (C: N ~10) (Ferguson et al., 2003). This suggests therefore that sediment from MI which had the lowest C: N ratio was more impacted by the marine algal biomass. Despite the relatively small amount of added algal biomass in this study, it is possible that labile organic matter, which is also highly nutritious, could increase the metal bioavailability in deposit-feeding animals. The greater content of the fresh algal organic matter vs. more degraded and less nutritious organic matter would likely alter the ingestion rates in *N. succinea*. Further, a study by Lee and Luoma (1998) showed that assimilation efficiency of ingested Cr ranged between 2.0 and 2.5% in the clam *Potamocorbula amurensis* feeding on sediment and between 11.6 – 12.2% for clams feeding on microalgae. Baumann and Fisher (2011b) found that the assimilation efficiency in the polychaete *N. succinea* of ingested As from algal cells was 72%, but only 1-12% when As was bound to unamended sediment. Sequential extraction procedures showed that 80% of ^{73}As was extracted from radiolabeled algae in the first three steps (Fig. 2), similar to its assimilation efficiency from algal cells.

Using approaches relying on chemical extractions of toxic metals to study metal bioavailability does not mimic the physiological processes that take place in the polychaete guts. Instead they serve as a proxy estimating the pool of metal that could be assimilated by polychaetes. Furthermore, ^{73}As AEs are higher in *N. succinea* feeding on 30 d old algae-labeled sediment than for those feeding on 30 d old directly-labeled sediment (Fig. 4). This difference is even more pronounced in fresh sediments (Baumann and Fisher, 2011b). The greater bioavailability of ^{73}As from sediment mixed with labeled algae is consistent with the greater percentage of ^{73}As extracted in the first two steps of the sequential extraction procedure from these mixed sediments (Fig. 4).

Combined metal pools extracted in steps 1(by $MgCl_2$) and 2 (by NaOAc) have indeed been demonstrated to be positively related to bioavailability to *N. succinea* for As, Cd, and Cr (Baumann and Fisher, 2011b). Diks and Allen (1983) related Cu fractionation in sediment with its bioavailability to freshwater worms, where Cu bioavailability showed a positive relationship

to Cu in the “adsorbed/exchanged” fraction, extracted in the same manner as the exchangeable fraction in the present study. Fan et al. (2002) similarly found that assimilation efficiencies of Cd in mussels and clams increased with Cd fractionation in the exchangeable pool ($MgCl_2$ extracted).

Radiotracers are useful for investigating sedimentary metal bioavailability in deposit-feeding polychaetes. Radiolabeled sediments can be used to experimentally to determine metal assimilation efficiencies, and they can be used to determined operationally-defined radiotracer fractionation by sequential extraction methodology. Future studies are required to evaluate the application of this method for other metal contaminants and other deposit-feeding animals. This approach can ultimately lead to improved modeling of sedimentary metal transfer in the benthic food chains.

TABLES

Table 1. Concentration of radiotracers expressed in units of radioactivity Bq/g and moles in sediment from all three locations at the time of the experiment

Label	Equilibration time (d)	Bq/g (wet wt)			nmol/g (wet wt)		
		^{73}As	^{109}Cd	^{51}Cr	^{73}As	^{109}Cd	^{51}Cr
	2	200	59	71	10	44	49.2
direct	30	209	36	59	10.2	26.6	47.4
	90	104	31		5	22.2	
algae	30	68	47	353	3.3	34.8	232.2

Table 2. Geochemical properties such as elemental concentrations and porosity (φ) in surface (0-1cm) sediment, salinity of the overlying water collected from Baltimore Harbor (BH), Elizabeth River (ER) and Mare Island (MI). Elemental composition of Earth's crust and metal to Al ratios are also provided as a reference for potential contamination.

	units	Location							
		Concentrations				metal/Al ratio			
		BH	ER	MI	Earth Crust	BH	ER	MI	Earth Crust
Fe	mg g^{-1}	4.8	23.2	3.2	36	2.4	987.2	1.2	0.5
Al		1987 [a]	23.5 [a]	2657 [a]	6.9E+04	1.00	1.00	1.00	1.00
Ca		134	8.6	718		0.07	0.4	0.3	
Mn		94.7	281	53.9	525	0.05	12.0	0.02	0.01
As	µg g^{-1}	47.2	6.4	1.4	787	0.02	0.3	0.001	0.01
Cd		1	0.5	2.4	2.0E+05	0.0005	0.02	0.0009	2.9
Cr		322.7	33.3	47.4	71.2	0.16	1.4	0.018	0.001
C		5	2	1.5					
N	wt %	0.3	0.1	0.1					
S		0.5	0.6	0.4					
atomic C:N		18	19	14					
Salinity	ppt	8.5	19.5	23					
Φ		0.8	0.7	0.8					

Concentration of Al is not representative of the total; HF extraction of sediment was not conducted

FIGURES

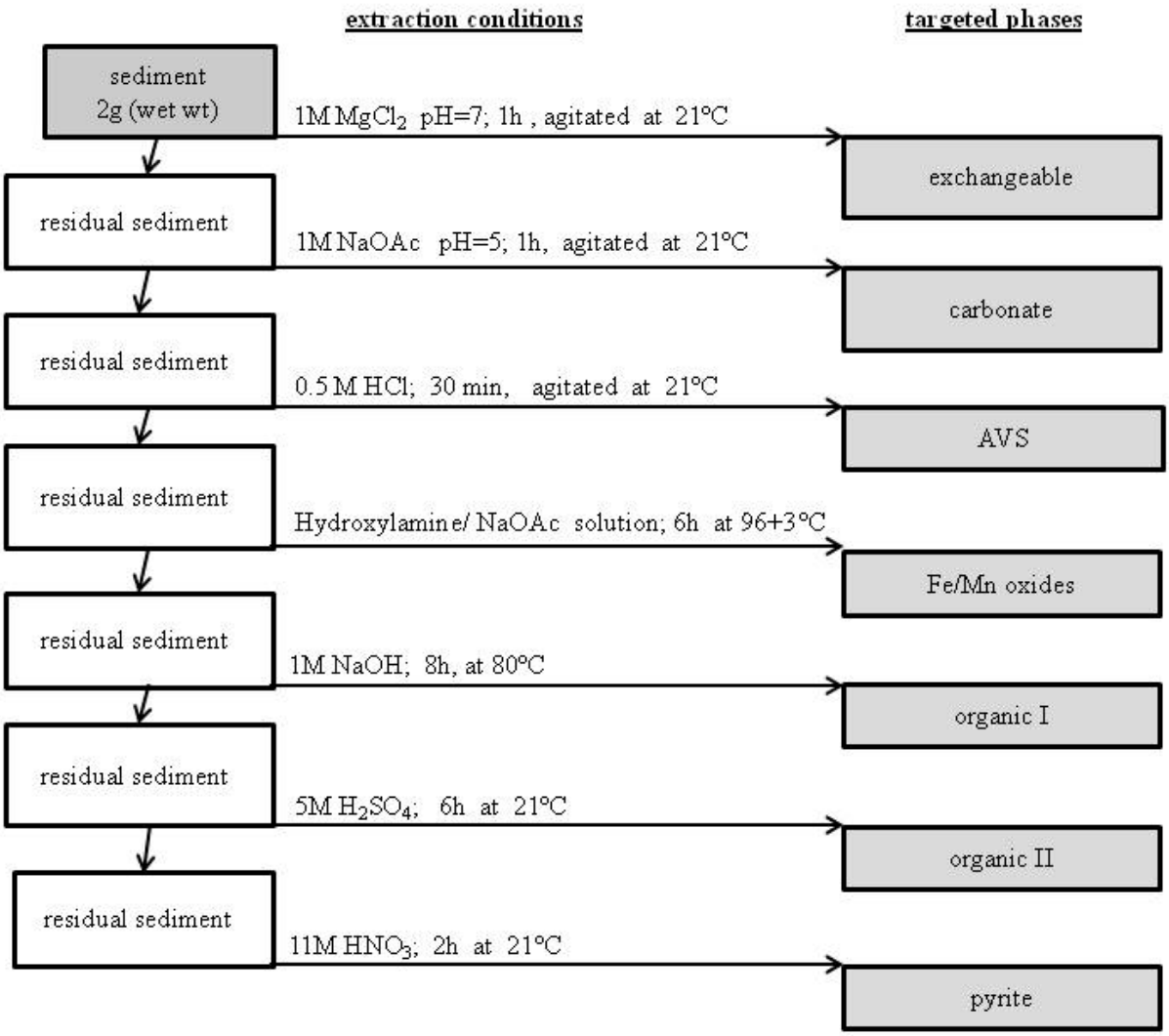

Fig. 1. Scheme showing the steps of the sequential extraction procedure used in this study.

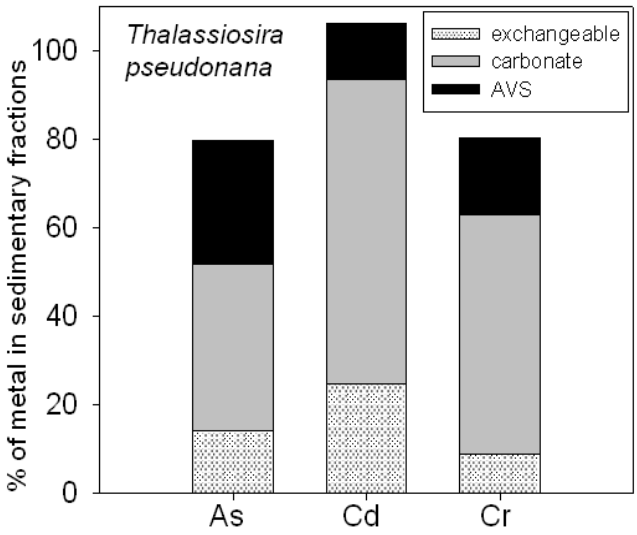

Fig. 2. Per cent of total extracted 73As, 109Cd and 51Cr from pure algal cells in the first three steps (exchangeable, carbonate and AVS phases) of the sequential extraction procedure.

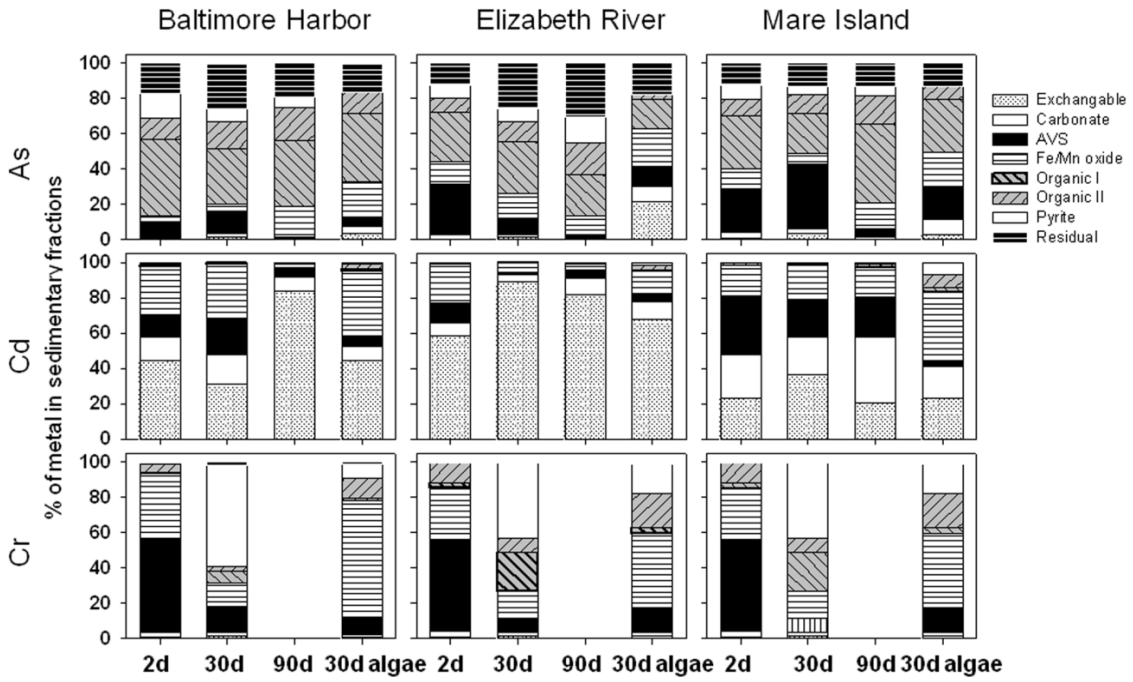

Fig. 3. Phase speciation of ^{73}As, ^{109}Cd and ^{51}Cr expressed as per cent of metal in sequentially extracted geochemical fractions from estuarine sediments that were equilibrated with the radiotracers added directly for 2, 30 and 90 days after and with radiotracers added via previously radiolabeled algae for 30 days

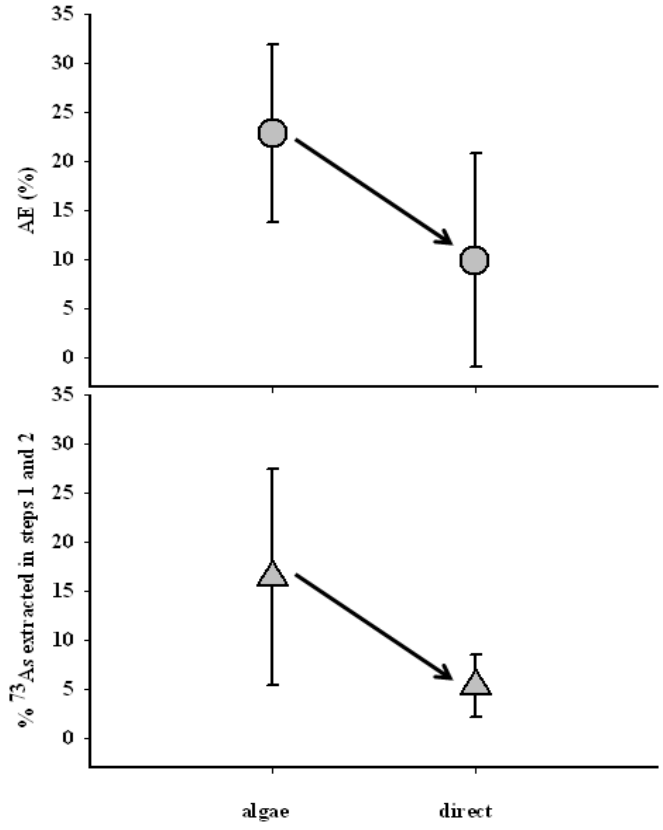

Fig. 4. Mean assimilation efficiencies (± 1 standard deviation; grey circles; top panel) of ^{73}As in *Nereis succinea* fed sediments mixed with radiolabeled algae incubated for 30 days (left) and sediments that were labeled by a direct addition of the radiotracer and aged for 30 days (right). Mean percentage (± 1 standard deviation; grey triangles; bottom panel) of ^{73}As extracted in the first two steps of the sequential extraction procedure (i.e., magnesium chloride and sodium acetate) from sediments mixed with radiolabeled algae that were incubated for 30 days (left) and sediments that were labeled by a direct addition of the radiotracer and aged for 30 days (right). Average values of AEs and extracted in steps 1 and 2 ^{73}As represent all of the sediment types i.e., BH, ER and MI, except AEs for ER sediment (not determined).

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Salinity Effects on the Bioavailability of Aqueous Metals for the Estuarine Killifish *Fundulus heteroclitus*

ABSTRACT:

Estuarine organisms experience varying salinity conditions on a daily and seasonal basis, and these fluctuations could influence the amount of metal accumulated from the aqueous phase. We experimentally assessed the role of salinity (0, 2, 6, 12, and 25 ppt) on the uptake of As, Cd, Cr, inorganic Hg (Hg(II)) and methylmercury (MeHg) into the euryhaline killifish (*Fundulus heteroclitus*) from the aqueous phase using gamma-emitting radioisotopes. Patterns of metal uptake as a function of salinity varied by metal. Chromium showed no relationship with salinity, Cd, which was most affected by salinity showed an inverse relationship, and As, Hg(II), and MeHg uptake increased as salinity increased from 0 ppt to 25 ppt. Arsenic (at salinities ≤ 6 ppt) and Cr were regulated by the fish, whereas Cd, Hg(II), and MeHg were not. Cadmium, Hg(II), and MeHg chloro-complex, increasing bioavailability for Hg(II) and MeHg, and reducing bioavailability for Cd. Concentration factors (CFs) were > 1 at all salinities for Cd, Hg(II) and MeHg, indicating that the fish were more enriched in the metal than the surrounding water, whereas As and Cr CFs were < 1 at all salinities. Uptake rate constants (k_us) were highest for MeHg (0.79 to 2.29 L g^{-1} d^{-1}), followed by Hg(II), Cd, Cr, and lowest for As (0.0004 to 0.0008 L g^{-1} d^{-1}). Tissue distribution of each metal was determined by dissections. Data for Cd showed that as salinity increased the concentration of this metal increased in the viscera, whilst it decreased in the head and gills, suggesting that drinking to osmoregulate may account for a portion of Cd uptake from the aqueous phase in marine fish.

Keywords: *Fundulus heteroclitus*, Metals, Salinity, Bioavailability, Uptake rates

INTRODUCTION

Field studies have shown that fish caught in heavily urbanized and industrialized estuaries have elevated metal levels [1,2]. Like other aquatic organisms, fish are exposed to waterborne metals through the aqueous phase and their diet [3-6]. Water properties including dissolved organic matter concentration and salinity have been shown to affect metal bioavailability from the dissolved phase for a diverse range of aquatic organisms, including phytoplankton, for which the bioconcentration step is typically greatest [7]. The trophic transfer of metals from phytoplankton to zooplankton to fish has been shown to account for a substantial portion of a fish's body burden for a diverse range of metals [3,5,6]. However, the uptake of dissolved metal still needs to be quantified, because if a food source is in limited supply, or the fish is an infrequent feeder then dissolved exposure may become an important uptake route.

Studies have shown that salinity can play an important role in influencing the bioavailability of some metals to aquatic organisms due to chloro-complexation of the metal ion; Cd uptake has an inverse relationship with salinity [8,9], whereas inorganic Hg (Hg(II)) and methylmercury (MeHg) accumulation shows no consistent relationship with salinity. Some studies show greater Hg(II) and MeHg accumulation with decreasing salinity, while other studies show greater accumulation as salinity increases [10-12]. Cationic and anionic competition also increases with salinity for biological uptake sites. For example, cationic competition between Cd^{2+} and Ca^{2+} has been documented, with both elements sharing the same uptake channel in fish [13,14]. Competition between anionic arsenate (As(V)) and PO_4 influences As(V) uptake, as noted in phytoplankton [15]. As a result, metal uptake as a function of salinity varies by metal.

Aqueous exposure studies have quantified metal uptake into fish [3-5,16,17], but there is a paucity of information on how metal uptake in fish varies with salinity. Studies have reported the influence of salinity on the acquisition of Cu in *Fundulus heteroclitus* [18], Ag in the gulf toadfish (*Opsanus beta*) [19], Cd in the tidewater silverside (*Menidia beryllina*) [9], and Cd and Zn in the black sea bream (*Acanthopagrus schlegeli*) [20]. In estuarine systems salinity fluctuates with the daily tidal cycle, potentially influencing the rate of metal uptake from the aqueous phase. Salinity can also show seasonal variability, influenced by the amount of freshwater entering the system during the spring freshet or periods of drought. Understanding this relationship can help model the relative importance of dietary versus dissolved uptake under varying salinity conditions using a well-developed metal bioaccumulation model [21,22].

We investigated the influence of salinity (0, 2, 6, 12, and 25 ppt) on the uptake of three metals (Cd, Cr(III), and Hg (as Hg(II), and MeHg)) and a metalloid (As(V)) into killifish (*Fundulus heteroclitus*) from the aqueous phase. Killifish are a potential bioindicator of regional metal contamination due to their wide distribution [23] and limited swimming range. Furthermore, they are euryhaline [23], have varying sensitivity to different contaminants, and may serve as a conduit for the transfer of metals to higher trophic levels including crabs, fish, sharks and birds [24-26]. Uptake was monitored using gamma-emitting radioisotopes, and concentration factors (CFs) and uptake rate constants (k_us) were calculated for each metal at each experimental salinity. Fish were then dissected to assess whether the tissue distribution of each metal varied with salinity.

Metals were selected based on their chemical characteristics and environmental interest. The chosen trace elements have varying protein associations due to their binding preferences for nitrogen or sulfur; As, Cd, and Cr are borderline metals/metalloid, whereas Hg is a sulfur-

seeking Class B metal [27]. Cadmium, Hg(II), and MeHg are known to be chloro-complexed, whereas As and Cr are not [28]. These metals, except for As, are particle-reactive, cationic, and can passively sorb to organism surfaces. They are found at elevated concentrations in industrialized estuaries [1,2], and are of interest from marine ecosystem and public health perspectives.

MATERIALS AND METHODS

Fish

Field collected (Taylor River, Hampton, NH, USA; salinity = 25 to 30 ppt) juvenile killifish, *Fundulus heteroclitus*, (mean wet weight 1.86 g ± 0.4 SD) were obtained from Aquatic Research Organisms (Hampton, NH, USA). Fish were acclimated to experimental conditions for at least 4 weeks prior to the beginning of experiments, and fed a diet of TetraCichlid™ cichlid flakes (Tetra Holding Inc., Blacksburg, VA, USA) and frozen bloodworms daily. Fish were starved for 36 h prior to the start of the experiments to allow for complete gut clearance, and not fed throughout the experiments so the aqueous phase was the only source of metals for the fish.

Water and experimental conditions

Experiments were conducted using 0.2 µm sterile-filtered (Millipak 200, Millipore, Bedford, MA, USA) Hudson River Water (HRW; salinity = 0 ppt), collected in Poughkeepsie (NY, USA), and adjusted to a salinity of 2, 6, 12 or 25 ppt using Instant Ocean™ (Aquarium Systems Inc., Mentor, OH, USA). Hudson River water was used because the dissolved organic carbon (DOC) concentration is high enough that any added DOC associated with Instant Ocean™ is negligible against background values. All water parameters (pH, temperature, DOC concentration) were essentially equal among treatments at the start of experiments, so salinity

was the only variable (Table 1). Dissolved organic carbon concentration was analyzed using a Shimadzu TOC-5000 total organic carbon analyzer (Columbia, MD, USA), and chloride ion concentration was analyzed using a Dionex DX-500 ion chromatograph with an IonPac AS4A-SC anion-exchange column, and sodium carbonate/bicarbonate eluent (Sunnyvale, CA, USA). All experiments were carried out in glass beakers which had been combusted at 450°C to remove any organic matter. All experiments were conducted at 18 ± 0.5°C, and held on a 14:10 h light:dark cycle.

Metal uptake

For each salinity, 1.25 L HRW was amended with Instant Ocean™ and sodium hydroxide until the required salinity and pH was reached. Water (250 ml) was poured into individual 1 L beakers (*n* = 5 per salinity; *n* = 25 per experiment), after which radioisotopes were added and left to equilibrate for 8 h. We used high specific activity gamma-emitting radioisotopes (259 kBq/µg ^{73}As, 427 kBq/µg ^{109}Cd, 3610 kBq/µg ^{51}Cr, 81 kBq/µg ^{203}Hg(II), and 74 kBq/µg MeHg) in the present study. ^{73}Arsenic ($t_{½}$ = 80.3 d) and ^{109}Cd ($t_{½}$ = 462.6 d) were purchased from the U.S. Department of Energy (Los Alamos National Laboratory, Los Alamos, NM, USA) and held in 0.1 M HCl, ^{51}Cr ($t_{½}$ = 27.7 d) was purchased from PerkinElmer (Boston, MA, USA) and held in 0.5 M HCl, and ^{203}Hg(II) ($t_{½}$ = 46.6 d) was purchased from Eckert & Ziegler Isotope Products (Valencia, CA, USA) and held in 1 M HCl. ^{73}Arsenic was obtained as As(V), ^{51}Cr as Cr(III), and ^{203}Hg as Hg(II). Inorganic mercury (Hg(II)) was methylated in our lab to $CH_3{}^{203}$Hg(II), methylmercury, following a procedure described elsewhere [29-31], and held in deionized water (MilliQ, Millipore, Bedford, MA, USA). Radioisotopes were added in microliter quantities, and sodium hydroxide was added at equal molar concentrations to neutralize the acid. Per replicate (250 ml) each fish was exposed to 23.6 kBq ^{73}As, 8.6 kBq ^{109}Cd, 23.6 kBq ^{51}Cr, 2.1 kBq

^{203}Hg(II), and 1.6 kBq MeHg; corresponding to the following metal concentrations: 4.98 nM ^{73}As, 0.73 nM ^{109}Cd, 0.51 nM ^{51}Cr, 0.51 nM ^{203}Hg(II), and 0.42 nM MeHg. The pH remained unchanged after radioisotope additions. Gamma analysis allowed us to count ^{73}As and ^{51}Cr simultaneously, so these radioisotopes were added together, whereas fish were exposed to ^{109}Cd, ^{203}Hg(II), and MeHg individually.

After radioisotope equilibration, one fish was added per beaker and the uptake of each radioisotope was monitored for 48 h for MeHg, or 72 h for ^{73}As, ^{109}Cd, ^{51}Cr, and ^{203}Hg(II). The exposure period was shorter for MeHg to allow an exposure time long enough to monitor metal uptake, while reducing the risk of metal drawdown in the exposure medium. At each sample time, after removing the fish from the beaker and just prior to radioanalysis, the fish received two rinses of unlabeled water to remove any liquid containing radioisotope adhering to the body surface. A 1 ml water sample was taken at each time point to determine ambient radioisotope concentration in the dissolved phase; these values were used to calculate concentration factors and uptake rate constants for each metal in the fish. At the end of uptake, the fish were euthanized using 450 ppm MS-222 (ethyl 3-aminobenzoate methanesulfonate), dissected into head, gills, viscera, and body (backbone, fins, fillet, and skin) to assess tissue distribution, and dried for 4 d at 60°C to obtain dry weights.

Uptake rate constants (k_u, L g^{-1} d^{-1}) were calculated by regressing the ratio of radioactivity in fish divided by the radioactivity in 1 L water against time; the resulting slope was divided by the gram dry weight of the fish to obtain the k_u. Concentration factors (CF) were determined as radioactivity per gram dry weight of fish divided by radioactivity per gram water, calculated at 10 h for Cr, 14 h for MeHg, and 72 h for As, Cd, and Hg(II). For all metals except MeHg, these times corresponded to the maximum CFs. For MeHg, there was significant removal

of the radioisotope by the fish, so CFs were determined after 14 h of exposure. Dry weight CFs can be converted to wet weight CFs by dividing by 4 (dry weight is approximately 25% of the wet weight value). All mean metal uptake rates and tissue distributions among salinity treatments were analyzed statistically using *t*-tests. Significance was determined at the 0.05 and 0.01 confidence levels.

Radioanalyses

Radioactivity in the fish was counted non-invasively using a Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector. This allows the same fish to be counted throughout uptake, therefore reducing biological variability. Counting time did not exceed 5 min to reduce stress on the fish, and generally obtain propagated counting errors ≤ 5%. For ^{73}As and ^{51}Cr , however, propagated errors reached up to 25% due to low uptake of the radioisotopes. Water and dissected fish tissue were counted in an intercalibrated LKB Pharmacia-Wallac 1282 CompuGamma CS gamma counter (Turku, Finland) for 5 min. The γ-emission of ^{73}As was detected at 53 keV, ^{109}Cd at 22 keV, ^{51}Cr at 320 keV, and ^{203}Hg at 279 keV. All sample counts were adjusted for radioactive decay and background radioactivity.

RESULTS

Metal uptake as a function of salinity

Figure 1 shows the accumulation of metals by *Fundulus heteroclitus* throughout the uptake period. For all metals and salinities uptake was greatest at the start of exposure, and then slowed throughout the remaining exposure period. This was particularly evident for As and Cr. The uptake of Cr peaked at 10 h for all salinities, after which its concentration decreased over time. Cadmium uptake had an inverse relationship with salinity, whereas As, Hg(II), and MeHg

uptake increased with salinity. Arsenic uptake peaked at 10 h and leveled off thereafter for the three lowest salinities (0, 2, and 6 ppt), however uptake kept increasing at 12 and 25 ppt. The relationship between uptake and salinity for MeHg is not as pronounced as for Hg(II); MeHg uptake varied with salinity until 14 h of exposure and decreased thereafter so that by the end of uptake the amount accumulated was similar for all salinities. At the end of the 72 h exposure period the amount of metal accumulated increased by 4.3-fold for As and 3.3-fold for Hg(II), and decreased by 8.5-fold for Cd when salinity was increased from 0 to 25 ppt. The amount of MeHg accumulated increased by 1.8-fold from 0 to 25 ppt after 14 h of exposure. No metal was totally removed from the water by the end of exposure. The percentage of metal associated with fish at the end of uptake ranged from 0.03 to 0.11% for As, 0.17 to 1.5% for Cd, 4.9 to 27% for Hg(II), and 55 to 62% for MeHg. At the 10 h peak 0.08 to 0.12% of Cr was associated with fish. No toxic effects of metal exposure (death, excess mucus production, abnormal swimming behavior) were observed in fish during the experiments.

Figure 2 shows metal concentration factors (CFs) as a function of salinity for *F. heteroclitus* after 10 h (Cr), 14 h (MeHg) and 72 h (As, Cd, and Hg(II)) exposure. Cadmium, Hg(II), and MeHg were more enriched in the fish than the surrounding water (CFs > 1), whereas As and Cr were not (CFs < 1). Concentration factors were 4.1-fold higher for As, 5.6-fold higher for Hg(II), and 2.4-fold higher for MeHg at 25 ppt than at 0 ppt. Cadmium CFs were 10-fold higher at 0 ppt than at 25 ppt. Concentration factors for Cr ranged between 0.44 at 2 ppt and 0.69 at 25 ppt. There was a strong positive linear relationship between salinity and CF for As ($p < 0.01$), and no relationship between salinity and CF for Cr and MeHg ($p > 0.05$). There was no statistically significant relationship between Cd CFs and salinity ($p > 0.05$); the largest decline in CF was between 0 and 2 ppt, and after 12 ppt salinity had limited influence on uptake. Inorganic

Hg had a significant positive relationship between salinity and CF ($p < 0.05$); CFs increased with salinity between 0 to 12 ppt above which there was only a small increase (Fig. 2). Overall, CFs were highest for MeHg, followed by Hg(II), Cd, Cr, and lowest for As at all times during the uptake period.

Table 2 shows the uptake rate constants (k_u, L g^{-1} d^{-1}) for each metal as a function of salinity for *F. heteroclitus* calculated after 2 h (Hg(II), MeHg), 10 h (As, Cr), and 24 h (Cd) exposure. Uptake rate constants were highest for MeHg (0.79 to 2.29), followed by Hg(II) (0.049 to 0.220), Cd (0.0005 to 0.0051), Cr (0.0011 to 0.0016), and As (0.0004 to 0.0008). Arsenic, Cd, Cr, and Hg(II) showed no significant relationship between salinity and k_u ($p > 0.05$), whereas MeHg had a strong positive linear relationship ($p < 0.01$). Arsenic uptake at 25 ppt continued until the end of exposure, but the k_u was 6-fold lower than that observed in the first 10 h. Cadmium k_us declined sharply between 0 (0.0051) and 2 ppt (0.0017), but showed little difference with salinity above 12 ppt. Inorganic Hg k_us increased with salinity between 0 to 6 ppt, and decreased thereafter in the first 2 h of uptake; whereas k_u peaked at 12 ppt and decreased thereafter between 2 to 13 h. While MeHg k_us significantly increased with salinity in the first 2 h of exposure, this relationship was not noted thereafter ($p > 0.05$) (Table 2).

Tissue distribution and corresponding metal concentrations

Table 3 shows the tissue distribution of each metal at the end of exposure for each salinity. Table 4 presents the corresponding regression equations and r^2 values, therefore describing the relationship between the fraction of metal in individual tissue compartments and salinity. For Cr and Hg(II), the tissue distribution was not influenced by salinity ($p > 0.05$ for each tissue compartment). Arsenic was mainly associated with the body (54 to 64%), and least

with the gills (3 to 8%). Chromium was either associated with the head (28 to 40%) or viscera (24 to 46%), and least with the body (8 to 16%). Inorganic Hg was predominantly associated with the body (38 to 41%), followed by similar distributions between the head and gills (27 to 28% and 23 to 30% respectively), and lowest in the viscera (4 to 8%) regardless of salinity. For Cd, the percentage of metal associated with the viscera increased with salinity, from 3% at 0 ppt to 57% at 25 ppt; while the percentage associated with the head and gills decreased from 32 to 11% and 57 to 25%, respectively, as salinity increased ($p < 0.01$ for each tissue compartment). There was no relationship between Cd associated with the body compartment and salinity (7 to 12%, $p > 0.05$). For MeHg, the percentage associated with the viscera increased slightly from 11% at 0 ppt to 19% at 25 ppt, and this relationship was found to be statistically significant ($p < 0.01$); however there was no relationship between the percentage of MeHg associated with the body and salinity ($p > 0.05$). The greatest percentage of MeHg was associated with the body (45 to 49%).

Table 5 shows the radioactivity concentration of metals in fish tissue at the end of exposure on a weight normalized basis. Body compartment radioactivity concentrations were highest for MeHg (even though MeHg had the lowest concentration in the experimental water throughout uptake), followed by Hg(II), Cd, Cr, and lowest for As. For Cd, Hg(II), and MeHg, the radioactivity concentrations were highest in the gills at all salinities, whereas Cr was distributed between the gills and viscera. The concentration of Cd decreased in the head and gills with increasing salinity, and the concentration nearly doubled in the viscera as salinity increased from 0 ppt to 25 ppt. Arsenic was concentrated in the gills or viscera regardless of salinity, but as salinity increased from 0 ppt to 25 ppt the concentration of metal associated with the head, viscera and body also increased.

DISCUSSION

Metal uptake as a function of salinity

The influence of salinity on the bioavailability of metals from the aqueous phase varied among the metals. For all metals, the rapid uptake at the start of exposure at all salinities presumably represented the binding of labile metal to the most reactive sites on the surface of the fish, which is thought to predominantly be at the gills [13,32]. The more gradual increase after the initial sharp uptake could have resulted from one or a combination of such factors as increased saturation of the initial binding sites followed by metal binding to less reactive sites, reduced metal bioavailability due to metal complexation with dissolved organic matter, or internal regulation of metal within the fish.

Arsenic had the lowest uptake of all metals, but CFs increased 4.1-fold across the salinity range examined. Arsenic was added as arsenate (As(V)), the dominant form of inorganic As in marine and brackish waters [33]; both As(V) and organism body surfaces are negatively charged, which could account for the very low uptake and CFs < 1. Experimental CF and k_u values for As could not be found in the literature to compare with our values, but in the field higher As concentrations in herring (*Clupea harengus*; 2.2-fold higher), cod (*Gadus morhua*; 8.1-fold higher) and flounder (*Platichthys flesus*; 3.1-fold higher) from higher salinity water were reported [34]. Nevertheless, it is recognized that As is largely acquired via diet [33] and the extent to which salinity directly affects As absorption from the aqueous phase in the field is not known. In the present study, As uptake reached equilibrium within the first 10 h of exposure at 0, 2, and 6 ppt, suggesting a regulatory mechanism at lower salinities, where the rate of As uptake equaled the rate of As loss. A possible explanation for the increase in As uptake with salinity is

that marine fish drink to osmoregulate and entrained As subsequently passed over the gut lining into the body. This was supported by an increase in As concentration in the viscera from 19 to 82 Bq g^{-1} as salinity increased from 0 to 25 ppt (Table 5).

Chromium was the only metal in which salinity had no influence on uptake; like As, it does not chloro-complex in seawater, instead it binds to oxygen forming oxyanions [28]. It was added as trivalent Cr (Cr(III)) which is highly particle-reactive, yet uptake was still low (resulting in CFs < 1), suggesting Cr(III) does not readily cross biological membranes in living organisms. In one experiment that was performed, a dead fish was also placed into water containing ^{51}Cr(III) and within 1 h very high counts (over an order of magnitude higher than in living fish) were associated with this dead fish, consistent with the high reactivity of Cr(III) for particles and the inability of this dead animal to regulate or lose Cr from its body, in contrast to living fish (and other marine organisms) which showed little net uptake (this report; [35]). In comparison, while hexavalent Cr (Cr(VI)) is not as particle-reactive as Cr(III), it more readily crosses biological membranes and accumulates in marine organisms [35]. At all salinities uptake of Cr(III) peaked after 10 h of exposure, indicating that Cr taken up as Cr(III) is also regulated in fish. In fact, equilibrium may have been reached for both As and Cr before 10 h of exposure, but due to low radioactivity uptake was not accurately determined within the first few hours of exposure. In other studies, Cr uptake from the aqueous phase (38 ppt) reached equilibrium within 2 to 3 d for the sea bream (*Sparus auratus*) [17], after several days for the dogfish (*Scyliorhinus canicula*) [16], and did not reach equilibrium after 2 weeks for the turbot (*Psetta maxima*) [16]. For the dogfish and turbot CFs were < 1 throughout the 2 week exposure period. The range of k_u values calculated for the sea bream (0.006 to 0.013 L g^{-1} wet weight d^{-1}) [17] was somewhat higher than the values noted in this study.

Cadmium, Hg(II), and MeHg are all chloro-complexed in seawater [28]. Cadmium showed an inverse relationship with salinity, while Hg(II) and MeHg uptake increased as salinity increased from 0 ppt to 25 ppt. For Cd and both species of Hg it appears that chloro-complexation influenced uptake from 0 to 12 ppt, and between 12 and 25 ppt it had limited effect (Fig. 2). This indicates that nearly all of the free Cd and Hg ions were bound to chloride at salinities ≥ 12 ppt. Methylmercury accumulation was not appreciably affected by salinity after 14 h, and by 48 h there is little difference between salinities. By the end of the 48 h exposure, < 66% of the metal had been taken up, so a drawdown of total dissolved MeHg could not account for this observation. At the start of exposure (0 to 2 h) there was a rapid uptake of MeHg at 25 ppt, after which uptake slowed; this decreased uptake could be due to some of the remaining MeHg being bound to dissolved organic matter (DOM) with a lower bioavailability. Methylmercury uptake kept increasing more slowly over time at lower salinities until the concentration of MeHg in the fish had matched that at the 25 ppt level (Fig. 1). The extent to which MeHg was bound to DOM was not determined in our study.

The present study showed that there is preferential uptake of Hg(II) and MeHg in the mercuric chloride form, compared to the hydroxide form found in freshwater. The octanol-water partition coefficient (k_{ow}) of Hg(II) and MeHg are higher when bound to chloride (CH_3HgCl = 1.7, $HgCl_2$ = 3.33) than when bound to hydroxide (CH_3HgOH = 0.07, $Hg(OH)_2$ = 0.05); suggesting that Hg(II) and MeHg should penetrate biological membranes more readily in marine systems [36,37]. The k_{ow} of Hg(II) is nearly double that of MeHg when bound to chloride, suggesting that Hg(II) should penetrate membranes more readily than MeHg when chloro-complexed, while the similar low k_{ow}'s for both Hg species bound to hydroxide suggest comparable binding to membranes might occur in freshwater. However, the k_u value for MeHg is

16.1 times higher than that of Hg(II) at 0 ppt and 20.6 times higher at 25 ppt. A study by Pickhardt and Fisher [38] using freshwater phytoplankton showed both passive and active uptake of MeHg by cells, while Hg(II) was only taken up passively, resulting in a greater accumulation of MeHg in the cytoplasm. If uptake across the gill epithelium was a passive process then we would expect greater uptake of Hg(II) than MeHg in saline water, based on k_{ow} values, but higher MeHg k_u values suggest that MeHg uptake across the gills is an energy mediated process as seen in other studies [5,39]. While we are unable to compare our Hg(II) and MeHg k_u values to other fish studies using the same species across a salinity gradient, the k_u values of both Hg species were lower in freshwater fish than in marine fish [4,5]. However, uptake rate and toxicity data for aquatic invertebrates showed that Hg uptake increased with decreasing salinity [10,40], the reverse of what has been observed in fish.

Cadmium uptake decreased as salinity increased due to a change in Cd speciation with increasing salinity; in freshwater Cd is bioavailable in the free ion Cd^{2+} form, whereas in brackish and higher salinity waters it is present as $CdCl_2$ and $CdCl_3^-$ [20], reducing its bioavailability to organisms. Cadmium chloride has a very low k_{ow} (0.21) [37] compared to $HgCl_2$ and CH_3HgCl, so it is unable to penetrate into biological membranes easily due to its low lipophilicity. Other Cd bioaccumulation and toxicity studies using fish and invertebrates support our findings of higher Cd accumulation at lower salinities [8,9,20,40].

Dissolved organic carbon (DOC) measurements were not made after the beginning of the experiments and it is possible that the DOC concentration in the experimental waters increased over time due to organic carbon release from fish. This increased DOC may have decreased the bioavailability of the metals over time, although we are unaware of studies that have demonstrated different organic carbon releases from fish as a result of salinity. As noted

previously, no excess mucus production was noted among any of the treatments. Therefore, it is unlikely that differences in metal uptake among salinity treatments resulted from DOC effects.

Distribution of metals in fish tissue

As salinity increased from 0 ppt to 25 ppt there was change in the tissue distribution of Cd within *Fundulus heteroclitus*. In freshwater, Cd was predominantly associated with the gills, while in seawater it was associated with the viscera, comparable to the findings of an earlier study [20]. The percentage of Cd associated with the head and gills decreased inversely with salinity and that associated with the viscera increased; but the overall uptake rate of Cd into the fish was higher at lower salinities. Marine fish drink to osmoregulate and this could be an important uptake mechanism for Cd in these fish. Another study has shown that the drinking rate decreased as salinity decreased [20], which would support this conclusion. However, Ca uptake also increases at lower salinities [20], and studies have shown that Cd and Ca share the same uptake pathway and therefore there is competitive uptake [13]; as a result both of these factors probably play a role.

The greater accumulation of As at higher salinities may also be explained by marine fish drinking to osmoregulate. When comparing the radioactivity concentrations of As in fish tissues the concentration was 1.7-times higher in the gills, and 4.3-times higher in the viscera at 25 ppt than at 0 ppt (Table 5), suggesting that the viscera (presumably the intestine) is a more important uptake site than the gills at higher salinities. The increased accumulation of Hg(II) with increasing salinity can not be explained by drinking; as the salinity increased from 0 ppt to 25 ppt the Hg(II) concentration in the gills increased 3.5-fold and the viscera 2.3 fold (Table 5). This suggests that the gill was still the dominant uptake site at higher salinities, and the increase

of Hg(II) in all tissue compartments (except the anomaly in the viscera at 2 ppt) was due to an increase in the absolute overall concentration of Hg(II) in the fish. After 48 h of exposure, the MeHg concentrations were similar at all salinities for each tissue compartment; this was a consequence of the fact that after this exposure period the fish had accumulated a similar amount of MeHg at all salinities (Fig. 1).

There are limited tissue distribution data with which to compare our results because most bioaccumulation studies have a depuration component before dissections, allowing potential movement of metals between tissue compartments. The tissue distribution of Cr showed no relationship with salinity, but the viscera was shown to be an important uptake site. Another study using the turbot (*Psetta maxima*) showed that after a 14 d aqueous exposure 53% of Cr was associated with the digestive tract [16]. At the end of uptake As, Hg(II), and MeHg were predominantly associated with the body (backbone, fillet, fins, and skin). Assuming that tissue distribution is similar in larger predatory fish, this could pose a risk to human consumers of contaminated seafood. These metals were distributed around the body once they had been taken up by the gills, presumably via the blood, and entered the fillet where they are bound to protein.

Table 1. Chloride ion concentrations, dissolved organic carbon (DOC) concentrations, and pH values for experimental waters. Values represent means ± 1 standard deviation; *n* = 3; HRW = Hudson River Water.

	Salinity (ppt)	Chloride (mM)	DOC (µM)	pH
HRW	0	0.85 ± 0.001	233 ± 32	7.85
HRW + 2	2	36 ± 0.05	245 ± 19	7.98
HRW + 6	6	124 ± 0.10	223 ± 14	8.00
HRW + 12	12	237 ± 0.18	250 ± 43	8.01
HRW + 25	25	500 ± 0.52	254 ± 9.0	8.04

Table 2. As, Cd, Cr, Hg(II), and MeHg uptake rate constants (k_u, L g^{-1} d^{-1}) in killifish (*Fundulus heteroclitus*) at 0, 2, 6, 12, and 25 ppt salinity. Values represent means ± 1 standard error; n = 5 per salinity. ND: not determined

		Salinity (ppt)				
Metal	Time (h)	0	2	6	12	25
As	0 - 10	0.0005 ± 0.0002	0.0004 ± 0.0001	0.0008 ± 0.0003	0.0006 ± 0.0001	0.0006 ± 0.0001
	10 - 72	ND	ND	ND	ND	0.0001 ± 0.00001
Cd	0 - 24	0.0051 ± 0.0006	0.0017 ± 0.0001	0.0010 ± 0.0001	0.0006 ± 0.0001	0.0005 ± 0.0001
Cr	0 - 10	0.0016 ± 0.0003	0.0011 ± 0.0002	0.0014 ± 0.0004	0.0013 ± 0.0004	0.0016 ± 0.0004
Hg(II)	0 - 2	0.049 ± 0.003	0.053 ± 0.005	0.220 ± 0.083	0.212 ± 0.077	0.111 ± 0.014
	2 - 13	0.008 ± 0.001	0.051 ± 0.026	0.056 ± 0.026	0.191 ± 0.098	0.033 ± 0.009
MeHg	0 - 2	0.79 ± 0.06	0.99 ± 0.14	1.30 ± 0.13	1.70 ± 0.62	2.29 ± 0.21
	2 - 14	0.17 ± 0.02	0.42 ± 0.05	0.34 ± 0.02	0.37 ± 0.10	0.36 ± 0.08

Table 3. Tissue distribution of As, Cd, Cr, Hg(II), and MeHg at the end of uptake after aqueous metal exposure at different salinities. Values represent the percentage of total body burden associated with each tissue compartment (head, gills, viscera, body). Values represent means ± 1 standard error; n = 5 per salinity. Regression equations and r^2 values describing the relationship between percentage associated with each tissue compartment and salinity are shown in Table 4.

		Salinity (ppt)				
Metal	Compartment	0	2	6	12	25
As	Head	23 ± 4	25 ± 2	21 ± 2	21 ± 1	18 ± 0.9
	Gills	8 ± 4	5 ± 2	5 ± 0.9	5 ± 3	3 ± 0.6
	Viscera	15 ± 4	16 ± 2	14 ± 2	15 ± 1	15 ± 2
	Body	54 ± 7	54 ± 4	60 ± 2	59 ± 2	64 ± 2
Cd	Head	32 ± 1	26 ± 1	23 ± 1	20 ± 2	11 ± 1
	Gills	57 ± 0.9	55 ± 2	51 ± 2	48 ± 1	25 ± 2
	Viscera	3 ± 0.7	7 ± 1	19 ± 4	25 ± 3	57 ± 2
	Body	8 ± 0.7	12 ± 2	7 ± 1	7 ± 0.9	7 ± 2
Cr	Head	40 ± 4	29 ± 7	28 ± 4	32 ± 3	33 ± 5
	Gills	23 ± 2	11 ± 3	15 ± 3	25 ± 4	19 ± 6
	Viscera	24 ± 5	46 ± 10	41 ± 6	29 ± 4	40 ± 8
	Body	13 ± 2	14 ± 2	16 ± 2	14 ± 0.9	8 ± 2
Hg(II)	Head	27 ± 1	28 ± 0.4	27 ± 0.3	28 ± 0.6	27 ± 1
	Gills	27 ± 2	23 ± 2	30 ± 1	28 ± 0.7	27 ± 2
	Viscera	6 ± 0.8	8 ± 4	5 ± 0.3	4 ± 0.4	7 ± 0.9
	Body	40 ± 2	41 ± 3	38 ± 2	40 ± 0.4	39 ± 3
MeHg	Head	24 ± 0.8	25 ± 1	23 ± 0.7	23 ± 0.8	22 ± 0.5
	Gills	16 ± 1	18 ± 0.7	16 ± 0.9	16 ± 1	14 ± 0.5
	Viscera	11 ± 0.7	12 ± 0.5	15 ± 1	16 ± 1	19 ± 0.9
	Body	49 ± 1	45 ± 2	46 ± 0.4	45 ± 1	45 ± 1

Table 4. Regression equations and r^2 values describing the relationship between salinity and fraction of metal in each tissue compartment for killifish (*Fundulus heteroclitus*) using data shown in Table 3. Statistically significant differences (by *t*-test) between salinity and the percent of metal in each tissue compartment are represented by * ($p<0.05$) and ** ($p<0.01$).

Metal	Tissue	Equation	r^2
As	Head	y = -0.215x + 23.623	0.83*
	Gills	y = -0.136x + 6.4528	0.55
	Viscera	y = -0.028x + 15.225	0.12
	Body	y = 0.379x + 54.699	0.86*
Cd	Head	y = -0.742x + 28.861	0.95**
	Gills	y = -1.276x + 58.579	0.95**
	Viscera	y = 2.111x + 3.497	0.99**
	Body	y = -0.09x + 9.0626	0.23
Cr	Head	y = -0.068x + 33.053	0.02
	Gills	y = 0.101x + 18.057	0.03
	Viscera	y = 0.176x + 33.933	0.04
	Body	y = -0.209x + 14.958	0.61
Hg(II)	Head	y = -0.019x + 27.557	0.09
	Gills	y = 0.079x + 26.185	0.09
	Viscera	y = -0.019x + 6.2048	0.01
	Body	y = -0.040x + 40.053	0.19
MeHg	Head	y = -0.108x + 24.558	0.82*
	Gills	y = -0.118x + 16.973	0.80*
	Viscera	y = 0.298x + 11.823	0.92**
	Body	y = -0.072x + 46.646	0.27

Table 5. Radioactivity concentrations of metals in fish tissues (head, gills, viscera, body) after aqueous exposure at varying salinities. Values represent means ± 1 standard error; *n* = 5 per salinity. Units are kBq g^{-1} for Cd, Hg(II), and MeHg, and Bq g^{-1} for As and Cr.

		Salinity (ppt)				
Metal	Compartment	0	2	6	12	25
As	Head	12 ± 4.5	18 ± 4.7	24 ± 7.3	28 ± 5.1	46 ± 2.3
	Gills	30 ± 10	27 ± 7.2	41 ± 11	70 ± 41	50 ± 9.5
	Viscera	19 ± 7.8	27 ± 11	30 ± 7.8	48 ± 10	82 ± 11
	Body	13 ± 5.4	19 ± 6.9	31 ± 9.2	37 ± 5.9	64 ± 4.0
Cd	Head	0.59 ± 0.1	0.20 ± 0.01	0.09 ± 0.01	0.06 ± 0.004	0.03 ± 0.001
	Gills	8.7 ± 1.2	3.6 ± 0.4	1.7 ± 0.2	1.2 ± 0.2	0.59 ± 0.07
	Viscera	0.17 ± 0.04	0.14 ± 0.03	0.23 ± 0.05	0.22 ± 0.03	0.33 ± 0.07
	Body	0.07 ± 0.02	0.04 ± 0.01	0.02 ± 0.002	0.01 ± 0.001	0.01 ± 0.005
Cr	Head	51 ± 8.8	36 ± 10	22 ± 3.5	30 ± 4.4	44 ± 12
	Gills	274 ± 64	109 ± 19	95 ± 12	173 ± 29	121 ± 24
	Viscera	69 ± 17	153 ± 57	78 ± 27	61 ± 9.0	152 ± 83
	Body	6.5 ± 0.6	8.5 ± 1.9	5.6 ± 0.6	5.8 ± 0.4	4.5 ± 1.2
Hg(II)	Head	0.14 ± 0.03	0.26 ± 0.05	0.32 ± 0.04	0.41 ± 0.10	0.46 ± 0.04
	Gills	1.2 ± 0.3	2.2 ± 0.4	3.4 ± 0.4	3.6 ± 0.1	4.2 ± 0.4
	Viscera	0.11 ± 0.02	0.65 ± 0.41	0.14 ± 0.02	0.17 ± 0.03	0.26 ± 0.05
	Body	0.10 ± 0.02	0.18 ± 0.04	0.20 ± 0.03	0.26 ± 0.07	0.29 ± 0.02
MeHg	Head	1.6 ± 0.03	1.6 ± 0.09	1.6 ± 0.1	1.6 ± 0.2	1.5 ± 0.1
	Gills	10 ± 0.8	13 ± 1.2	11 ± 0.9	10 ± 1.0	8.1 ± 0.6
	Viscera	2.6 ± 0.3	2.3 ± 0.2	3.2 ± 0.3	3.0 ± 0.5	2.5 ± 0.2
	Body	1.5 ± 0.03	1.3 ± 0.06	1.4 ± 0.1	1.4 ± 0.2	1.4 ± 0.08

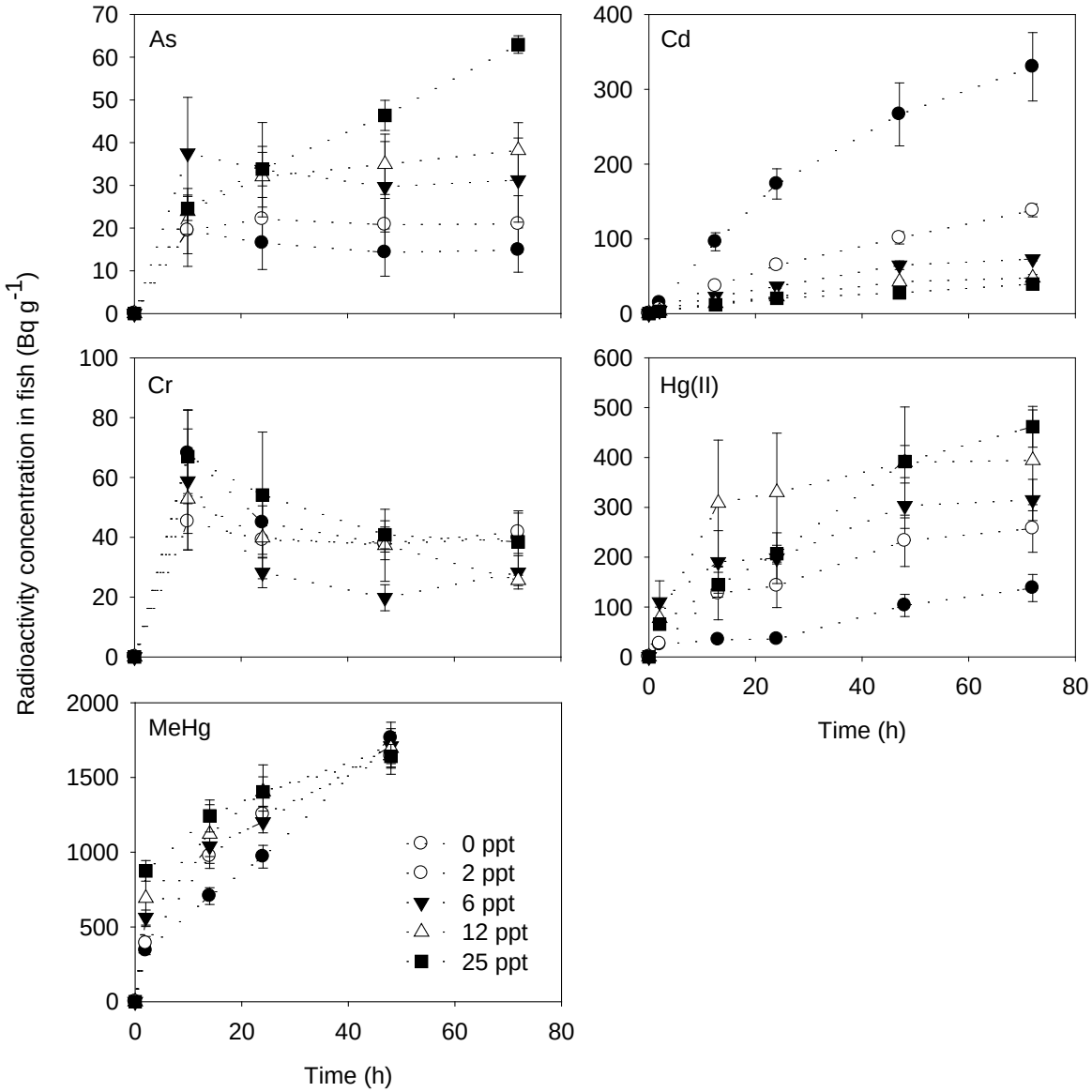

Figure 1. Accumulation of aqueous metals (Bq g^{-1} dry weight) at varying salinities (ppt) over a 48 h (MeHg) or 72 h (As, Cd, Cr, and Hg(II)) uptake period in killifish (*Fundulus heteroclitus*). Values represent means ± 1 standard error; n = 5 per salinity.

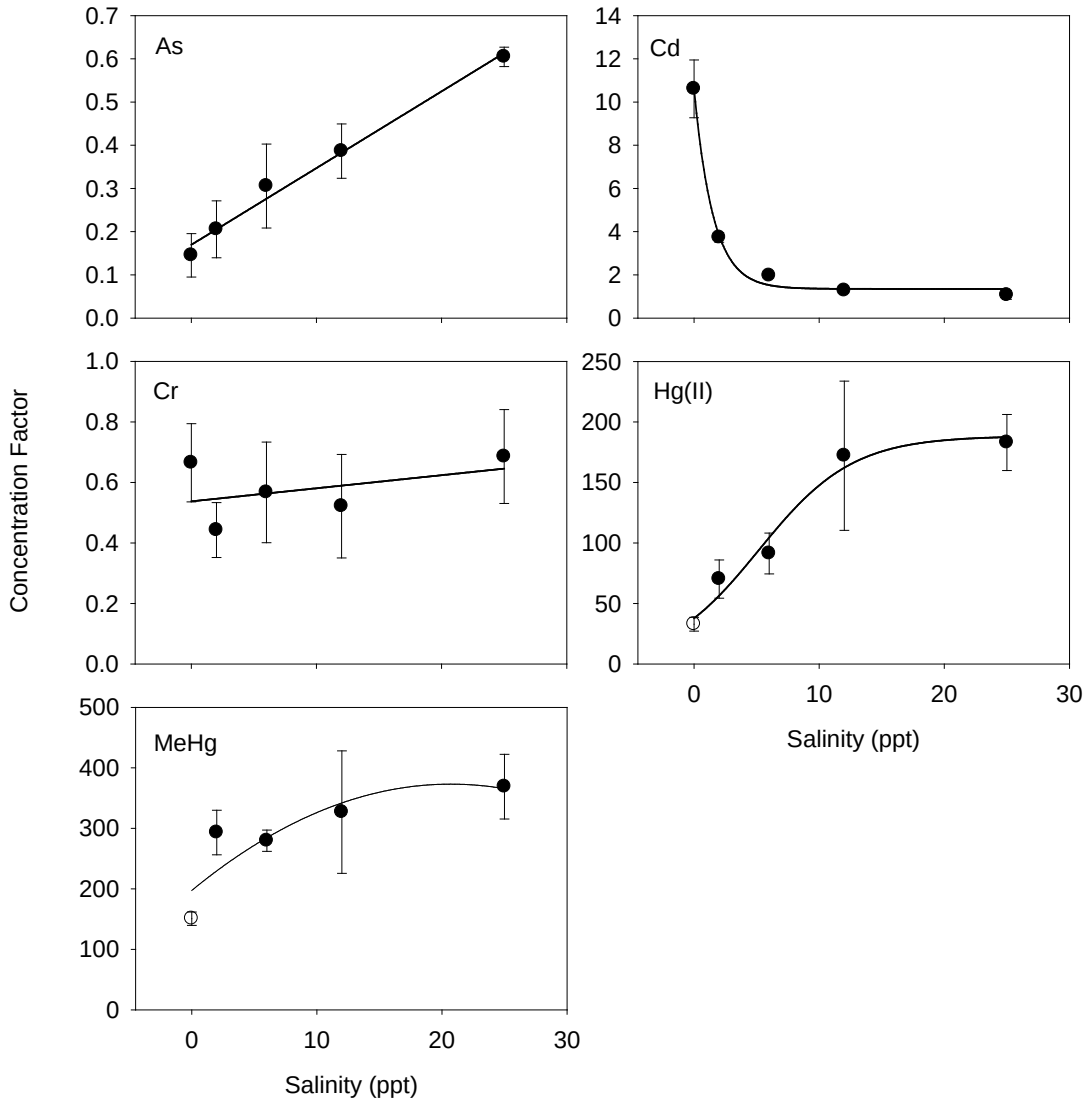

Figure 2.

Metal concentration factors (CFs) as a function of salinity for killifish (*Fundulus heteroclitus*) after 10 h (Cr), 14 h (MeHg) or 72 h (As, Cd, Hg(II)) exposure. Values represent means ± 1 standard error; n = 5 per salinity. Regression equations and r^2 values describing the relationship between CF and salinity are as follows: As y = 0.018x + 0.170, r^2 = 0.99; Cd y = $5.042e^{-0.076x}$, r^2 = 0.67; Cr y = 0.004x + 0.537, r^2 = 0.19; Hg(II) y = $-0.357x^2 + 14.999x + 32.678$, r^2 = 0.99; MeHg y = $-0.410x^2 + 16.966x + 197.450$, r^2 = 0.76.

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Influence of humic acid on the uptake of aqueous metals by the killifish *Fundulus heteroclitus*

ABSTRACT:

This study investigated the role of humic acids over a concentration range of 0-20 mg L^{-1} on the uptake of three metals (Cd, Cr, and Hg (as inorganic Hg (Hg(II)) and methylmercury (MeHg)) and a metalloid (As) from the aqueous phase by the killifish (*Fundulus heteroclitus*). Cadmium uptake showed no relationship with humic acid concentration, whereas Cr, Hg(II), and MeHg uptake showed an inverse relationship, and As uptake increased with increasing humic acid concentration. Concentration factors (CFs) were >1 for Cd, Hg(II), and MeHg at all humic acid concentrations, indicating the killifish were more enriched in the metal than the experimental media, whereas As and Cr generally had CFs <1 at the end of a 72 h exposure. The uptake of As and Cr were regulated by killifish, whereas Cd, Hg(II) and MeHg were not. Uptake rate constants (k_us; ml g^{-1} d^{-1}) were highest for MeHg (91-3936), followed by Hg(II), Cd, Cr, and lowest for As (0.17-0.29). Dissection data revealed that the gills generally had the highest concentration of all metals under all humic acid treatments. This study concludes that changes in humic acid concentration can influence the accumulation of aqueous metals in killifish and needs to be considered when modeling metal bioaccumulation.

INTRODUCTION

It has been well demonstrated that dissolved organic matter (DOM) tends to diminish metal uptake and toxicity in aquatic organisms [1,2]. Studies originally focused on lower trophic levels, including phytoplankton [3], which typically have the highest bioconcentration [4]. Metal uptake by plankton is generally reduced in the presence of DOM, because the metal binds to organic ligands in the water, therefore reducing the concentration of the free-ion form which is thought to be more bioavailable [1]. The general applicability of this pattern to other organisms is questionable. For example, studies of bivalve mollusks found that metal bioavailability can either increase or decrease with increasing DOM concentration [5-8].

Studies investigating the protective role of DOM for fish employ the Biotic Ligand Model (BLM) in freshwater systems. The BLM takes into account water chemistry (pH, metal complexation with DOM, and cationic competition) and its impact on metal speciation, and therefore bioavailability to fish [9,10]. The BLM has become an influential tool in developing water quality criteria, and has been used to assess the toxicity of several metals including Ag, Cd, Cu, Ni, and Zn in freshwater fish [11-13].

The gills account for approximately 50% of the external body surface in fish [14]. As a result, the gills are an important uptake site for dissolved metal, either due to metal passively binding to the gill surface, or entering the gills through uptake channels [15]. Furthermore, studies have investigated the binding affinity of metals to gills compared to the binding affinity of metals to DOM and calculated stability constants [16]. To date, most studies investigating the role of DOM on metal accumulation in fish have focused on whether the presence of DOM

reduces metal uptake, uptake mechanisms, and the resulting physiological changes in the gills [2,14,17].

The objective of this study was to investigate the role of humic acid concentration (0, 2.5, 5, 10, 20 mg L^{-1}) on the accumulation of three metals (Cd, Cr(III), and Hg (as Hg(II), and MeHg, methylmercury)) and a metalloid (As(V)) from the aqueous phase by the killifish (*Fundulus heteroclitus*). Uptake was monitored for 72 h, and concentration factors (CFs), and uptake rate constants (k_us) were calculated for each metal and humic acid concentration. At the end of uptake fish were dissected to investigate whether the tissue distribution of each metal was influenced by humic acid concentration in the experimental water. Killifish were the chosen experimental organism as they are a small ubiquitous fish in coastal waters from the Gulf of St. Lawrence to northeastern Florida [18], and have varying sensitivity to aquatic contaminants. Furthermore, kiilifish are found in fresh, brackish, and saltwater [18], which experiences a large variation in DOM concentration. Humic acid was the chosen DOM because it is an effective chelating agent in natural waters e.g. in freshwater >90% of Hg and Cu is bound to humic materials [19].

The chosen trace elements are not biologically essential, and are toxic at elevated concentrations. They can all be found in high concentration in industrialized coastal areas due to anthropogenic activities [20]. The chosen elements have varying chemical characteristics; Cd, Cr(III), and Hg are cationic, particle reactive and passively sorb to organism surfaces, whereas As(V) is anionic and not particle reactive. Furthermore, Hg is a sulfur-seeking Class B metal, whereas As, Cd, and Cr are borderline metals/metalloid with similar binding affinities for O, S, and N [21].

MATERIALS AND METHODS

Fish acclimation

Field collected (Taylor River, Hampton, NH, USA; salinity = 25 to 30 ppt) killifish, *Fundulus heteroclitus*, (mean wet weight 2.06 g ± 0.31 standard deviation) purchased from Aquatic Research Organisms (Hampton, NH, USA) were used in this study. Fish were held at the experimental salinity (1 ppt) for at least 4 weeks prior to the start of experiments, and fed a daily diet of TetraCichlid™ cichlid flakes (Tetra Holding Inc., Blacksburg, VA, USA) and frozen bloodworms. Fish were not fed for 36 h prior to the start of the experiments to allow for gut clearance, or throughout the metal exposure periods, so metal exposure to killifish was only from the aqueous phase. Fish were held at 18 ± 0.5°C in a temperature controlled incubator on a 14:10 h light:dark cycle.

Water and experimental conditions

Experiments were carried out in 0.2 µm sterile filtered deionized water (MilliQ, Millipore, Bedford, MA, USA) amended to 1 ppt using Instant Ocean™ (Aquarium Systems Inc., Mentor, OH, USA), and either 0, 2.5, 5, 10, or 20 mg L^{-1} Suwannee River (GA, USA) humic acid. The humic acid was purchased from the International Humic Substances Society (St. Paul, MN, USA) as Suwannee River humic acid standard batch 2.

All experiments were carried out in 1 L glass beakers which were combusted at 450°C for at least 5 h to burn off any residual organic matter. All water parameters (pH, temperature, salinity) remained constant for each experiment, so the concentration of humic acid was the only variable (Table 1). The dissolved organic carbon (DOC) concentrations were measured using a Shimadzu TOC-5000 total organic carbon analyzer (Columbia, MD, USA). The chloride ion

concentration was measured using a Dionex DX-500 ion chromatograph with an IonPac AS4A-SC anion-exchange column and sodium carbonate/bicarbonate eluent (Sunnyvale, CA, USA).

Experimental procedure

For each humic acid concentration, 1.25 L of deionized water was adjusted to pH 10 using sodium hydroxide (1M NaOH; Fisher Scientific, Fair Lawn, NJ, USA) because humic acid dissolves more readily at a higher pH [22]. Humic acid was added to the required concentration and dissolved by sonicating for 15 minutes. The pH was then adjusted back to 7.8-8 using trace metal grade nitric acid (2N HNO_3; GFS Chemicals Inc., Columbus, OH, USA). Salinity was adjusted to 1 ppt using Instant Ocean™, and samples were collected for DOC and chloride ion analysis. 250 ml of water was poured into each 1 L beaker (n = 5 per concentration, n = 25 per experiment) and left for several hours. Each beaker was then radiolabeled and left to equilibrate for 12 h.

^{73}Arsenic (as As(V), $t_{½}$ = 80.3 d, in 0.1 M HCl) and ^{109}Cd (as Cd(II), $t_{½}$ = 462.6 d, in 0.1 M HCl) were obtained from the U.S. Department of Energy (Los Alamos National Laboratory, Los Alamos, NM, USA), ^{51}Cr (as Cr(III), $t_{½}$ = 27.7 d, in 0.5 M HCl) from PerkinElmer (Boston, MA, USA), and ^{203}Hg (as Hg(II), $t_{½}$ = 46.6 d, in 1 M HCl) from Eckert and Ziegler Isotope Products (Valencia, CA, USA). Inorganic mercury was methylated in our lab to $CH_3{}^{203}$Hg(II) (methylmercury, MeHg) following a well described method [23-25] and held in deionized water. Equimolar concentrations of sodium hydroxide in microliter quantities were added to experimental water to neutralize the acid associated with the radioisotopes, so the pH remained unchanged. ^{73}Arsenic and ^{51}Cr were added together, whereas ^{109}Cd, ^{203}Hg(II), and MeHg were

added individually. Each fish was exposed to the following radioisotope additions (per 250 ml water): 23.6 kBq ^{73}As, 9.3 kBq ^{109}Cd, 23.6 kBq ^{51}Cr, 4.5 kBq ^{203}Hg(II), and 4.8 kBq MeHg; this equals the following added metal concentrations: 4.69 nM ^{73}As, 0.73 nM ^{109}Cd, 0.43 nM ^{51}Cr, 0.72 nM ^{203}Hg(II), and 0.70 nM MeHg.

One fish was added per beaker and the uptake of each metal was monitored at regular intervals over 72 h. At each sampling time during this uptake period the fish received two 20 second rinses in nonradiolabeled water to remove excess radioisotope adhered to the body surface, and a 1 ml sample of water was collected from each beaker to determine the dissolved radionuclide concentration in order to calculate concentration factors and uptake rate constants. At the end of the 72 h uptake period the fish were euthanized using MS-222 (Sigma-Aldrich Inc., St. Louis, MO, USA), dissected into head, gills, viscera, and body (backbone, fillet, skin), radioassayed, and dried at 60°C for 4 d to assess tissue distribution. No fish died or exhibited toxicity effects (abnormal swimming behavior or excess mucus production) throughout the experiments.

Uptake rate constants (k_u; ml g^{-1} d^{-1}) were calculated by dividing the radioactivity in fish (g dry weight) by the radioactivity in 1 ml water and regressing this value against time; the resulting slope is the k_u. Concentration factors (CFs) were calculated by dividing the radioactivity per g fish (dry weight) by the radioactivity in 1 ml water. Concentration factors were calculated at 47.5 h for MeHg and at 72 h for As, Cd, Cr, and Hg(II). Killifish dry weight is 25% of the wet weight value; wet weight k_us and CFs can be calculated by dividing the dry weight value by 4. The statistical significance of CFs, k_us, and tissue distribution as a function of humic acid concentration were determined using linear regression analysis. Significance was determined at the $p<0.05$ or $p<0.01$ confidence level.

Radioanalyses

Fish were radioassayed noninvasively in a Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector; this reduces the biological variability by allowing the radioactivity in the same individual fish to be monitored throughout the 72 h uptake period. Fish were radioassayed for no longer than 5 minutes to reduce stress on the fish while still obtaining propagated counting errors ≤ 5% for most samples. However, propagated counting errors for ^{73}As and ^{51}Cr could reach 25% due to limited uptake of the radioisotopes. Radioactivity in the water samples and dissected fish tissue were radioassayed for 5 minutes using an inter-calibrated LKB Pharmacia-Wallac 1282 CompuGamma CS gamma counter (Turku, Finland). All counts were corrected for background radioactivity and radioactive decay. The γ-emissions were detected at 22, 53, 279, and 320 keV for ^{109}Cd (x-ray of daughter product ^{109}Ag), ^{73}As, ^{203}Hg, and ^{51}Cr respectively.

RESULTS

The influence of humic acid concentration on metal uptake

Figure 1 shows the accumulation of As, Cd, Cr, Hg(II), and MeHg by killifish throughout the 72 h uptake period as a function of humic acid concentration. Arsenic uptake increased with humic acid concentration (although As accumulation at 5 mg L^{-1} was 1.3–fold lower than at 2.5 mg L^{-1}); fish exposed to As with 20 mg L^{-1} humic acid accumulated 2.6-times more As than fish exposed with 0 mg L^{-1} at the end of uptake. Chromium uptake decreased as humic acid concentration increased; as the humic acid concentration increased from 0 to 2.5 mg L^{-1}, the accumulation of Cr at the end of uptake decreased by 3–fold, and Cr accumulation at the end of uptake was similar at 5, 10, and 20 mg L^{-1} (26-29 Bq g^{-1}). Arsenic and Cr uptake stabilized, and decreased for Cr at some humic acid concentrations, by the end of uptake, indicating equilibrium

may have been reached. Cadmium uptake increased linearly throughout the 72 h and no relationship with humic acid concentration was observed. The uptake of Hg(II) at all humic acid concentrations showed a sigmoidal uptake pattern. By the end of uptake no clear relationship between Hg(II) accumulation and humic acid concentration could be noted, but fish exposed at higher humic acid concentrations (10 and 20 mg L^{-1}) accumulated less Hg(II). Methylmercury showed rapid uptake during the first 2.5 h of exposure (particularly at 0 mg L^{-1}), and uptake slowed thereafter. Like Hg(II), no clear relationship between MeHg accumulation and humic acid concentration was noted, but MeHg accumulation was lower at higher humic acid concentrations. At the end of uptake 0.03-0.07% of As, 1.9-2.4% of Cd, 0.06-0.3% of Cr, 8-21% of Hg(II), and 50-86% of MeHg was associated with the fish, indicating that no metal was totally removed from the experimental water throughout uptake.

Concentrations factors (CFs) in fish were >1 throughout uptake at all humic acid concentrations for Hg(II) and MeHg, indicating that the fish were more enriched in the metal than the surrounding experimental water, whereas As had CFs <1 throughout uptake regardless of humic acid concentration, indicating the experimental water was more enriched in As than the fish. Chromium CFs were >1 at 0 mg L^{-1} throughout uptake, but <1 at 2.5-20 mg L^{-1}. Cadmium CFs did not exceed 1 until 12 h exposure at 0, 2.5, 10, and 20 mg L^{-1} (data not shown). Figure 2 shows CFs of each metal as a function of humic acid concentration after 47.5 h (MeHg) or 72 h (As, Cd, Cr, Hg(II)) exposure. CFs were highest for MeHg, followed by Hg(II), Cd, Cr, and lowest for As. Concentration factors were 6.8-times lower for Cr, 2.4-times lower for Hg(II), and 6-times lower for MeHg at 20 mg L^{-1} than at 0 mg L^{-1}, whereas As CFs were 2.5-times higher at 20 mg L^{-1} than at 0 mg L^{-1}. Cadmium CFs ranged from 10 to 12.5. There was a statistically significant relationship between humic acid concentration and CF for Cr, Hg(II), and MeHg

($p<0.01$ for each), and As ($p<0.05$), whereas there was no relationship between humic acid concentration and CF for Cd ($p>0.05$).

Table 2 shows the uptake rate constants (k_u) for each metal as a function of humic acid concentration at varying time periods during the 72 h uptake. Uptake rate constants were highest for MeHg, followed by Hg(II), Cd, Cr, and lowest for As. Arsenic and Cd showed no statistically significant relationship between humic acid concentration and k_u ($p>0.05$). Arsenic k_us ranged between 0.17 ml g^{-1} d^{-1} at 5 mg L^{-1} and 0.29 ml g^{-1} d^{-1} at 10 mg L^{-1} during the first 9.5 h of exposure, and 0.04 ml g^{-1} d^{-1} at 0 mg L^{-1} and 0.28 ml g^{-1} d^{-1} at 20 mg L^{-1} between 9.5-24 h of exposure. The Cd k_us were lowest at 0 mg L^{-1} (3.0 ml g^{-1} d^{-1}) and highest at 10 mg L^{-1} (6.3 ml g^{-1} d^{-1}). Chromium k_us decreased from 4.7 to 0.68 ml g^{-1} d^{-1} as humic acid concentration increased from 0 to 20 mg L^{-1}, and this relationship was statistically significant ($p<0.01$). The greatest decrease in Cr k_us was noted between 0 and 2.5 mg L^{-1}; the k_u at 2.5 mg L^{-1} was 2.8-times lower than at 0 mg L^{-1}. The uptake of Hg(II) occurred in four stages; the greatest uptake occurred between 24 to 47 h of exposure (38 to 77 ml g^{-1} d^{-1}) at all humic acid concentrations. At each stage of uptake the k_u value decreased as humic acid concentration increased, and at each stage the relationship was found to be statistically significant ($p<0.05$ at 0-2.5 h, $p<0.01$ for the following three stages). The k_u of MeHg decreased as humic acid concentration increased, and this relationship was statistically significant ($p<0.01$). At 0 and 2.5 mg L^{-1} the highest k_u was observed between 0-2.5 h of exposure, whereas for 5, 10, and 20 mg L^{-1} the highest k_u was observed between 23.5-47.5 h of exposure. Like Cr, the largest decrease in MeHg k_us was noted between 0 and 2.5 mg L^{-1}; at 0-2.5 h the k_u was 6.4-times lower, at 2.5-23.5 h the k_u was 5.1-times lower, and at 23.5-47.5 h the k_u was 2.2-times lower at 2.5 mg L^{-1}.

Tissue distribution and corresponding metal concentrations

Table 3 shows the tissue distribution at the end of 72 h exposure for each metal and humic acid concentration. The percentage of As associated with the head and gills decreased from 31 to 22% and 17 to 6% respectively, and increased from 44 to 56% in the body as humic acid concentration increased from 0 to 20 mg L^{-1}, and these relationships were found to be statistically significant ($p<0.05$ for head and body, $p<0.01$ for gills). Arsenic was associated least with the viscera (8-17%) ($p>0.05$). Cadmium was mainly associated with the gills (53-62%), and least with the viscera (2-11%) and body (6%). There was no statistically significant relationship between the percentage of Cd in any tissue compartment and humic acid concentration ($p>0.05$). As humic acid concentration increased from 0 to 20 mg L^{-1}, the percentage of Cr associated with the head and gills decreased from 65 to 18% and 26 to 9% respectively, and increased in the viscera and body from 3 to 53% and 6 to 20% respectively; for all tissue compartments this relationship was statistically significant ($p<0.01$). Inorganic Hg was mainly associated with the body (37-43%), followed by the gills (23-30%) and head (26-28%), and least with the viscera (6-7%), at each humic acid concentration. The decrease in Hg(II) associated with the gills, and increase in Hg(II) associated with the body as humic acid concentration increased was statistically significant ($p<0.05$ and $p<0.01$ respectively). Methylmercury was mainly associated with the body (46-50%), followed by the head (22-25%), and then nearly evenly distributed between the gills (10-14%) and viscera (14-18%). The slight decrease in MeHg associated with the viscera as humic acid concentration increased was statistically significant ($p<0.01$).

Table 4 shows the weight-normalized radioactivity concentrations of As, Cd, Cr, Hg(II), and MeHg in each tissue compartment at the end of 72 h exposure. The highest concentration of As, Cd, Cr (except at 20 mg $L^{-}1$), Hg(II), and MeHg was found in the gills regardless of humic

acid concentration. Arsenic concentration increased in the head, viscera, and body (14-29, 9-56, and 8.8-34 Bq g^{-1} respectively) as humic acid concentration increased from 0 to 20 mg L^{-1}; for each tissue compartment this relationship was statistically significant ($p<0.05$). The Cr concentration decreased from 939 to 42 Bq g^{-1} in the gills ($p<0.01$), and 228 to 15 Bq g^{-1} in the head ($p<0.01$), and increased from 23 to 105 Bq g^{-1} in the viscera ($p>0.05$), as humic acid concentration increased from 0 to 20 mg L^{-1}. The decrease of Hg(II) associated with the head, gills, and body, and MeHg associated with the head, viscera, and body were statistically significant ($p<0.01$ for all tissue compartments, except Hg(II) body ($p<0.05$)).

DISCUSSION

Metal uptake as a function of humic acid concentration

The importance of humic acid concentration on metal uptake from the aqueous phase by killifish varied by metal. Arsenic uptake increased with humic acid concentration but uptake was so low that the CFs were <1 at all humic acid concentrations. The low CFs observed in this study can be attributed to both As (as arsenate) and the body surface of the fish being negatively charged, and therefore repelling each other. The very low accumulation of As after 47 h of exposure at all humic acid concentrations was due to internal regulation of As within the fish, where the rate of As uptake nearly equaled the rate of loss. This same accumulation pattern was observed when As uptake was measured as a function of salinity [26]. The significant relationship between As CFs and humic acid concentration could possibly be attributed to As binding to a component of the humic acid that is taken up through uptake channels in the gills and As was taken up with it, or to arsenate being reduced to a more available form such as arsenite in the presence of humic acid [27]. After the experimental media was radiolabeled it

was left to equilibrate for 12 h, so some arsenate could have been converted to arsenite during this time period, however, we did not determine the speciation of As in the experimental water. The increase in As accumulation with increasing humic acid concentration is unlikely to be environmentally important because the CFs never exceeded 1. Experimental CF and k_u values could not be found in the literature as a function of DOM concentration, but the range of values calculated in this present study are similar to (CFs) or slightly lower (k_us) than those calculated when As uptake was measured as a function of salinity [26].

Chromium uptake decreased inversely with humic acid concentration, conforming to the BLM. Chromium was added as trivalent Cr (Cr(III)), which is highly particle reactive; the CF at 2.5 mg L^{-1} was 3.7-fold lower than at 0 mg L^{-1}. At humic acid concentrations ≥ 5 mg L^{-1} there was no difference in CFs (0.28 to 0.33), suggesting that all available Cr was bound to humic acid at concentrations ≥ 5 mg L^{-1}. The decrease in CFs and k_us in the presence of humic acid also suggests that Cr(III) has a greater binding affinity for humic acid than fish gills. Although Cr(III) is highly particle reactive, the low CFs indicate it does not readily pass across biological membranes. In comparison, hexavalent Cr (Cr(VI)) is not as particle reactive, but can readily cross biological membranes in aquatic organisms [28]. In the presence of humic acid, equilibrium was reached within the first 24 h of exposure and within 48 h when no humic acid was added, indicating internal regulation within the fish. In other studies Cr uptake reached equilibrium within the first 10 h of exposure in killifish, 2 d in the sea bream (*Sparus auratus*), 9 d in the spotted dogfish (*Scyliorhinus canicula*) and did not reach equilibrium in the turbot (*Psetta maxima*) throughout a 14 d exposure period [26,29,30]. Nevertheless, CFs were <1 for killifish, dogfish, and turbot throughout the uptake periods tested. The k_us calculated in the presence of humic acid in this study are within the range calculated for killifish when Cr uptake

was investigated in Hudson River water with naturally occurring DOC at a comparable concentration to the 5 mg L^{-1} humic acid concentration [26].

Cadmium was the only metal for which humic acid concentration had no influence on metal uptake. Prior studies found the presence of DOM did not have a protective role against metal binding at the gills in rainbow trout (*Oncorhynchus mykiss*) [2,31]. This is presumably due to the lower binding affinity of Cd to DOC (log $K_{Cd\text{-}DOC}$ = 7.4) compared to fish gills (log $K_{Cd\text{-}gill}$ = 8.6) [32]. In comparison, metals such as Cu which are less reactive for fish gills when complexed with DOC have a stronger binding affinity for DOC than fish gills (log $K_{Cu\text{-}DOC}$ = 9.1, log $K_{Cu\text{-}gill}$ = 7.4) [32]. At 0, 2.5, 10, and 20 mg L^{-1} humic acid concentrations, CFs ranged from 10 to 12.5, comparable to another killifish study where Cd uptake was monitored at 0 ppt [26]. The CFs calculated in the present study (at 1 ppt) can be attributed to the increased bioavailability of Cd in the free-ion Cd^{2+} form; at higher salinities CFs decrease to 1 due to the chlorocomplexation of Cd reducing metal accumulation [26]. The data for Cd at 5 mg L^{-1} were not shown in the figures and tables; at the end of uptake killifish had accumulated 3.4 to 4.2-fold less Cd than at lower or higher humic acid concentrations (96 ± 56 Bq g^{-1}), the CF was 2.7 and the k_u was 0.81 ± 0.44 ml g^{-1} d^{-1}. The reason for this pronounced decrease at 5 mg L^{-1} compared to other humic acid concentrations is not apparent to us, and while every precaution was taken, contamination cannot be ruled out. Interestingly, the percentage of Cd associated with the gills decreased to 40%, and increased to 21% in the viscera at 5 mg L^{-1}, suggesting a possible binding to particulate matter during the Cd exposure period and dietary uptake.

Like Cr, the uptake of Hg(II) and MeHg decreased inversely with the humic acid concentration, conforming to the BLM. For Hg(II) this relationship was nearly linear, whereas for MeHg the concentration of humic acid had little effect above 10 mg L^{-1} (Fig. 2), indicating

that all the available MeHg was bound to humic acid at this concentration. The inverse relationship between Hg(II) and MeHg uptake and ambient DOM is consistent with findings for two species of freshwater fish [33] and a freshwater diatom [34]. At a humic acid concentration of 0 mg L^{-1}, the decline in MeHg accumulation after 47.5 h of exposure reflected the fact that 86% of the MeHg had been taken up by 72 h, and the remaining dissolved MeHg could have been bound to DOM in the experimental water and no longer bioavailable. In comparison, Hg(II) uptake was greatest between 24 to 47.5 hours of exposure at all humic acid concentrations, including in water to which no humic acid was added. The reason for this delayed uptake pattern is not apparent to us and was not observed in a parallel study investigating the effects of salinity on Hg(II) uptake in killifish [26].

The CFs were 2.4- and 6.0-fold lower for Hg(II) and MeHg at 20 mg L^{-1} than at 0 mg L^{-1} consistent with the idea that both Hg forms have a similar or stronger binding affinity for DOM than to killifish gills. A study of rainbow trout calculated equally strong binding constants of Hg(II) for natural organic matter (NOM) and gills (log $K_{\text{Hg-NOM}}$ and log $K_{\text{Hg-gill}}$ = 18.0) [35]. Given mercury's strong affinity for sulfur, the complexation of Hg by humic acids may be dominated by its binding to thiol components, even though these are not typically enriched in humic acids, including those used in this study [36]. Although we are unable to compare the Hg(II) and MeHg k_us calculated in this study with other fish studies across a DOM concentration gradient, our values are comparable to other values calculated in other studies for freshwater and marine fish [26,33,37].

Distribution and concentration of metals in fish tissue

For all metals at all humic acid concentrations (except Cr at 20 mg L^{-1}), the concentration of metal was highest in the gills (Table 4), which is presumed to be the predominant uptake site for dissolved metals to fish. Prior studies addressing the influence of DOM on metal distribution in fish tissues are limited for the metals investigated in this study, and earlier experiments assessed tissue distributions under different DOM and different experimental conditions. Consequently, comparison of the tissue distribution data for the metals with findings from other studies is limited.

The sharp decline in Cr concentration in the head and especially gill tissue with increasing humic acid concentration is presumably attributable to the binding of Cr to the humic acid, thereby reducing the bioavailable free metal ion. This resulted in a significant decline in the total body burden of Cr. There was no significant trend in Cr concentrations in the viscera or body with increasing humic acid levels. Consequently, the proportion of the total body burden of Cr in the head and gills declined significantly and increase accordingly in viscera and body. In comparison, cadmium tissue distribution did not vary with humic acid concentration. The same tissue distribution was observed in killifish in another study monitoring the aqueous uptake of Cd at 0 and 2 ppt [26].

Unlike Cd and Cr, As, Hg(II) and MeHg were redistributed throughout the killifish and accumulated in the body (presumably the fillet). The tissue distribution of Hg(II) and MeHg did not vary with humic acid concentration, and the lower radioactivity concentration in individual tissue compartments (except MeHg in the gills) at higher humic acid concentrations is attributed to a lower overall bioavailability of Hg(II) and MeHg for killifish at higher humic acid concentrations, resulting in lower total body burdens. There were no consistent trends in the increase in As concentrations in the head, viscera, and body with increasing humic acid

concentrations. The tissue distribution of As, Hg(II) and MeHg observed in this study are comparable to another study using killifish where the aqueous uptake of As, Hg(II) and MeHg were monitored at 0 and 2 ppt [26].

The importance of humic acid concentration

Bioaccumulation studies often focus on the trophic transfer of metals because the diet is considered to be the dominant exposure route [33,37,38]. This study as well as another [26] concludes that slight changes in water chemistry can influence the accumulation of aqueous metals in killifish and needs to be considered to account for metal bioaccumulation in these fish. If the fish is an infrequent feeder, or migrates between fresh, brackish and saltwater with varying DOM concentrations, then the rate of metal uptake from the aqueous phase can change. This can influence the overall concentration of metal in the fish at steady-state and the importance of aqueous exposure as a source of metals to fish.

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Table 1. Water properties. Chloride ion concentrations, dissolved organic carbon (DOC) concentrations, % of humic acid (HA) composed of DOC, and pH of experimental waters. To calculate % HA, the 0 mg L^{-1} DOC value was subtracted from the DOC value for each HA concentration to remove the DOC associated with the deionized water. Chloride ion concentration (*n* = 3) and DOC concentration (*n* = 12) values represent means ± 1 standard deviation. ND = not determined.

HA (mg L^{-1})	Chlorinity (mM)	DOC (mg L^{-1})	% of HA	pH
0	16.9 ± 0.02	0.31 ± 0.08	ND	7.94
2.5	16.7 ± 0.03	1.36 ± 0.16	42	7.90
5	16.8 ± 0.01	2.40 ± 0.21	42	7.95
10	16.9 ± 0.02	4.27 ± 0.27	40	7.90
20	16.8 ± 0.01	8.69 ± 0.54	42	7.94

Table 2. Metal uptake rate constants (k_u; ml g^{-1} d^{-1}) in killifish at 0, 2.5, 5, 10, and 20 mg L^{-1} humic acid concentrations. Values represent means ± 1 standard error; n = 5 per humic acid concentration. Statistically significant differences between humic acid concentration and k_u are represented by * ($p<0.05$) and ** ($p<0.01$). ND = not determined.

		Humic acid concentration (mg L^{-1})				
Metal	Time (h)	0	2.5	5	10	20
As	0-9.5	0.20 ± 0.03	0.21 ± 0.03	0.17 ± 0.07	0.29 ± 0.09	0.24 ± 0.05
	9.5-24	0.04 ± 0.02	0.16 ± 0.12	0.11 ± 0.05	0.27 ± 0.21	0.28 ± 0.16
Cd	0-24.5	3.0 ± 1.1	3.6 ± 0.7	ND	6.3 ± 3.0	3.3 ± 0.7
Cr	0-9.5**	4.7 ± 0.8	1.7 ± 0.3	0.92 ± 0.06	0.58 ± 0.08	0.68 ± 0.11
Hg(II)	0-2.5*	48 ± 15	13 ± 1.1	10 ± 0.8	9.1 ± 1.4	7.8 ± 0.5
	2.5-24**	10 ± 1.4	4.2 ± 0.5	3.9 ± 0.6	3.2 ± 0.5	1.9 ± 0.2
	24-47**	77 ± 18	71 ± 5.8	74 ± 5.7	59 ± 6.8	38 ± 0.8
	47-72**	49 ± 4.9	32 ± 3.2	33 ± 3.1	26 ± 5.1	19 ± 3.2
MeHg	0-2.5**	3936 ± 350	611 ± 67	404 ± 91	188 ± 22	91 ± 7.5
	2.5-23.5**	792 ± 119	156 ± 13	201 ± 43	97 ± 11	71 ± 9.0
	23.5-47.5**	1163 ± 199	517 ± 53	746 ± 144	357 ± 25	304 ± 32

Table 3. Tissue distribution of metals in killifish as a function of humic acid concentration at the end of 72 h aqueous exposure. Values (means ± 1 standard error) represent the percentage of metal associated with each tissue compartment (head, gills, viscera, and body). *n* = 5 per humic acid concentration. Statistically significant differences between humic acid concentration and the percent of metal in each tissue compartment are represented by * ($p<0.05$) and ** ($p<0.01$). ND = not determined.

		Humic acid concentration (mg L^{-1})				
	Compartment	0	2.5	5	10	20
As	Head*	31 ± 3	27 ± 3	26 ± 3	25 ± 3	22 ± 2
	Gills**	17 ± 2	7 ± 2	8 ± 1	6 ± 0.7	6 ± 1
	Viscera	8 ± 3	14 ± 2	17 ± 3	17 ± 2	16 ± 2
	Body*	44 ± 3	52 ± 3	49 ± 3	52 ± 2	56 ± 3
Cd	Head	32 ± 1	32 ± 2	ND	30 ± 4	29 ± 3
	Gills	59 ± 2	60 ± 4	ND	53 ± 5	62 ± 2
	Viscera	3 ± 0.8	2 ± 0.3	ND	11 ± 8	3 ± 0.8
	Body	6 ± 1	6 ± 2	ND	6 ± 0.5	6 ± 1
Cr	Head**	65 ± 2	49 ± 6	38 ± 7	40 ± 10	18 ± 5
	Gills**	26 ± 2	17 ± 5	22 ± 4	12 ± 1	9 ± 4
	Viscera**	3 ± 0.8	25 ± 9	24 ± 12	35 ± 8	53 ± 14
	Body**	6 ± 1	9 ± 1	16 ± 3	13 ± 2	20 ± 6
Hg(II)	Head	27 ± 0.8	28 ± 2	26 ± 0.9	26 ± 0.2	27 ± 0.6
	Gills*	30 ± 2	26 ± 2	28 ± 1	25 ± 1	23 ± 2
	Viscera	6 ± 0.5	6 ± 0.6	6 ± 0.7	6 ± 0.5	7 ± 0.9
	Body**	37 ± 2	40 ± 0.6	40 ± 1	43 ± 1	43 ± 1
MeHg	Head	22 ± 1	22 ± 1	25 ± 1	22 ± 0.8	24 ± 0.5
	Gills	10 ± 1	14 ± 0.9	13 ± 2	13 ± 2	14 ± 1
	Viscera**	18 ± 0.9	16 ± 1	16 ± 0.9	15 ± 0.5	14 ± 0.7
	Body	50 ± 1	48 ± 2	46 ± 2	50 ± 2	48 ± 1

Table 4. Radioactivity concentrations of metals in fish tissues (head, gills, viscera, and body) as a function of humic acid concentration after 72 h aqueous exposure. Values represent means ± 1 standard error; *n* = 5 per humic acid concentration. Units are Bq g^{-1} for As and Cr, and kBq g^{-1} for Cd, Hg(II), and methylmercury (MeHg). Statistically significant differences between humic acid concentration and the radioactivity concentration in each tissue compartment are represented by * ($p<0.05$) and ** ($p<0.01$). ND = not determined.

		Humic acid concentration (mg L^{-1})				
	Compartment	0	2.5	5	10	20
As	Head*	14 ± 1.3	19 ± 5.1	15 ± 2.9	22 ± 5.4	29 ± 9.3
	Gills	84 ± 18	48 ± 10	36 ± 8.7	60 ± 23	58 ± 17
	Viscera*	9.0 ± 4.2	28 ± 11	21 ± 7.7	28 ± 11	56 ± 27
	Body*	8.8 ± 1.0	18 ± 6.5	13 ± 4.6	25 ± 8.7	34 ± 14
Cd	Head	0.60 ± 0.09	0.75 ± 0.04	ND	0.69 ± 0.10	0.59 ± 0.06
	Gills	9.3 ± 1.0	14 ± 3.9	ND	10 ± 0.7	11 ± 1.5
	Viscera	0.17 ± 0.05	0.14 ± 0.02	ND	0.89 ± 0.77	0.22 ± 0.08
	Body	0.05 ± 0.01	0.05 ± 0.02	ND	0.06 ± 0.008	0.05 ± 0.01
Cr	Head**	228 ± 33	51 ± 9.1	25 ± 5.3	33 ± 7.3	15 ± 5.5
	Gills**	939 ± 141	185 ± 33	116 ± 24	88 ± 12	42 ± 21
	Viscera	23 ± 4.7	90 ± 41	27 ± 10	47 ± 13	105 ± 47
	Body	9.0 ± 1.4	4.1 ± 1.0	4.8 ± 1.3	5.4 ± 1.2	6.8 ± 1.9
Hg(II)	Head**	0.85 ± 0.06	0.82 ± 0.06	0.88 ± 0.04	0.78 ± 0.04	0.67 ± 0.02
	Gills**	8.4 ± 0.7	6.8 ± 0.3	7.6 ± 0.6	7.2 ± 0.7	5.3 ± 0.5
	Viscera	0.52 ± 0.12	0.49 ± 0.08	0.62 ± 0.09	0.45 ± 0.06	0.34 ± 0.03
	Body*	0.53 ± 0.03	0.54 ± 0.03	0.60 ± 0.02	0.54 ± 0.04	0.46 ± 0.02
MeHg	Head**	5.2 ± 0.3	4.6 ± 0.2	5.3 ± 0.3	3.5 ± 0.2	3.9 ± 0.3
	Gills	23 ± 2.5	25 ± 1.6	23 ± 1.1	18 ± 2.0	24 ± 2.3
	Viscera**	12 ± 1.2	8.0 ± 0.8	9.5 ± 1.8	5.4 ± 0.5	6.1 ± 1.4
	Body**	5.1 ± 0.2	4.4 ± 0.4	4.7 ± 0.3	3.3 ± 0.2	3.4 ± 0.3

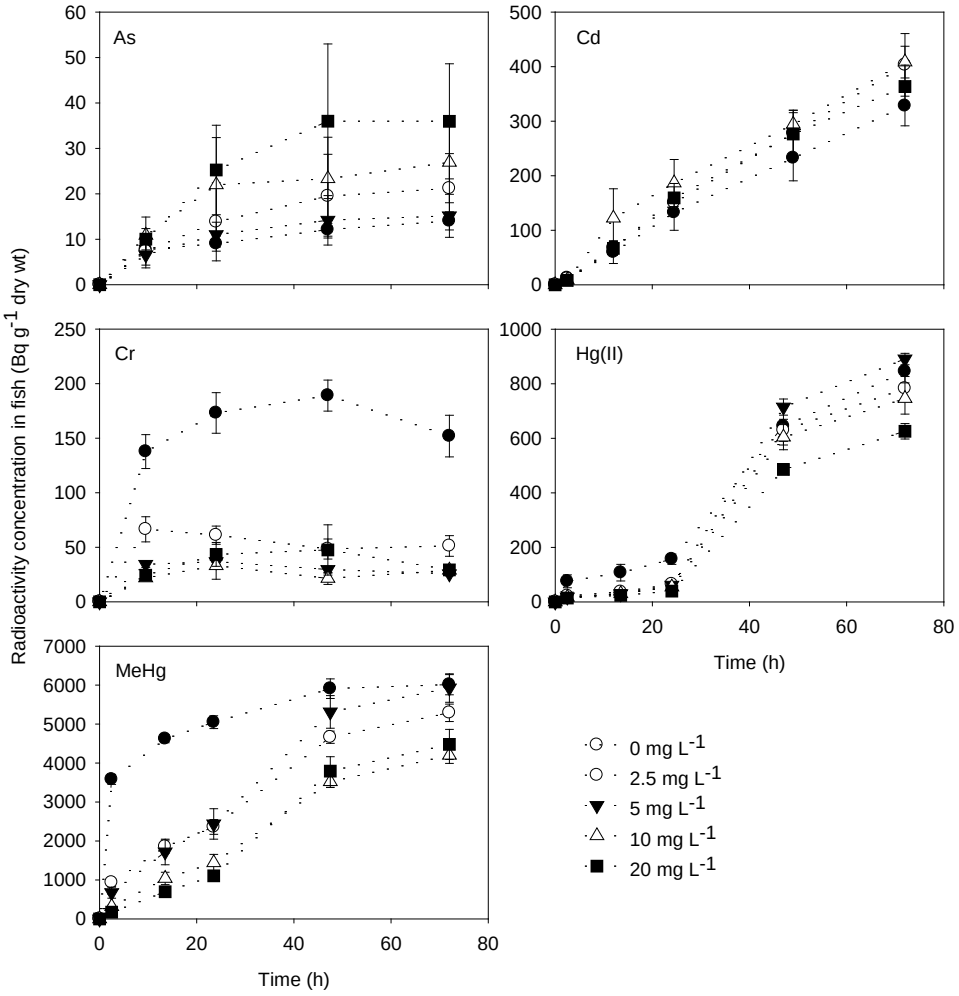

Figure 1. Accumulation of aqueous metals (Bq g^{-1} dry weight) in killfish (*Fundulus heteroclitus*) at varying humic acid concentrations (mg L^{-1}). Values represent means ± 1 standard error; n = 5

per humic acid concentration.

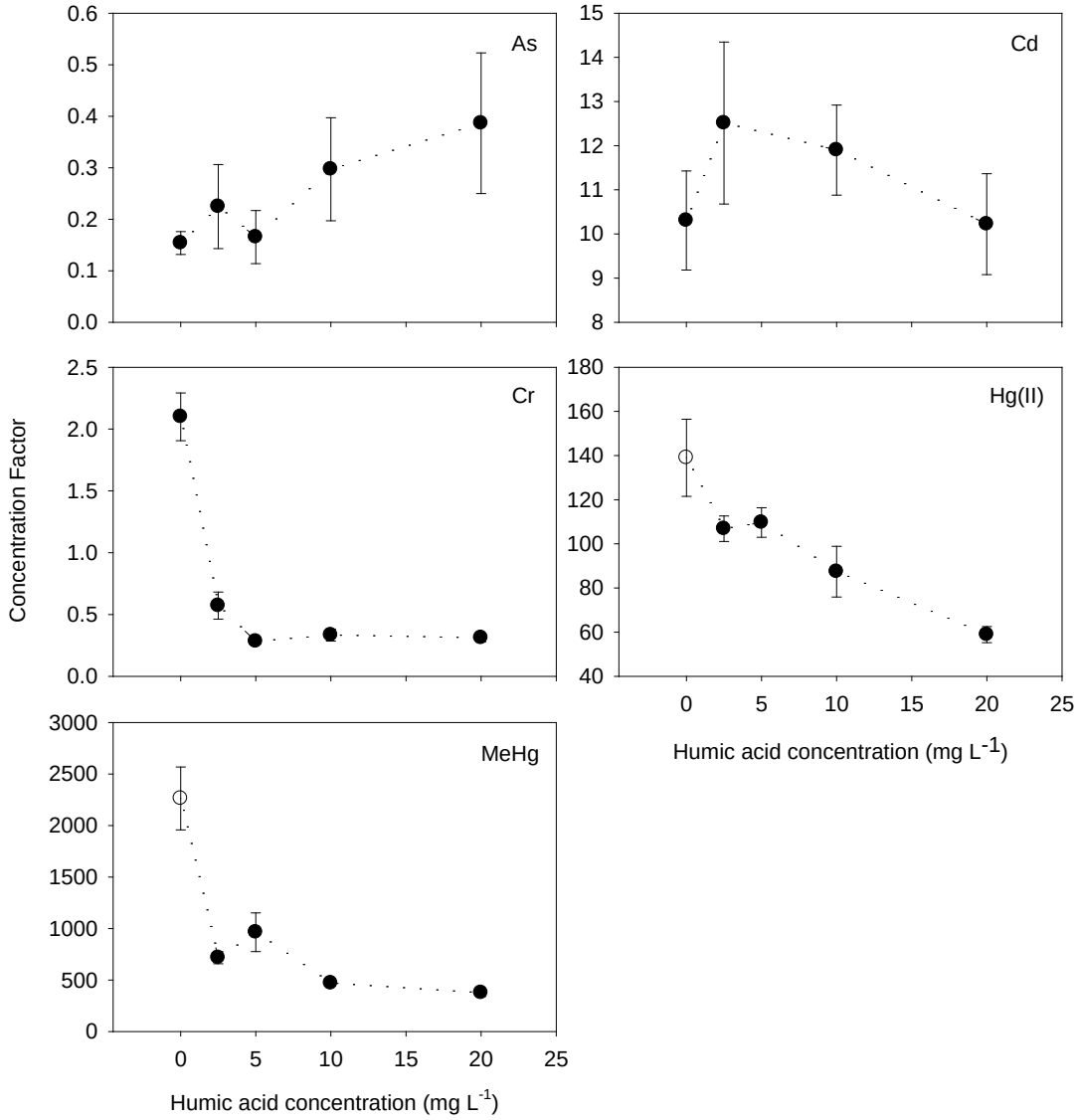

Figure 2. Metal dry weight concentration factors (CFs) in killifish (*Fundulus heteroclitus*) after 47.5 h (methylmercury [MeHg]) or 72 h (As, Cd, Cr, Hg(II)) aqueous uptake as a function of humic acid concentration. Values represent means ± 1 standard error; n = 5 per humic acid concentration.

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Bioaccumulation of As, Cd, Cr, Hg(II), and MeHg in killifish (*Fundulus heteroclitus*) from amphipod and worm prey

Abstract

Elevated metal levels in fish are a concern for the fish themselves, their predators, and possibly humans who consume contaminated seafood. Metal bioaccumulation models often rely on assimilation efficiencies (AEs) of ingested metals and loss rate constants after dietary exposure (k_{ef}s). These models can be used to better understand processes regulating metal accumulation and can be used to make site-specific predictions of metal concentrations in animal tissues. Fish often consume a varied diet, and prey choice can influence these two parameters. We investigated the trophic transfer of As, Cd, Cr, Hg(II), and methylmercury (MeHg) from a benthic amphipod (*Leptocheirus plumulosus*) and an oligochaete (*Lumbriculus variegatus*) to killifish (*Fundulus heteroclitus*) using gamma-emitting radioisotopes. Except for MeHg, AEs varied between prey type. AEs were highest for MeHg (92%) and lowest for Cd (2.9-4.5%) and Cr (0.2-4%). Hg(II) showed the largest AE difference between prey type (14% amphipods, 24% worms). For Cd and Hg(II) k_{ef}s were higher after consuming amphipods than consuming worms. Tissue distribution data shows that Cd and Hg(II) were mainly associated with the intestine, whereas As and MeHg were transported throughout the body. Calculated trophic transfer factors (TTFs) suggest that MeHg is likely to biomagnify at this trophic step at all ingestion rates, whereas As, Cd, Cr, and Hg(II) will not. Data collected in this study and others indicate that using one prey item to calculate AE and k_{ef} could lead to an over- or underestimation of these parameters.

Keywords: metals, fish, dietary exposure, assimilation efficiency, trophic transfer

1. Introduction

It is well known that metals can bioaccumulate in aquatic food chains and in some cases biomagnify as well (Mathews and Fisher, 2008a, 2008b; Mathews et al., 2008; Wang, 2002; Wang and Wong, 2003; Xu and Wang, 2002). Trophic transfer can lead to elevated metal concentrations in higher trophic level organisms, particularly fish, which can impact the health of the fish themselves, their predators, and ultimately human consumers of contaminated seafood. Of the metals examined thus far, methylmercury (MeHg) is of particular concern because with high enough doses it can cause neurological disorders (Chang et al., 2008; Harada, 1995), and possibly myocardial infarction in humans (Guallar et al., 2002). MeHg can accumulate in marine and freshwater fish to a level that can exceed the health based maximum permitted level for human consumption, resulting in advisories being issued regarding seafood consumption.

Fish, like other aquatic organisms, accumulate metals through aqueous and dietary exposure routes (Pickhardt et al., 2006; Wang and Wong, 2003; Xu and Wang, 2002). Recent studies have shown that the diet is the dominant exposure route for many metals (Mathews and Fisher, 2009; Pickhardt et al., 2006; Xu and Wang, 2002). It is therefore important to understand those factors which influence metal assimilation and loss following dietary exposure, enhancing this trophic transfer. Studies have shown that prey choice and subcellular partitioning within the prey has a direct influence on metal assimilation efficiencies (defined as the percentage of ingested metal crossing the gut lining) in fish (Wang and Wong, 2003; Xu and Wang, 2002; Zhang and Wang, 2006).

Fish can accumulate metals through planktonic and benthic food chains. Within the planktonic food chain, by far the largest metal enrichment step occurs in phytoplankton which

lies at the base of most aquatic food chains (Fisher and Reinfelder, 1995). Planktonic organisms can therefore serve as an enriched source of metals to fish. In addition, estuarine sediments are enriched in metals, and concentrations may reach extremely high levels in industrialized areas (Kennish, 1998). Sediment-bound metals are bioavailable to benthic organisms, including worms (Wang et al., 1998, 1999), which in turn can act as a conduit for the transfer of metals to higher level consumers.

Estuaries are an important nursery ground for juvenile fish, providing protection and an abundant source of food. However, estuaries are often found next to heavily populated and industrialized areas, and the resulting metal discharges can lead to elevated body burdens in fish. To further understand the importance of prey choice on metal accumulation in fish, we investigated the trophic transfer of three trace metals (Cd, Cr(III), and Hg (as inorganic Hg (Hg(II)), and MeHg)) and a metalloid (As(V)) through a representative estuarine food chain to killifish (*Fundulus heteroclitus*) using gamma-emitting radioisotopes. Killifish was the chosen predator because it is a potential biomonitor of regional metal contamination due to their wide distribution; are euryhaline, withstanding fluctuating salinities associated with estuaries (Abraham, 1985); tolerant of high pollutant levels, and a conduit for the transfer of metals to higher trophic levels including crabs, fish, sharks, and birds (Kneib, 1986; Rountree and Able, 1996; Tupper and Able, 2000). Comparisons were made between two prey types, the California blackworm (*Lumbriculus variegatus*), an oligochaete, radiolabeled via an aqueous exposure; and the benthic amphipod *Leptocheirus plumulosus*, radiolabeled via a phytoplankton diet. Higher assimilation efficiencies would be expected after consuming worms, because amphipods have a chitinous exoskeleton and metals bound to this will pass through the fish unassimilated, as it is harder to digest (Reinfelder and Fisher, 1994). Using these two representative estuarine food

chains we compared the uptake and loss kinetics of the chosen metals after fish were fed a single pulse of radiolabeled prey. Kinetic parameters (assimilation efficiency, loss rate constant), and tissue distribution at the end of depuration were measured.

The metals studied were chosen based on their chemical characteristics and environmental interest. These metals have varying binding preferences for oxygen, nitrogen and sulfur, influencing their association with proteins. Hg is a Class B metal (sulfur-seeking), while As, Cd, and Cr are borderline metals (Nieboer and Richardson, 1980). Finally, these metals are often found at elevated concentrations in contaminated estuarine waters (Kennish, 1997).

2. Materials and methods

2.1. Water and experimental conditions

Phytoplankton, amphipods and killifish were held in 0.2 µm sterile filtered (Millipak 200, Millipore, Bedford, MA, USA) Baltimore Harbor (Baltimore, MD, USA) water throughout the experiments (salinity = 7.6; dissolved organic carbon concentration = 219 µM ± 20.5 SD, n = 3), and maintained at 18 ± 1°C on a 14/10 h light/dark cycle. Worms were held in 0.2 µm sterile filtered Stony Brook tap water in the dark at 4°C, which was changed daily prior to and during experiments. The background dissolved concentrations of metals in Baltimore Harbor water, as determined by ICP-MS, were 13.0 nM As, 2.1 nM Cd, 3.7 nM Cr, and 0.014 nM total Hg.

2.2. Prey organisms and fish

California blackworms (*Lumbriculus variegatus*; 2.5-3 cm long) were obtained from a local aquarium store (Magic Isle Aquarium, Centereach, NY, USA). The worms were not fed, as they live off stored energy reserves. Amphipods (*Leptocheirus plumulosus*; 4-7 mm) were obtained from Aquatic Research Organisms (Hampton, NH, USA), acclimated to Baltimore Harbor water, and fed a daily diet of the green algae *Dunaliella tertiolecta*. Amphipods were not fed for 2 hours prior to feeding on radiolabeled phytoplankton to allow the gut to empty.

Field collected (Taylor River, Hampton, NH, USA) juvenile killifish (*Fundulus heteroclitus*; mean wet weight 2.5 g ± 0.5 SD) were obtained from Aquatic Research Organisms (Hampton, NH, USA), acclimated to experimental conditions for at least 4 weeks prior to experiments, and fed a daily diet of frozen bloodworms (Hikari Sales USA Inc., Hayward, CA, USA) and TetraCichlid™ cichlid flakes (Tetra Holding Inc., Blacksburg, VA, USA). Four days prior to the experiments killifish were fed either blackworms or amphipods to acclimate them to live prey. All fish were starved for 36 hours prior to feeding on radiolabeled prey to allow for gut clearance.

2.3. Metal uptake from amphipods

The euryhaline green algae *Dunaliella tertiolecta* (CCMP 1320) was uniformly radiolabeled for 4 days in 2.2 L Baltimore Harbor water amended with nutrients at f/2 concentrations (Guillard and Ryther, 1962), but modified so no Cu, Zn and EDTA (ethylenediaminetetraacetic acid) were added. When labeling with ^{73}As, phosphate was added at a reduced concentration (f/200) to minimize competition for uptake (Sanders and Windom, 1980). Following uptake, the percentage of radioisotope associated with the phytoplankton

(mean ± 1 SE, n = 4) was 62 ± 1% for ^{73}As, 13 ± 1% for ^{109}Cd, 68 ± 2% for ^{51}Cr, 78 ± 5% for ^{203}Hg(II), and 52 ± 2% for MeHg. The phytoplankton cells were filtered through a 0.2 µm polycarbonate membrane, rinsed with unlabeled water, and resuspended in 1 L Baltimore Harbor water. Algal cells (4 x 10^5 ml^{-1}) were distributed between 10 containers, and 25 amphipods were added to each 100 ml, and allowed to feed for 2 days. Amphipods were then removed, rinsed to remove surface droplets containing radioisotope, and fed to killifish. Prior to, and during feeding of radiolabeled amphipods, fish were held in groups of 5 individuals per container (n = 25 per treatment). The fish ate immediately, but initial radioactivity in the fish was not assessed for 30 minutes to prevent regurgitation of food if stressed from handling. No feces were produced during the feeding period, so the start of depuration was when the fish ate. After the initial radioassay, fish were returned to individual containers with 600 ml aerated non-radiolabeled Baltimore Harbor to depurate for 9 days, and fed non-radiolabeled bloodworms to purge their guts of unassimilated radiolabeled food. Radioactivity in the fish was assessed at regular intervals for the first 2 days, and then once a day for the following 7 days to monitor metal loss. At each sample time, feces and water were collected, and water was changed after 24 hours, and then every other day. Five fish were euthanized using 450 ppm MS-222 (tricaine methane sulphonate, $C_9H_{11}O_2N + CH_3SO_3H$) at regular intervals throughout depuration (4 hours and 1, 3, 6 and 9 days) to monitor how metal body distribution changed over 9 days after feeding. Fish were dissected into head, gills, intestine, liver, other viscera, skeleton (backbone and fins), fillet, and skin. After obtaining radioactive counts, tissue samples were dried at 60°C for 4 days to obtain dry weights. 25 fish were exposed to radiolabeled food at the start of the experiment, but not all ate. As a result n = 24 for Cd, Hg(II), and MeHg, and 25 for As and Cr. Four fish were sacrificed at the first dissection time for Cd, Hg(II), and MeHg, and 5 fish thereafter.

2.4. Metal uptake from worms

To radiolabel blackworms, radioisotopes were added to 300 ml of Stony Brook tap water and allowed to equilibrate for 1 hour, after which 60 ml was poured into two containers, and 30 worms added to each. Worms were radiolabeled for 48 hours, with a water change after the first 24 hours. At the end of labeling, the worms were removed, rinsed and held in non-radiolabeled tap water for 5 minutes, and fed to killifish. The worms did not accumulate As during labeling. Prior to, and during feeding of radiolabeled worms, all fish were held in a single large container (n = 10), but after the initial radioassay fish were transferred to individual containers. Not all fish ate so the resulting n values are as follows: 10 for Cd, 5 for Cr, 7 for Hg(II), and 8 for MeHg. The depuration procedure was identical to that described for amphipods. All fish were dissected at the end of depuration into head, gills, viscera, liver, skeleton (backbone and fins), fillet, and skin.

2.5. Radioisotopes and analyses

High specific activity gamma-emitting radioisotopes were used in these experiments. ^{73}As (t½ = 80.3 days) and ^{109}Cd (t½ = 462.6 days) were purchased from the U.S. Department of Energy (Los Alamos National Laboratory, Los Alamos, NM, USA), ^{51}Cr (t½ = 27.7 days) was purchased from PerkinElmer (Boston, MA, USA), and ^{203}Hg(II) (t½ = 46.6 days) was obtained from Georgia State University (GA, USA). ^{73}As was purchased as As(V) and ^{51}Cr as Cr(III). Methylmercury (MeHg), $CH_3{}^{203}$Hg(II), was synthesized in our lab from ^{203}Hg(II) following a method described elsewhere (Bancon-Montigny et al., 2004; Imura et al., 1971; Rouleau and

Block 1997). ^{73}As and ^{109}Cd were dissolved in 0.1 M HCl, ^{51}Cr in 0.5 M HCl, ^{203}Hg(II) in 1 M HCl, and $CH_3{}^{203}$Hg(II) in deionized water. Radioisotopes were added in microliter amounts, and sodium hydroxide (NaOH) was added at equimolar concentrations to neutralize the acid. The pH of radiolabeled water was monitored and remained unaffected by the radioisotope additions. ^{73}As and ^{51}Cr were double-labeled, while ^{109}Cd, ^{203}Hg(II), and MeHg were single-labeled. For the trophic transfer using amphipods each algal replicate (550 ml media) was exposed to 76.4 kBq ^{73}As, 51.3 kBq ^{109}Cd, 72.8 kBq ^{51}Cr, 12.7 kBq ^{203}Hg(II), and 17.9 kBq MeHg. This equals the following metal concentrations: 2.77 nM ^{73}As (21% of background), 1.23 nM ^{109}Cd (59% of background), 0.45 nM ^{51}Cr (12% of background), 1.3 nM ^{203}Hg(II), and 1.44 nM MeHg (both greatly exceeding background Hg). For the trophic transfer using blackworms each worm replicate (60 ml tap water) was exposed to 26.5 kBq ^{73}As, 2.3 kBq ^{109}Cd, 12.2 kBq ^{51}Cr, 3.5 kBq ^{203}Hg(II), and 2 kBq MeHg daily. This equals the following metal concentrations: 1.5 nM ^{73}As (12% of background), 0.45 nM ^{109}Cd (21% of background), 4 nM ^{51}Cr (108% of background), 0.4 nM ^{203}Hg(II), and 1.25 nM MeHg (both greatly exceeding background Hg).

Blackworms, amphipods and live fish were counted in a Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector. Counting times did not exceed 5 minutes to obtain propagated counting errors ≤ 5%, and minimize stress on the fish. After gut clearance propagated counting errors could reach 25% due to decreased radioactivity in the samples. Phytoplankton, water, feces and dissected fish tissue were counted in an inter-calibrated LKB Pharmacia-Wallac 1282 CompuGamma CS gamma counter (Turku, Finland) for 5 minutes (^{73}As, ^{109}Cd, and ^{51}Cr), or 10 minutes (^{203}Hg). The γ-emission of ^{73}As was detected at 53 keV, ^{109}Cd at 22 keV, ^{51}Cr at 320 keV, and ^{203}Hg at 279 keV. All counts were adjusted for background radioactivity and radioactive decay.

2.6. Modeling metal bioaccumulation in killifish

The bioaccumulation of metals in aquatic organisms can be calculated using the following equation, which takes into account metal uptake and loss following aqueous and dietary exposures. This was originally described by Thomann (1981), and modified by Wang et al. (1996) and Reinfelder et al. (1998), and has been tested using zooplankton (Fisher et al., 2000), bivalves (Fisher et al., 1996; Wang et al., 1996), and fish (Pickhardt et al., 2006). Under steady-state conditions the equation to describe the steady-state concentration of metal in fish (C_{ss}, in μg g^{-1}) is:

$$C_{ss} = (k_u \cdot C_w)/(g + k_{ew}) + (\mathrm{AE} \cdot \mathrm{IR} \cdot C_f)/(g + k_{ef}) \qquad (1)$$

where k_u is the uptake rate constant of metal from the dissolved phase (L g^{-1} d^{-1}), C_w is the metal concentration in the dissolved phase (μg L^{-1}), g is the growth rate constant (d^{-1}), k_{ew} is the metal loss rate constant after aqueous exposure (d^{-1}), AE is the assimilation efficiency of ingested metal in the fish (fraction), IR is the weight-specific ingestion rate (g g^{-1} d^{-1}), C_f is the metal concentration in food (μg g^{-1}), and k_{ef} is the metal loss rate constant after dietary exposure (d^{-1}).

AE and k_{ef} were calculated for individual fish after feeding on amphipods or worm prey by fitting an exponential regression, describing retention of metal in the fish between the 48 h to 216 h of depuration. The AE was determined to be the *y*-intercept, and the k_{ef} the slope of the curve. For fish feeding on amphipods AE and k_{ef} values were calculated using fish sacrificed after 144 and 216 h of depuration (n = 10 per metal). IR values were obtained from the literature and ranged from 0.01-0.1 g^{-1} g^{-1} d^{-1} (Garnier-Laplace et al., 2000; Pauly, 1989). The biological

half-life ($tb_{½}$) of a metal in the fish is defined as the time it takes for 50% of the metal to be excreted, and is calculated as follows:

$$tb_{1/2} = \ln 2/k_{ef} \qquad (2)$$

Eq. 1 can be rearranged to calculate the trophic transfer factor (TTF), which indicates the likelihood that a metal will biomagnify based upon the ratio of metal in the fish compared to its prey. This ratio is calculated as follows:

$$TTF = (AE \cdot IR)/k_{ef} \qquad (3)$$

A TTF < 1 indicates that biomagnification at that step in the food chain is unlikely, whereas a TTF > 1 indicates biomagnification is likely (Reinfelder et al., 1998). The statistical significance of comparisons of metal kinetic parameters in individual fish between the two prey types and relating body length to AE values were determined using *t*-tests. Significance was evaluated at either the $p<0.05$ or $p<0.01$ confidence level.

3. Results

3.1. Assimilation of metals

The AE for MeHg greatly exceeded those for all other metals for both prey types (Table 1). After feeding on amphipods AEs were highest for MeHg (92%), followed by Hg(II) (14%), As (9.4%), Cd (4.5%), and lowest for Cr (0.2%). After feeding on worms AEs were highest for MeHg (92%), followed by Hg(II) (24%), Cr (4%), and Cd (2.9%). The AE of As was not calculated because the worms did not accumulate As during radiolabeling. When comparing AEs with prey type, killifish assimilated more Hg(II) (1.7-fold) and Cr (20-fold) when feeding on

worms, and more Cd (1.6-fold) when feeding on amphipods. There was a significant relationship between prey choice and AE for Cd ($p<0.05$) and Cr ($p<0.01$), but no significant relationship for Hg(II) and MeHg ($p>0.05$). Unlike Hg(II), the assimilation of MeHg did not vary with prey type. The AE for Cr after feeding on amphipods was given as the percentage retained after 48 hours of depuration because exponential regressions could not be fitted to the data due to elimination of the radioisotope.

Fig. 1 shows the relationship between fish length and AE for both prey types. For all metals fish size had little influence on AE. There was no significant relationship ($p>0.05$) between fish length and AE for all metals and both prey types, except for worms labeled with MeHg ($p<0.05$). However if the smallest fish labeled with MeHg from a worm diet is removed from the regression analysis there is no significant relationship ($p>0.05$), and the r^2 is reduced to 0.075.

3.2. Retention and removal of metals

After feeding on either radiolabeled amphipods or worms all metals were eliminated from killifish following a two-compartment loss pattern. During the first 24 h of depuration rapid loss corresponded to gut clearance of unassimilated metal, and the slower loss for the remainder of depuration corresponded to the physiological turnover of assimilated metal (Fig. 2). Within the first 24 hours 89% of As, 94% of Cd, 99.8% of Cr, 86% of Hg(II), and 8% of MeHg had been eliminated from killifish after feeding on amphipods; and 96% of Cd, 95% of Cr, 73% of Hg(II), and 8% of MeHg had been eliminated after feeding on worms. At the end of the 9 day depuration period the fraction of the original metal retained in killifish was 0.9% As, 2.7% Cd, 0.3% Cr,

2.5% Hg(II), and 87% MeHg after feeding on amphipods, and 2% Cd, 2.5 % Cr, 14% Hg(II), and 86% MeHg after feeding on worms (Fig. 2). Loss rate constants (k_{ef}s) were highest for As (0.287 d^{-1}) and lowest for MeHg (0.008 d^{-1}) after feeding on amphipods, and highest for Hg(II) (0.067 d^{-1}) and lowest for MeHg (0.007 d^{-1}) after feeding on worms. For Cd and Hg(II) k_{ef}s were higher when fed radiolabeled amphipods, and there was no difference between MeHg k_{ef}s and prey type. There was no significant relationship between prey type and k_{ef} for Cd and MeHg ($p>0.05$), but there was for Hg(II) ($p<0.01$). k_{ef} could not be calculated for Cr after feeding on amphipods due to near complete elimination of the radioisotope (Table 1).

3.3. Tissue distribution and concentration of metals

Fig. 3 shows the tissue distribution of Cd, Hg(II), and MeHg in killifish at the end of the depuration period after feeding on a single radiolabeled amphipod or worm meal. Cd and Hg(II) were mainly associated with the viscera (86-94% Cd, 78-82% Hg(II)), whereas MeHg was predominantly associated with the fillet (35-36%), followed by the viscera (15-18%) and head (16-17%). Each metal's tissue distribution was not influenced by prey choice. Cr tissue distribution could not be determined for both prey types due to low detection in the fish. Table 2 shows the radioactivity concentration (Bq g^{-1} dry wt) of Cd, Hg(II), and MeHg in tissue compartments at the end of depuration after feeding on radiolabeled worms. For all 3 metals the radioactivity concentration was highest in the viscera (excluding liver), and MeHg was the only metal to appreciably accumulate in the fillet. No comparison could be made between radioactivity concentration in fish tissue and prey type because the prey organisms were exposed to different metal concentrations.

Table 3 shows the movement of As, Cd, Hg(II), and MeHg between tissue compartments throughout the 9 day depuration. Cd and Hg(II) were mainly associated with the intestine, whereas As and MeHg were transported around the body. The Cd associated with the intestine decreased from 94 to 85% throughout depuration, while that associated with the fillet and skin increased from 1.3 to 3.6%, and 1.5 to 7.8% respectively. The Hg(II) associated with the intestine decreased from 93 to 73%, that associated with the head increased from 0.6 to 7.4%, and the fillet from 0.3 to 3.3%. The association of As with the intestine and viscera decreased throughout the depuration (22 to 4.7% and 41 to 7.7%, respectively), and increased in the head (8.7 to 18%), fillet (11 to 42%) and skin (4.8 to 12%). The MeHg associated with the intestine decreased from 62 to 14%, and increased in the head, fillet and skin (6.9 to 17%, 10 to 36%, and 4.9 to 11%, respectively). Fish dissected after 4 hours of depuration showed that all metals were associated with the intestine. When the percentage of metal associated with the intestine was regressed against time the elimination rate of As, Cd, Hg(II), and MeHg was calculated to be 0.147, 0.012, 0.028, and 0.189 d^{-1}. MeHg and As associated with the intestine had a short $tb_{1/2}$ (3.7 and 4.7 days), while Hg(II) and Cd had a longer $tb_{1/2}$ (25 and 58 days). Assuming it takes 7 half-lives to remove essentially all of the metal, the resulting intestinal residence time is 26 days for MeHg, 33 days for As, 173 days for Hg(II), and 404 days for Cd. Table 4 shows the radioactivity concentrations (Bq g^{-1} dry wt) of As, Cd, Hg(II), and MeHg in tissue compartments on day 1, 3, 6, and 9 of depuration after feeding on amphipods. For Cd, Hg(II), and MeHg, the radioactivity concentration was highest in the intestine, and MeHg had appreciable accumulation in all tissue compartments. However, As concentration was highest in the viscera (excl. intestine and liver) throughout depuration. No comparison could be made between individual metals because the phytoplankton was exposed to different metal concentrations. This data cannot be

used to calculate metal efflux rates from tissue compartments, because killifish ate varying amounts of radiolabeled amphipods, resulting in the large standard error values observed.

3.4. Modeling dietary metal uptake in killifish

Fig. 4 shows the trophic transfer factors (TTFs) for each metal and prey type as a function of ingestion rate (IR). MeHg was the only metal to have a TTF >1 for all IRs and both prey types (TTFs = 1.2-13), indicating that MeHg would be expected to biomagnify at this trophic step. TTFs were <1 for As, Cd, Cr, and Hg(II), regardless of IR and prey type, indicating that these metals would not be expected to biomagnify at this trophic step. For Hg(II) and MeHg, TTFs were higher after consuming worms, whereas Cd TTF's were higher after consuming amphipods. There was a significant relationship between prey choice and TTF for Hg(II) ($p<0.01$), but not for Cd and MeHg ($p>0.05$).

The biological half-life ($tb_{1/2}$) of metals in killifish (Table 5) was highest for MeHg (87-99 days), followed by Cd (11–13 days), Cr (11 days), Hg(II) (5–10 days), and As (2.4 days). For Cd, Hg(II) and MeHg the $tb_{1/2}$ was greater for a worm diet than an amphipod diet. Assuming it takes 7 half-lives to remove all of the assimilated metal, Cd would be retained for 77 and 91 days, Hg(II) for 35 and 70 days, and MeHg for 609 days and 693 days, after feeding on amphipod and worm prey, respectively. As would be retained for a total of 17 days after feeding on amphipods, and Cr for 77 days after feeding on worms.

4. Discussion

4.1. Metal assimilation and retention

The general ranking of metal AEs in killifish (MeHg > Hg(II) > As > Cd > Cr after feeding on amphipods, and MeHg > Hg(II) > Cr $\geq$ Cd after feeding on worms) indicated that prey choice did not greatly affect this ranking. The AEs for MeHg reported here are comparable to other radiotracer studies using freshwater and marine fish fed zooplankton prey (Pickhardt et al., 2006; Wang and Wong, 2003), including killifish (Mathews and Fisher, 2008a). For both prey types, MeHg assimilation was much greater than Hg(II), being 6.6-fold higher after feeding on amphipods, and 3.8-fold higher after feeding on worms. This higher assimilation could be explained by fish gut chemistry, allowing MeHg to be readily solubilized from the ingested material, and transported across the intestine wall (Leaner and Mason, 2002a).

Hg(II) assimilation was 1.7-times higher when fish were fed worm prey. Previous studies have shown that AEs in juvenile fish are related to the percentage of metal associated with the soft body of the prey. Fish can not digest the chitinous amphipod exoskeleton, and therefore assimilate a limited amount of metal associated with the exoskeleton (Reinfelder and Fisher, 1994). It should be noted that the prey in our experiments were labeled by different methods, with amphipods receiving a dietary exposure, and worms an aqueous exposure due to logistical constraints. This could influence the AE in fish due to differences in body partitioning within the prey; however, worms were held in non-radiolabeled tap water for 5 minutes before feeding to fish, so all radioisotope should be internalized and not adsorbed to the body surface. Our Hg(II) AE after feeding on amphipods (14%) is comparable to other studies where fish were fed amphipods (8.5-9.8%) or brine shrimp (10%), but lower than in fish feeding on copepods (27%), or *Daphnia pulex* (42-51%) (Pickhardt et al., 2006; Wang and Wong, 2003). Copepods and *D.pulex* may accumulate more Hg(II) than the *Leptocheirus plumulosus* we used, efficiently

transferring more Hg(II) to higher trophic levels. The lower AE in the killifish could also be due to killifish having a shorter gut passage time than the mosquitofish (*Gambusia affinis*) fed *D. pulex* Pickhardt et al. (2006) examined (< 24 hours versus 48 hours), reducing the time Hg(II) is in contact with the intestine. To our knowledge this is the first study calculating AEs for MeHg and Hg(II) in fish after feeding on worms.

Cd assimilation showed little variation with prey choice (4.5% for amphipods, 2.9% for worms), but the small difference was statistically significant ($p<0.05$). This is the only metal investigated where the AE was higher after feeding on amphipods compared to worms. The AE for killifish when fed worm prey falls within the range found when the same oligochaete was fed to rainbow trout, *Oncorhynchus mykiss* (0.9-6.4%; Ng and Wood, 2008). For zooplankton prey, our AE values are comparable to *D. pulex* fed to killifish (Mathews and Fisher, 2008a), and copepods fed to silversides, *Menidia menidia* and *Menidia beryllina* (Reinfelder and Fisher, 1994). However, our Cd AE values are lower than zooplankton prey fed to mudskipper (*Periophthalmus cantonensis*,10-26%), glassy (*Ambassis urotaenia* 14-33%), sea bream (*Sparus auratus*, 21%) and striped bass (*Morone saxatilis*, 23-28%) (Baines et al., 2002; Mathews and Fisher 2008b; Ni et al., 2000). Higher Cd AEs could be explained by longer gut passage times in these fish increasing time for absorption across the gut lining, or the variation in bioavailability of Cd among zooplankton prey items.

Cr AE was significantly higher when killifish were fed worm prey ($p<0.01$). No values could be found to compare AEs in killifish, or any other species when fed worm prey, but our AE value after feeding on amphipods is lower than AEs (4.2-19%) for other fish species fed zooplankton prey (Ni et al., 2000). Due to the very low AE Cr could be used as a tracer to measure gut passage time, as shown in copepods (Reinfelder and Fisher, 1991) and bivalves

(Wang and Fisher, 1996). Differences in AE between amphipod and worm prey is due to higher accumulation of Cr in soft bodied worm tissue, whereas Cr bound to amphipod exoskeleton is not assimilated, consistent with Reinfelder and Fisher's (1994) findings.

To our knowledge this is the first study to calculate As AE from zooplankton prey. Because As was not accumulated in worms, no comparison between the two prey types could be made. This was probably due to competition with phosphate for uptake, and the addition of As as arsenate which is anionic and repels against the worm's body surface which carries a negative surface charge. However, previous studies have shown that As concentration in organisms decreases with increasing trophic level in freshwater and estuarine food chains (Chen and Folt, 2000; Culioli et al., 2009; Lindsay and Sanders, 1990).

In this study there was no significant relationship between fish body length and AE, most likely due to the narrow range of fish size used (most fish were 55-70 mm long). In other studies fish length was shown to influence AE. AEs calculated in juvenile striped bass at 20 mm and 39 mm showed an increase with body length, with Cd increasing from 23% to 28%, Se from 33% to 42%, and Zn from 23% to 40% (Baines et al., 2002). Another study also showed that Se and Zn AEs increase with body size, but Cd AE shows no relationship (Zhang and Wang, 2007). This increase in AE could be tied to a decrease in IR in larger size fish. A study using the mangrove snapper (*Lutjanus argentimaculatus*) concluded that AEs for Cd decreased from 24% to 7%, Se from 69% to 54%, and Zn from 43% to 17% as ingestion rates increased from 0.05 to 0.57 g g^{-1} d^{-1} (Xu and Wang, 2002).

Loss rate constants (k_{ef}s) calculated after feeding on amphipod and worm prey could be compared for Cd, Hg(II), and MeHg. For each metal the k_{ef} was always higher after feeding on

amphipod prey, but Hg(II) was the only metal where k_{ef}s varied significantly with prey choice. To our knowledge this is the first study to report k_{ef} values for As and Cr for any prey item in fish, and after feeding on worms for Cd, Hg(II), and MeHg. Cd, Hg(II), and MeHg k_{ef}s after feeding on amphipods could be compared to other literature values. Our k_{ef} for MeHg was comparable to other values for freshwater and marine fish (0.01-0.018 d^{-1}; Mathews and Fisher, 2008a; Pickhardt et al., 2006; Wang and Wong, 2003). The k_{ef} for Hg(II) (0.131 d^{-1}) observed in this study is slightly higher than for the marine sweetlips, *Plectorhinchus gibbosus* (0.096 d^{-1}; Wang and Wong, 2003), but much higher than recorded for the freshwater mosquitofish (0.025-0.033 d^{-1}) and redear sunfish (0.003-0.007 d^{-1}) (Pickhardt et al., 2006). The k_{ef} values for freshwater fish could be much lower than for marine fish due to osmoregulatory differences since marine fish actively drink to replace what is lost from tissues and salt removal across the gills to the surrounding seawater. As a result, the elimination rate of Hg(II) in marine fish could be higher if Hg(II) is also transported through the same ion-transport channels as what is used for salt removal (Andres et al., 2002). Hg(II) k_{ef} could also be higher because some Hg(II) remained attached to the intestine wall after 9 days of depuration, suggesting that metal loss should be monitored for a longer period of time. Wang and Wong (2003) noted that during the first 7 days of depuration the Hg(II) k_{ef} was 0.096 d^{-1}, but during 9-28 days after the start of depuration the k_{ef} decreased to 0.055 d^{-1}. Our Cd k_{ef} (0.064 d^{-1}) was within the range noted in the literature for other marine species (0.03-0.14 d^{-1}; Baines et al., 2002; Mathews and Fisher, 2008b; Xu and Wang, 2002), but higher than noted in another killifish study after feeding on *Daphnia pulex* (0.03 d^{-1}; Mathews and Fisher, 2008a).

4.2. Distribution of metals in fish tissue

The distribution of metal in fish tissue varied by metal, and there was a distinct difference between the two Hg species. Cd and Hg(II) remained associated with the initial site of exposure (intestine) throughout depuration, and very little was transported across the intestinal wall. However, As and MeHg was transported around the body. The type of prey ingested did not influence the body distribution of metal in killifish.

Other literature values for Cd body distribution show that there is great variability between fish species. Our study concluded that 85-94% of Cd remains associated with the intestine, while other studies which grouped all the internal organs together calculated 81% in the mangrove snapper (Xu and Wang, 2002), 50% in another killifish study (Mathews and Fisher, 2008a), and 20% in striped bass (Baines et al., 2002). This indicates that some fish are better protected against Cd uptake across the intestinal wall than others. Cd is known to share the same uptake pathway as Ca in the gills and gastrointestinal tract (Franklin et al., 2005). Studies using rainbow trout have shown that the low AE for Cd, and limited uptake into body tissues may be due to the stomach and intestine behaving as a protective barrier due to preferential uptake of Ca (Wood et al., 2006).

To our knowledge this is the first study to assess the body distribution of As after feeding on radiolabeled prey. Our results show that As rapidly decreases in the intestine and viscera throughout depuration, and accumulates in the head and fillet. Field studies have shown that As can accumulate in the fillet, and that as the salinity of seawater increases so does the concentration of As (Larsen and Francesconi, 2003). Furthermore, field studies have shown that the dominant form of As in fish tissue is arsenobetaine, which is obtained through the diet and accumulates in the fillet (Kirby and Maher, 2002), potentially posing a risk to higher trophic levels.

The two species of Hg we examined behaved very differently. Like Cd, Hg(II) remained associated with intestine throughout depuration, whereas MeHg was transported around the body. Pickhardt et al. (2006) also found that most Hg(II) was associated with the viscera in mosquitofish (92-96%) and redear sunfish (68-73%). For MeHg, Mathews and Fisher (2008a) concluded that 58% of the body burden was associated with the skin, fillet, and skeleton of killifish, which is comparable to our result of 53%. Pickhardt et al. (2006) found that 32-36% of MeHg in redear sunfish was associated with the fillet which matched our value, but only 11-14% in mosquitofish. Furthermore, previous studies have shown that intestinal bacteria in large piscivorous freshwater fish can methylate Hg (Rudd et al. 1980); the extent to which this occurs in smaller fish like *F. heteroclitus* remains unstudied, although our Hg(II) tissue distribution and efflux data indicate that such methylation was unlikely.

The difference in body distribution of Hg(II) and MeHg suggests the two species are processed in different ways within the digestive tract. MeHg is more easily solubilized than Hg(II) during digestion, and readily transported across the stomach and intestinal lining as a cysteine complex through an amino acid transport channel (Leaner and Mason, 2002a,b), where it is effectively transported to different tissue compartments via the blood (Leaner and Mason, 2004). The intestine had a high body burden of MeHg in the first few days, but rapidly decreased throughout the depuration period, as the MeHg was transported to other regions of the body. Our calculated efflux rate of MeHg from the intestine concluded it should take 26 days to transfer all the MeHg across the gut lining, which is similar to the value calculated by Oliveira Ribeiro et al. (1999). The higher proportion of MeHg associated with the fillet poses a risk to higher consumers, including humans.

4.3. Biomagnification of metals in killifish

Trophic transfer factors (TTFs) are used to calculate the potential for a metal to biomagnify at a particular step in the food chain. Of the metals examined MeHg was the only metal expected to biomagnify (TTF > 1) at all ingestion rates due to the high AE and low elimination rate. However, As, Cd, Cr, and Hg(II) will not biomagnify (TTF < 1), even at high ingestion rates, due to low assimilation and high elimination. To our knowledge this is the first study to calculate TTF values for As and Cr. The TTFs reported for MeHg in this study are comparable to those noted elsewhere for estuarine and marine fish (1-10; Mathews and Fisher, 2008a; Wang and Wong, 2003), and Hg(II) TTFs were also similar to other marine fish (<0.6; Wang and Wong, 2003). Our Cd TTF values (0.005-0.07) were comparable to what was noted in other studies using estuarine and marine fish, but at the lower end of the range (0-0.6; Mathews and Fisher, 2008a,b; Mathews et al., 2008; Xu and Wang, 2002); however, Cd has been shown to biomagnify (TTF > 1) in freshwater food webs (Croteau et al., 2005).

4.4. The importance of prey choice

Assimilation efficiencies and elimination rates are directly influenced by the type of prey item ingested. In this study more Hg(II) and Cr were assimilated after consuming worms, and more Cd after consuming amphipods. The assimilation of MeHg did not vary with prey type. Aside from zooplankton crustaceans, few studies have calculated metal AEs in fish using other prey types, and those studies focused mainly on Cd, Se, and Zn. For Cd AEs in clams > copepods ≈ fish viscera > mussels > barnacles; for Se copepods > clams > mussels > barnacles; and for Zn fish viscera > clams > copepods > mussels > barnacles (Zhang and Wang, 2006).

Further studies are needed to compare the AE of other metals in fish after consuming a wider variety of prey types.

Within zooplankton crustaceans AEs can vary from one crustacean group to the next. For example, AEs for mudskipper and glassy fed copepod prey are much lower (10 and 14%) than when fed *Artemia* (26 and 33%) prey (Ni et al., 2000). This suggests that metal bioavailabilty varies between crustacean groups.

Studies using piscivorous fish have shown that metal assimilation can either increase or decrease when fish muscle is consumed compared to invertebrate prey, indicating that metal bioavailability significantly changes. For example, when killifish were fed amphipods and worms in our study the AE of MeHg was 92%, but when fish were fed fish prey the AE was lower, ranging from 56-88% (Mathews and Fisher, 2008a; Wang and Wong, 2003). However, for Cd the AE increased when fed fish prey (23-45%; Mathews and Fisher 2008a,b; Mathews et al., 2008), compared to 2.9-4.5% for invertebrate prey (this study). Gut content analysis of field collected killifish indicates that this species consumes a wide range of prey items (Allen et al., 1994), and can occasionally be a piscivore (Able et al. 2007).

Fish consume a varied diet, and this study and others show that AE and k_{ef} can vary significantly with the type of prey ingested. To use one prey item to calculate AE and k_{ef} for the biokinetic model could lead to an over- or underestimation of these parameters. Ideally, the AE values should be paired with specific prey contents (perhaps as revealed through gut content analyses), although the latter would be expected to vary considerably among locations and seasons as they can influence the availability of prey. Combining prey-specific kinetic parameter data with gut content analysis would provide a more realistic model estimation of metal body

burdens in fish. Future studies are needed to calculate an accurate AE and k_{ef} for a diverse array of prey items.

Table 1. Assimilation efficiencies (AEs) and efflux rate constants (k_{ef}s) in killifish after feeding on amphipod and worm prey. Values represent means ± 1 SE; n = 10 for amphipods and 5–10 for worms. nd: not determined. Statistically significant differences (by *t*-test) between kinetic parameters and prey type are represented by * ($p<0.05$) and ** ($p<0.01$).

	Amphipods		Worms	
Metal	AE (%)	k_{ef} (d^{-1})	AE (%)	k_{ef} (d^{-1})
As	9.4 ± 0.9	0.287 ± 0.035	nd	nd
Cd	4.5 ± 0.6*	0.064 ± 0.010	2.9 ± 0.3*	0.054 ± 0.007
Cr	0.2 ± 0.06**	nd	4.0 ± 0.4**	0.064 ± 0.030
Hg(II)	14 ± 4	0.131 ± 0.011**	24 ± 2	0.067 ± 0.006**
MeHg	92 ± 2	0.008 ± 0.001	92 ± 4	0.007 ± 0.001

Table 2. Radioactivity concentrations (Bq g^{-1} dry wt) of Cd, Hg(II), and MeHg in fish tissues at the end of the 9 day depuration period after feeding on worms. Values represent means ± 1 SE; n = 5-10.

Tissue	Cd	Hg(II)	MeHg
Head	0.1 ± 0.09	13 ± 3	141 ± 24
Gills	2 ± 1	55 ± 18	210 ± 35
Viscera	122 ± 19	772 ± 106	505 ± 80
Liver	7 ± 3	43 ± 6	484 ± 82
Skeleton	0.4 ± 0.16	16 ± 5	162 ± 28
Fillet	0.2 ± 0.06	4 ± 1	205 ± 37
Skin	0.6 ± 0.35	13 ± 4	181 ± 34

Table 3. As, Cd, Hg(II) and MeHg partitioning in killifish fed radiolabeled amphipods after 1, 3, 6, and 9 days of depuration. Values represent the percentage of total body burden associated with each tissue compartment (means ± 1 SE). n = 5 per day and metal.

		Day			
Metal	Tissue	1	3	6	9
As	Head	8.7 ± 1.1	11 ± 1.5	14 ± 3.4	18 ± 1.6
	Gills	1.7 ± 0.4	1.7 ± 0.2	2.5 ± 0.6	4.0 ± 0.9
	Intestine	22 ± 2.9	5.9 ± 1.2	10 ± 2.3	4.7 ± 0.4
	Liver	7.1 ± 1.2	3.7 ± 0.3	2.9 ± 0.7	3.9 ± 1.2
	Other viscera	41 ± 6.0	47 ± 5.0	29 ± 14	7.7 ± 1.8
	Skeleton	3.7 ± 0.3	4.6 ± 0.9	6.3 ± 1.5	7.7 ± 0.7
	Fillet	11 ± 1.8	20 ± 2.5	26 ± 5.8	42 ± 2.4
	Skin	4.8 ± 0.5	6.1 ± 0.5	9.3 ± 2.2	12 ± 1.4
Cd	Head	1.5 ± 1.1	0.9 ± 0.4	1.7 ± 1.1	0.6 ± 0.3
	Gills	0.1 ± 0.04	0.3 ± 0.2	0.4 ± 0.3	0.1 ± 0.05
	Intestine	94 ± 2.0	91 ± 3.1	87 ± 6.5	85 ± 5.2
	Liver	0.4 ± 0.1	1.3 ± 0.2	1.5 ± 0.3	1.7 ± 0.7
	Other viscera	0.8 ± 0.5	0.6 ± 0.2	7.1 ± 6.2	0.9 ± 0.3
	Skeleton	0.4 ± 0.3	1.2 ± 0.6	0.5 ± 0.05	0.3 ± 0.2
	Fillet	1.3 ± 0.6	3.4 ± 2.3	1.0 ± 0.3	3.6 ± 1.8
	Skin	1.5 ± 0.7	1.3 ± 0.5	0.8 ± 0.2	7.8 ± 4.2
Hg(II)	Head	0.6 ± 0.08	2.4 ± 0.2	3.7 ± 0.6	7.4 ± 0.8
	Gills	0.3 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	2.1 ± 0.2
	Intestine	93 ± 3.5	80 ± 10	75 ± 9.3	73 ± 2.4
	Liver	0 ± 0	0.9 ± 0.1	1.7 ± 0.7	3.2 ± 0.4
	Other viscera	4.9 ± 3.5	11 ± 9.4	13 ± 9.4	4.7 ± 1.1
	Skeleton	0.3 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	2.8 ± 0.3
	Fillet	0.3 ± 0.1	2.1 ± 1.0	2.0 ± 0.5	3.3 ± 0.5
	Skin	0.6 ± 0.1	1.5 ± 0.5	2.6 ± 0.5	3.5 ± 0.5
MeHg	Head	6.9 ± 0.5	12 ± 0.6	15 ± 0.5	17 ± 1.2
	Gills	2.1 ± 0.2	2.5 ± 0.2	3.0 ± 0.1	2.5 ± 0.2
	Intestine	62 ± 1.7	41 ± 1.9	21 ± 2.0	14 ± 1.9
	Liver	7.8 ± 0.5	8.2 ± 0.3	9.2 ± 0.9	8.9 ± 0.5
	Other viscera	3.3 ± 0.2	4.7 ± 0.4	5.3 ± 0.6	4.0 ± 0.4
	Skeleton	3.0 ± 0.3	5.3 ± 0.2	6.5 ± 0.4	6.6 ± 0.2
	Fillet	10 ± 0.5	18 ± 1.3	22 ± 1.5	36 ± 2.1
	Skin	4.9 ± 0.4	8.3 ± 0.5	18 ± 0.4	11 ± 0.9

Table 4. Radioactivity concentrations (Bq g^{-1} dry wt) of As, Cd, Hg(II), and MeHg in fish tissues after feeding on amphipods. Tissue concentration calculated after 1, 3, 6, and 9 days of depuration. Values represent means ± 1 SE; n = 5 per day and metal.

Metal	Day	Head	Gills	Intestine	Liver	Other viscera	Skeleton	Fillet	Skin
As	1	100 ± 8	151 ± 23	2130 ± 637	766 ± 203	3267 ± 1240	100 ± 9	75 ± 10	99 ± 12
	3	35 ± 8	64 ± 22	148 ± 59	100 ± 38	1821 ± 619	35 ± 8	34 ± 8	41 ± 8
	6	32 ± 8	53 ± 17	217 ± 104	55 ± 16	2954 ± 2811	38 ± 12	38 ± 10	42 ± 15
	9	36 ± 12	58 ± 9	74 ± 24	77 ± 37	111 ± 23	31 ± 7	55 ± 21	40 ± 10
Cd	1	1 ± 0.3	3 ± 1	2015 ± 720	10 ± 3	7 ± 3	1 ± 0.5	1 ± 0.8	3 ± 1
	3	1 ± 0.5	5 ± 2	1232 ± 259	18 ± 5	5 ± 2	5 ± 3	5 ± 4	3 ± 1
	6	0.7 ± 0.4	0.9 ± 0.7	429 ± 71	7 ± 2	12 9	0.8 ± 0.1	0.4 ± 0.1	0.6 ± 0.2
	9	0.3 ± 0.1	0.6 ± 0.3	717 ± 309	12 ± 4	3 ± 1	0.4 ± 0.2	1 ± 0.6	8 ± 4
Hg(II)	1	2 ± 1	8 ± 5	2608 ± 458	0 ± 0	214 ± 170	2 ± 1	0.8 ± 0.5	4 ± 2
	3	7 ± 2	31 ± 9	2136 ± 271	24 ± 8	331 ± 306	8 ± 2	3 ± 1	11 ± 5
	6	5 ± 1	13 ± 4	973 ± 310	14 ± 4	134 ± 102	4 ± 1	1 ± 0	7 ± 2
	9	6 ± 2	15 ± 2	607 ± 50	24 ± 7	35 ± 11	6 ± 1	2 ± 1	6 ± 1
MeHg	1	337 ± 70	904 ± 166	23992 ± 3184	2718 ± 658	1518 ± 423	363 ± 70	343 ± 76	408 ± 89
	3	612 ± 90	1264 ± 217	17771 ± 1831	2647 ± 458	2046 ± 406	692 ± 90	643 ± 117	819 ± 123
	6	951 ± 81	1631 ± 115	8401 ± 939	3910 ± 474	2892 ± 356	999 ± 93	1143 ± 108	1314 ± 140
	9	878 ± 251	1153 ± 298	5416 ± 1481	3068 ± 926	2248 ± 646	889 ± 231	1184 ± 356	1089 ± 304

Table 5. Biological half-lives ($tb_{½}$; in days) of the metals examined after feeding on amphipods and worm prey. k_{ef} values used are found in Table 1. nd: not determined.

Metal	Amphipods	Worms
As	2.4	nd
Cd	11	13
Cr	nd	11
Hg(II)	5	10
MeHg	87	99

Figures

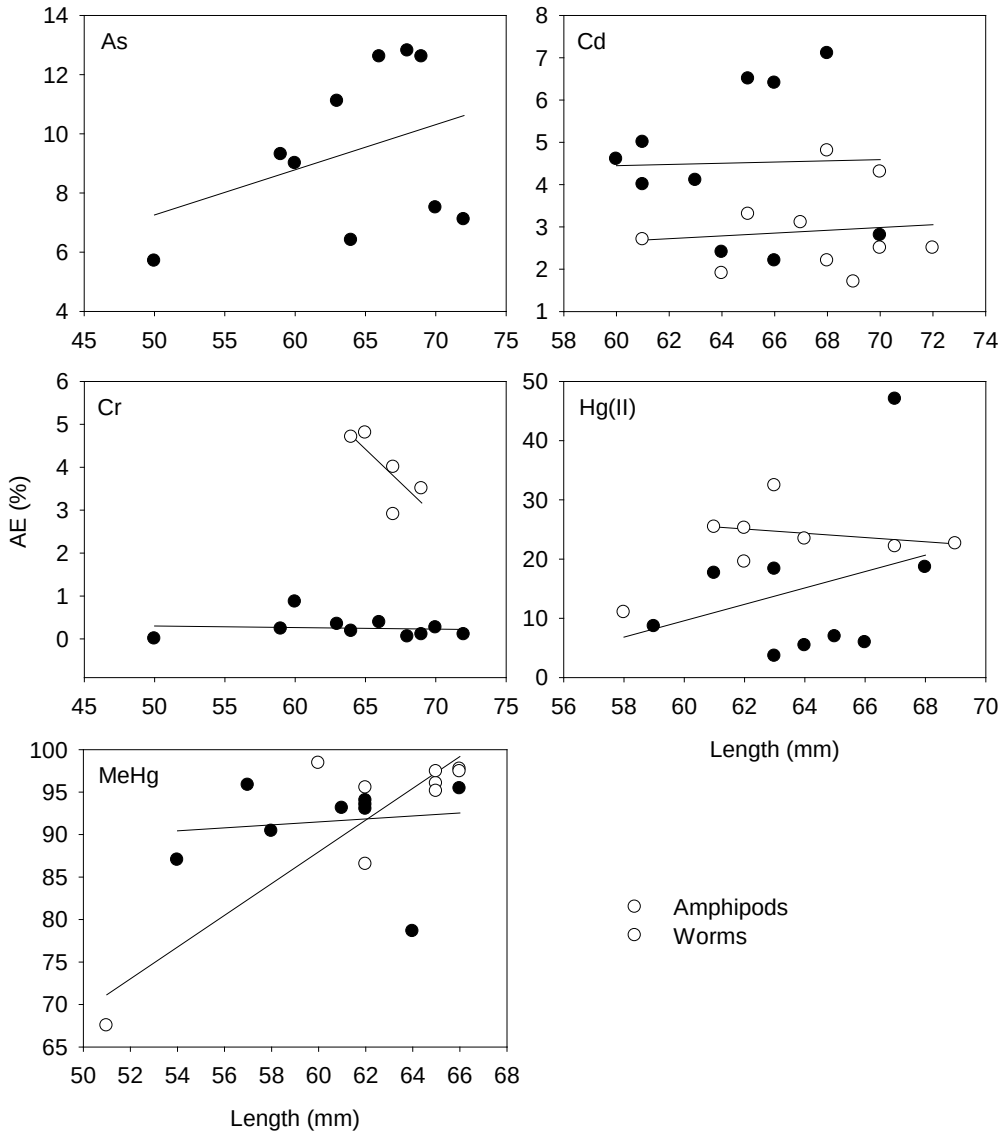

Fig. 1. Relationship between fish length and assimilation efficiency (AE) of the metals examined for both prey types (n = 10 for amphipods and 5-10 for worms). r^2 values describing this relationship are as follows: 0.134 for As, 0.001 for Cd, 0.009 for Cr, 0.128 for Hg(II), and 0.015 for MeHg after feeding on amphipods, and 0.012 for Cd, 0.576 for Cr, 0.068 for Hg(II), and 0.778 for MeHg after feeding on worms.

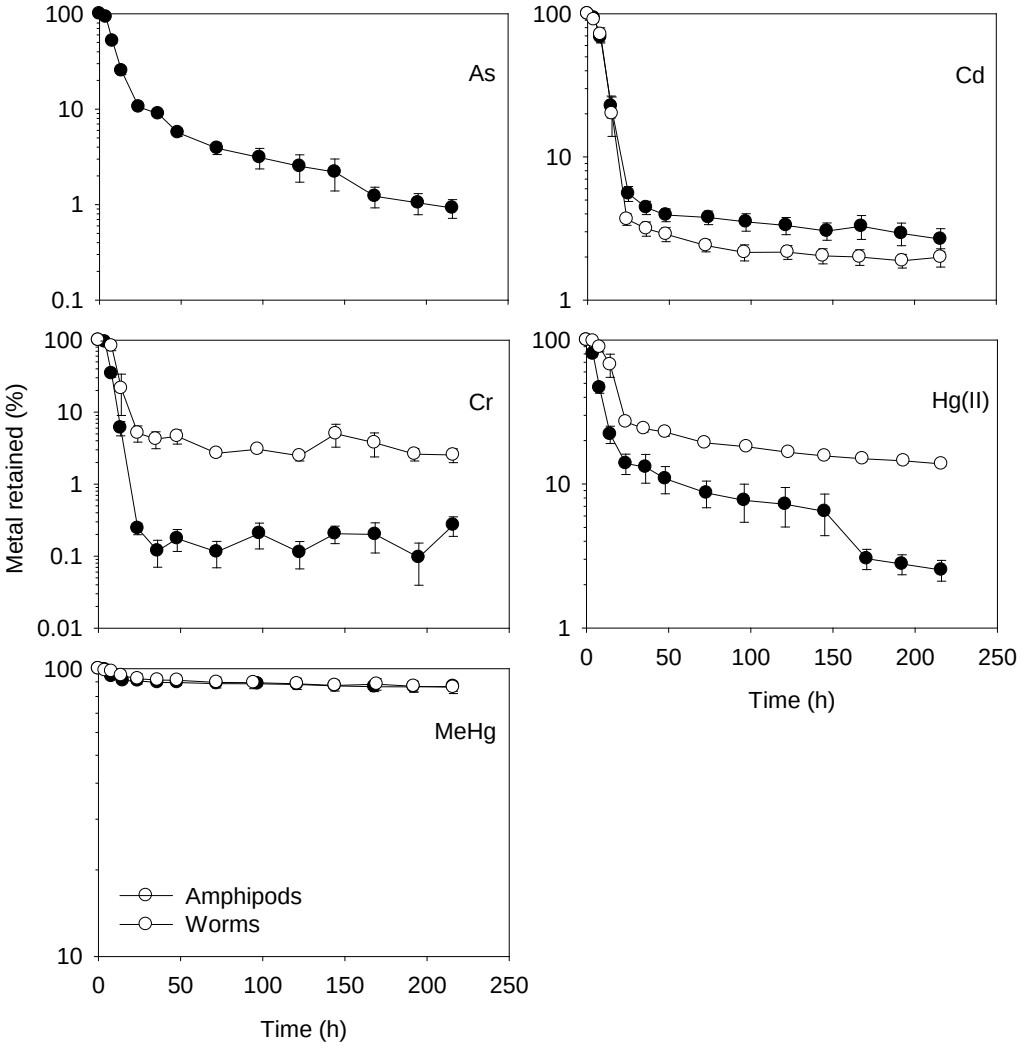

Fig. 2. Metal retention in killifish (*Fundulus heteroclitus*) over 9 days after feeding on radiolabeled amphipod (*Leptocheirus plumulosus*) or worm (*Lumbriculus variegatus*) prey. Values represent means ± 1 SE; n = 5–25 for amphipods and 5–10 for worms.

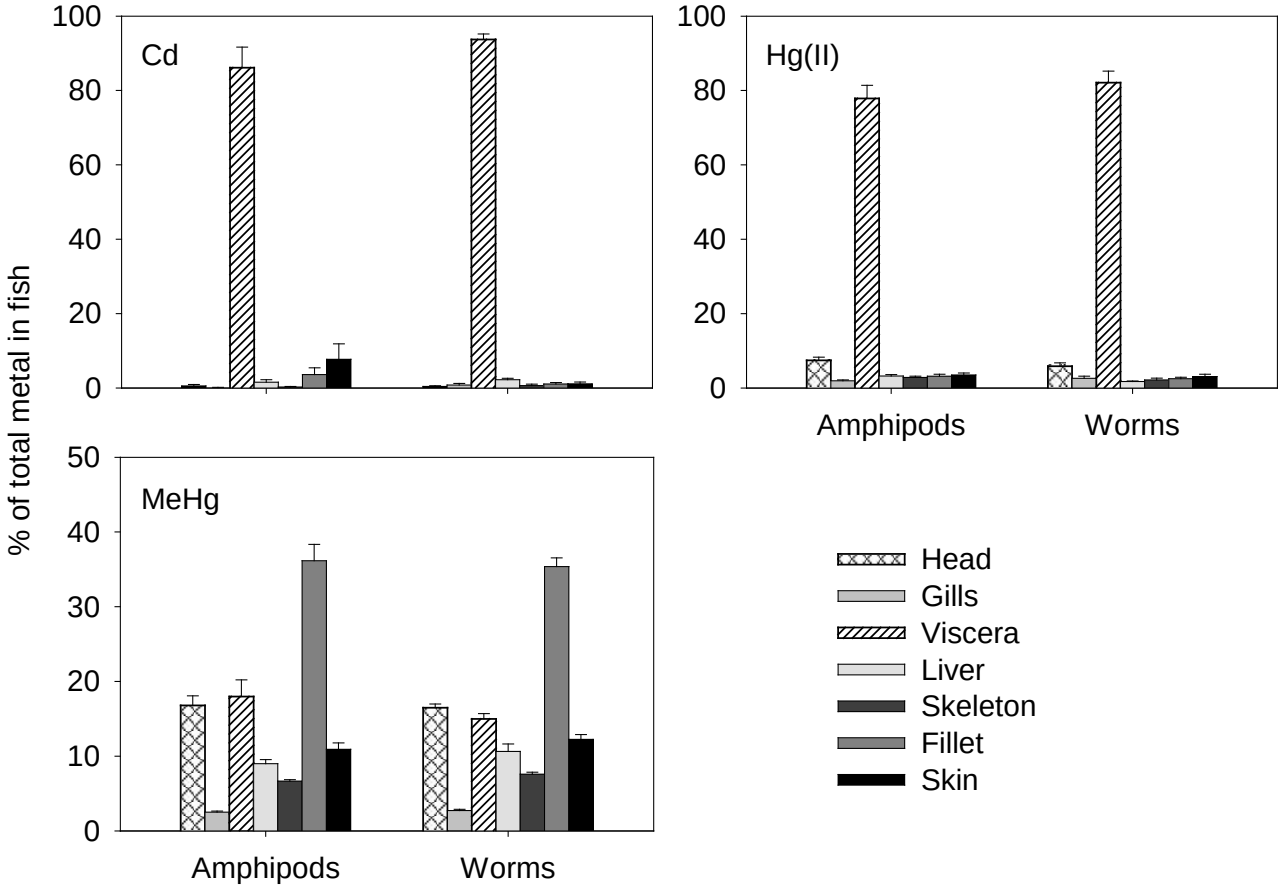

Fig. 3. Tissue distribution of Cd, Hg(II), and MeHg at the end of the 9 day depuration period after feeding on radiolabeled amphipods and worms. Bars represent the percentage of total body burden associated with each tissue compartment. Values represent means ± 1 SE; n = 5 for amphipods and 5–10 for worms.

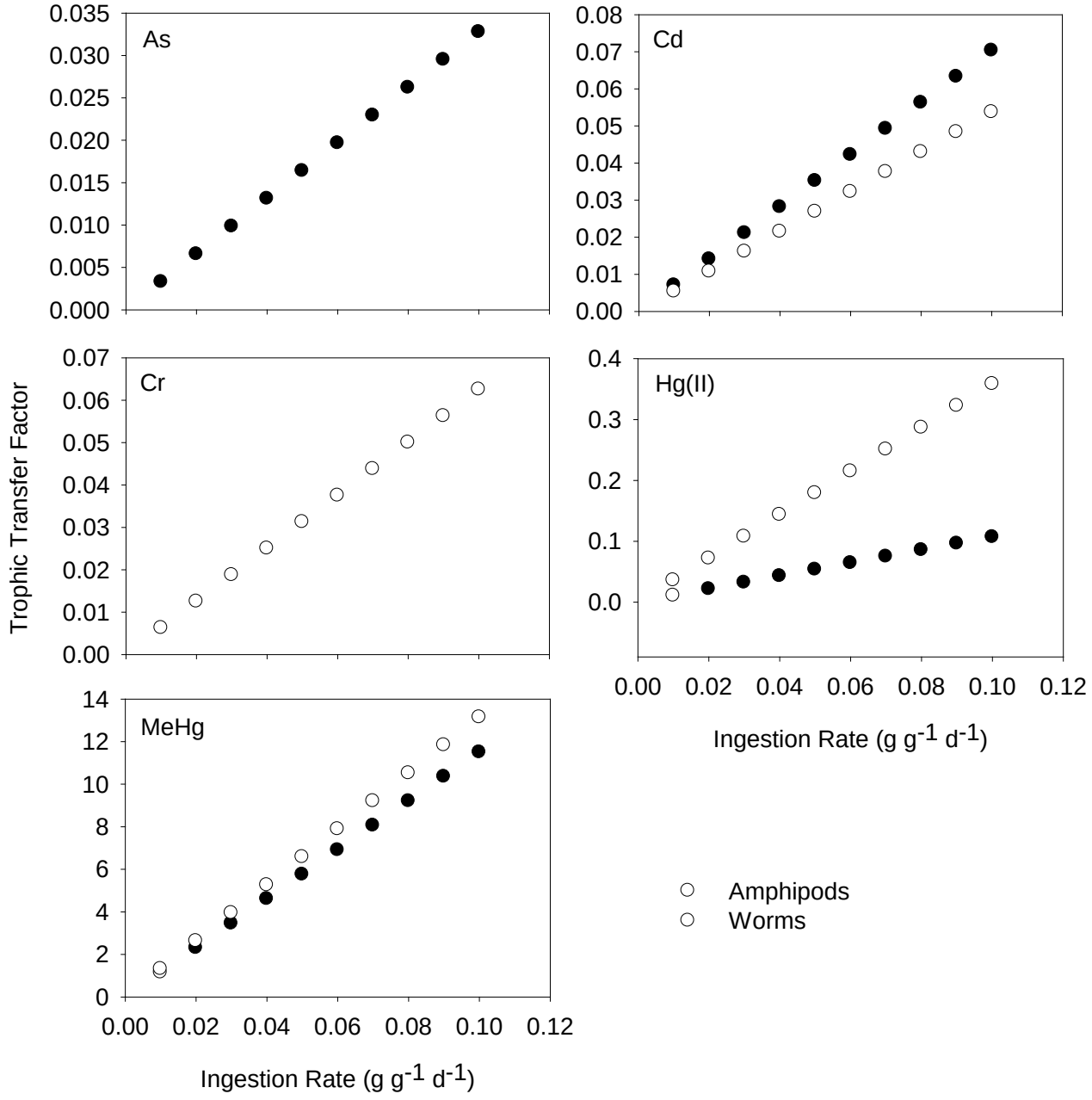

Fig. 4. Model-predicted As, Cd, Cr, Hg(II), and MeHg trophic transfer factors (TTFs) as a function of ingestion rate (IR) for killifish after feeding on amphipod and worm prey. AE and k_{ef} values used are in Table 1. Cr from amphipods was below detection.

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Bioavailability of sediment-bound and algal metals to killifish *Fundulus heteroclitus*

ABSTRACT: We experimentally assessed whether As(V), Cd, Cr(III), Hg(II), and methylmercury (MeHg) bound to sediment from three contaminated field sites (Baltimore Harbor, Elizabeth River, and Mare Island) and the green alga (*Dunaliella tertiolecta*) are bioavailable to killifish (*Fundulus heteroclitus*) using a radiotracer technique. Algae are a component of the killifish diet in salt marshes, and although killifish do not actively consume sediment, some is ingested through accidental uptake when attached to benthic prey. For both sediment and algae, assimilation efficiencies (AEs) of ingested metals were highest for MeHg followed by Hg(II), As, Cd, and lowest for Cr. Following sediment intubations AEs ranged from 0.01-0.03% (Cr) to 10-14% (MeHg), and ranged from 0.7% (Cr) to 82% (MeHg) following algal intubation. Following sediment intubations loss rate constants (k_{ef}s) were similar for As, Cd, and Hg(II), and lowest for MeHg, whereas following algal intubation k_{ef}s were highest for As, followed by Cr, Hg(II), Cd, and lowest for MeHg. At the end of depuration, tissue distribution data showed that Cd and Hg(II) remained primarily associated with the viscera, whereas As and MeHg were distributed throughout the body. Calculated trophic transfer factors (TTFs) showed that only MeHg bound to algae and Elizabeth River sediment is expected to biomagnify at this trophic step (TTF >1). Metals can accumulate to high concentrations in sediment in industrialized coastal areas, but this study indicates the risk of exposure to killifish from ingesting contaminated sediment is minimal.

INTRODUCTION

Sediments are a major repository for trace metals, especially in coastal regions, where metal concentrations can reach elevated levels in industrialized estuaries (Kennish 1997). Sediments are much more enriched in metals than the overlying water column. This provides a large source of metals to benthic organisms, which can potentially impact their health or that of their predators. Many factors influence whether a particular metal is bioavailable, and the mechanisms involved are still being studied. Field studies have shown that benthic organisms can accumulate metals to high concentrations, including in industrialized coastal waters and salt marshes where killifish reside (Kennish 1997, França et al. 2005).

The bioavailability of sediment-bound metal to benthic organisms is dependent on sediment geochemistry and phase-partitioning of the metal (Luoma 1989, Wang et al. 1999, Baumann & Fisher 2011), and the time of sediment exposure to the metal (Wang et al. 1999, Griscom et al. 2000, Baumann et al. 2011). Studies using radiotracers have shown that polychaetes, bivalves and amphipods can assimilate sediment-bound metals (Wang et al. 1999, Schlekat et al. 2000, Griscom et al. 2002a, Baumann & Fisher 2011), and these organisms can act as a conduit for the trophic transfer of metals to higher trophic levels including fish, birds, and potentially human consumers of seafood.

Within the planktonic food chain the largest enrichment step occurs at the bottom of the food chain, between the dissolved phase and phytoplankton (Fisher & Reinfelder 1995). Sinking planktonic debris enriched in metals can settle to sediments and serve as an additional source of

metal for benthic animals in addition to the metals that sorb directly to the sediments themselves (Baumann & Fisher 2011).

The killifish or mummichog (*Fundulus heteroclitus*) inhabits estuaries, bays, and salt marshes along the eastern seaboard of the United States from the Gulf of St. Lawrence to northeastern Florida (Abraham 1985). Killifish gut content analyses have shown they consume a varied diet including algae, amphipods, copepods, polychaetes, nematodes, molluscs, crabs, eggs, plant material, and detritus (Kneib & Stiven 1978, Allen et al. 1994, McMahon et al. 2005). While gut content analyses have not shown that killifish actively consume sediment, some sediment may be accidently ingested while feeding on benthic prey and the metals associated with this sediment may be bioavailable to killifish.

To investigate the bioavailability of sediment-bound and algal metals to killifish, we radiolabeled sediment from three contaminated field sites (Baltimore Harbor, Elizabeth River, and Mare Island) and the green alga, *Dunaliella tertiolecta*, with As, Cd, Cr, Hg(II), and methylmercury (MeHg). The transfer of these metals to killifish was assessed after the fish were intubated with sediment or algae. Following intubation, metal loss was monitored for 9 d, and kinetic parameters (assimilation efficiencies, or percentage of ingested material which crosses the gut lining, and loss rate constants) and tissue distributions of the ingested metal were determined. The kinetic parameters were used to calculate the trophic transfer factor (TTF), which describes the likelihood a metal will be transferred from food to fish at this trophic step. The three sediment locations were chosen because they are all contaminated, have differing organic carbon content, grain size distribution, and geochemical properties (Baumann & Fisher 2011), and are part of a larger project comparing these sites. *D. tertiolecta* was the chosen alga because it has no cell wall, therefore minimizing the digestive complication of a walled cell.

The three metals (Cd, Cr, Hg) and metalloid (As) were chosen based upon their chemical characteristics and environmental interest. Among the chosen trace elements, Hg is a Class B metal with a greater affinity for sulfur ligands than oxygen or nitrogen ligands, whereas As, Cd, and Cr are borderline metals (Nieboer & Richardson 1980). These metals are commonly found at elevated concentrations in estuarine sediments, particularly those near industry (Kennish 1997), and their bioavailability is of interest for management of coastal ecosystems.

MATERIALS AND METHODS

Study locations and sediment collection

Sediment was collected from two contaminated sites in the Chesapeake Bay, the Elizabeth River (ER; Norfolk, VA; 36°12’32”N, 76°20’09”W) in May 2006, and Baltimore Harbor (BH; Baltimore, MD; 39°12’25”N, 76°31’41”W) in June 2007, and one contaminated site in San Francisco Bay, the Mare Island naval complex (MI; Vallejo, CA; 38°04’23”N, 122°14’91”W) in October 2007 by box coring. Grain size distribution (described as percent coarse fraction (> 63µm)), organic carbon content, and background metal concentrations for each location are shown in Table 1. For grain size analysis, approximately 20g of sediment was dried at 60°C to obtain a total dry weight, rehydrated, and separated into coarse (> 63µm) and fine (< 63µm) fractions by wet sieving. The coarse fraction was then dried at 60°C for 48 h to obtain dry weights (n = 3 per field site). Organic carbon content was calculated by combusting dry sediment at 450°C for 6 h; the percent difference between the before and after dry weights was the percent organic carbon. Background metal concentrations were analyzed by ICP-MS at the Trace

Element Core Laboratory (Dartmouth College, Hanover, NH, USA) using an IAEA-433 reference standard.

Water from each field location was collected using a trace metal clean pump and the chemical properties (salinity, dissolved organic carbon (DOC) concentration, and background metal concentrations) are shown in Table 2. The Hg background water concentration was analyzed by the Trace Element Analysis Core Laboratory at Dartmouth College, and As, Cd, and Cr by Rutgers Inorganic Analytical Core Laboratory (Rutgers University, New Brunswick, NJ, USA) using ICP-MS. All water was 0.2 µm sterile-filtered (Millipak 200, Millipore, Bedford, MA, USA) before use. Sediment and water were held in the dark at 4°C until use.

Fish maintenance

Field collected (Taylor River, Hampton, NH, USA) killifish (*Fundulus heteroclitus*; 59.5 ± 2.2 mm (mean ± SD) long and a mean wet weight of 2.1 ± 0.3 g) were purchased from Aquatic Research Organisms (Hampton, NH, USA), and acclimated to experimental conditions for at least 4 wk prior to the start of the experiments. Fish were fed a diet of TetraCichlid™ cichlid flakes (Tetra Holding Inc., Blacksburg, VA, USA) and frozen bloodworms daily prior to the start of the experiments, and only bloodworms throughout the experiments. All fish were starved for 36 h prior to the start of the experiments to allow for total gut clearance. Fish were held at 18 ± 0.5°C, on a 14: 10 h light:dark cycle.

Metal uptake from sediment

To prepare radiolabeled sediment, approximately 6-6.5g of sediment from each of the three field locations were radiolabeled and left to age in the dark in sealed glass containers at room temperature (20 ± 1°C) for 7 d. Comparisons of wet and dry weights indicated that water comprised about 56% of the weight of sediment from BH, 63% from ER, and 52% from MI. Radioisotope additions per field location were 104 kBq ^{73}As, 40 kBq ^{109}Cd, 245 kBq ^{51}Cr, 33 kBq ^{203}Hg(II) and 33 kBq MeHg; this corresponds to the following metal concentrations: 511 nM ^{73}As, 103 nM ^{109}Cd, 54 nM ^{51}Cr, 195 nM ^{203}Hg(II) and 195 nM MeHg. At the end of the 7 d sediment aging period killifish were intubated with radiolabeled sediment (n = 5 per field site). To intubate killifish, radiolabeled sediment was added to a 3 cc syringe with a 16G1½ PrecisionGlide needle attached (Becton Dickinson and Co., Franklin Lakes, NJ, USA), and the needle was carefully slid into intramedic non-radiopaque polyethylene tubing (internal diameter 1.57 mm, 5 cm long; Clay Adams, Parsippany, NJ, USA). Fish were removed from the water, and the tubing was passed through the mouth and esophagus into the start of the intestine, where approximately 0.06-0.1 g of radiolabeled sediment was added. Fish were then returned to the water and not handled for 30 min to minimize stress and prevent sediment regurgitation. No feces were produced during this time, so the start of depuration was when the fish were intubated. No fish died or showed adverse effects (not feeding or abnormal swimming behavior) from this intubation procedure.

After determining their initial radioactivity, the fish were returned to individual containers with non-radiolabeled water collected from the same field location as the sediment, and fed non-radiolabeled bloodworms to purge their guts of radiolabeled sediment. Fish were radioassayed at regular intervals during the first 2 d, and then once a day for the following 7 d to evaluate metal loss. At each sample time, feces and a 1 ml water sample were collected. The

depuration water was changed after 1 d, and then every other day to minimize any radioisotope leaching into the dissolved phase, either due to release from feces or excretion from the fish. At the end of the 9 d depuration period the fish were euthanized using MS222 and dissected into head (including gills), viscera, and body (skeleton, fins, fillet, and skin). The radioactivity of each tissue compartment was determined, after which samples were dried at 60°C for 48 h to determine dry weights.

Statistical analyses were conducted using IBM SPSS statistics software (version 20; Armonk, NY, USA). One-way ANOVA and Tukey post-hoc tests were conducted to determine significant differences ($p<0.05$ or $p<0.01$) between sediment location and kinetic parameters (assimilation efficiencies and loss rate constants) for each metal.

Metal uptake from algae

The green alga *Dunaliella tertiolecta* (CCMP 1320) was uniformly radiolabeled with metals for 4 d in 1 L (2 flasks containing 500 ml each) Baltimore Harbor water, amended with nutrients at f/2 concentrations (Guillard & Ryther 1962), but modified so no Cu, Zn, and EDTA were added. Phosphate was added at an f/200 concentration for the ^{73}As treatment, due to competition for uptake between As and PO_4 (Sanders & Windom 1980). Radioisotope additions per 500 ml were 69 kBq ^{73}As, 20 kBq ^{109}Cd, 66 kBq ^{51}Cr, 4.2 kBq ^{203}Hg(II), and 4.2 kBq MeHg, corresponding to the following metal concentrations: 3.68 nM ^{73}As, 0.69 nM ^{109}Cd, 1.04 nM ^{51}Cr, 2.01 nM ^{203}Hg(II), and 2.01 nM MeHg. At the start of uptake, *D. tertiolecta* cell density (ml^{-1}) was 5.7×10^5 for ^{73}As and ^{51}Cr, 6.4×10^5 for ^{109}Cd, 3.9×10^5 for ^{203}Hg(II), and 7.6×10^5 for MeHg. Following uptake, the percentage of radioisotope associated with cells (n = 2) was

70% for ^{73}As, 55% for ^{109}Cd, 83% for ^{51}Cr, 79% for ^{203}Hg(II), and 55% for MeHg, and the cell density (ml^{-1}) increased to 3.1 x 10^{6} for ^{73}As and ^{51}Cr, 5.7 x 10^{6} for ^{109}Cd, 1 x 10^{6} for ^{203}Hg(II), and 6.9 x 10^{6} for MeHg. Cell counts were obtained using a Beckman Coulter™ Multisizer™ 3 Coulter Counter (Fullerton, CA, USA). The algal suspensions were filtered through 0.2 µm polycarbonate membranes, and rinsed four times with non-radiolabeled Baltimore Harbor water to remove excess radioisotope associated with the cell surface. The algal cells were then gently scraped off the filter using a scalpel and resuspended in 3 ml of non-radiolabeled Baltimore Harbor water to make an algal slurry. The intubation procedure followed that described for the sediment intubation (except a 1 cc tuberculin syringe (Becton Dickinson and Co., Franklin Lakes, NJ, USA) was used), and the fish were intubated with approximately 0.25 ml of the algal slurry (n = 10 for As, Cd, Cr, and Hg(II); n = 9 for MeHg). The depuration and dissection procedure followed what was previously described.

Radioisotopes and radioanalyses

High specific activity gamma-emitting radioisotopes were used in this study (10.97-13.97 µCi µg^{-1} ^{73}As, 13.88-14.43 µCi µg^{-1} ^{109}Cd, 67.58-340.8 µCi µg^{-1} ^{51}Cr, 0.55-3.22 µCi µg^{-1} ^{203}Hg(II), and 0.55-3.22 µCi µg^{-1} MeHg). ^{73}As ($t_{½}$ = 80.3 d, as As(V)) and ^{109}Cd ($t_{½}$ = 462.6 d), both dissolved in 0.1 M HCl were purchased from the Department of Energy (Los Alamos National Laboratory, Los Alamos, NM, USA), ^{51}Cr ($t_{½}$ = 27.7 d, as Cr(III), dissolved in 0.5 M HCl) was purchased from PerkinElmer (Boston, MA, USA), and ^{203}Hg(II) ($t_{½}$ = 46.6 d, as Hg(II), dissolved in 1 M HCl) was purchased from Eckert & Ziegler Isotope Products (Valencia, CA, USA). MeHg ($CH_3{}^{203}$Hg(II)) was synthesized in our lab using a method described in

Rouleau & Block (1997), and held in deionized water. The radioisotopes were added in microliter quantities, and equimolar concentrations of sodium hydroxide were added to neutralize the acid. The pH remained unchanged after radioisotope additions. ^{109}Cd, ^{203}Hg(II), and MeHg were single-labeled, while ^{73}As and ^{51}Cr were double labeled.

Live fish were radioassayed using a Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector for no longer than 5 min to minimize stress on the fish. Initially, the propagated counting error was ≤ 5%, but after gut clearance propagated counting errors could reach 25% due to much lower radioactivity caused by elimination of the radioisotope. Water, feces, and dissected fish tissue were radioassayed using an intercalibrated LKB Pharmacia-Wallac 1282 CompuGamma CS gamma-counter (Turku, Finland) for 5 min (^{73}As, ^{109}Cd, ^{51}Cr), or 10 min (^{203}Hg). The γ-emission of ^{109}Cd, ^{73}As, ^{203}Hg, and ^{51}Cr was detected at 22, 53, 279 and 320 keV respectively. All sample counts were adjusted for background radioactivity and radioactive decay.

Modeling metal bioaccumulation in killifish

The steady-state concentration of metals in aquatic organisms can be determined using a well described biokinetic model (Thomann 1981, Wang et al. 1996, Reinfelder et al. 1998). This model takes into account the uptake and loss of metals following aqueous and dietary exposure. We did not determine metal uptake from the aqueous phase in our study, but the dietary component of the model can be rearranged to calculate the trophic transfer factor (TTF). The TTF calculates the likelihood for a metal to biomagnify at a particular trophic step, based on the ratio of metal in a predator compared to metal in its prey. A TTF > 1 indicates that

biomagnification is likely at this trophic step, whereas a TTF < 1 indicates there is a low probability of biomagnification (Reinfelder et al. 1998). TTF is calculated as follows:

$$TTF = (AE \cdot IR)/k_{ef} \qquad \text{(Eq. 1)}$$

where AE is the assimilation efficiency of the ingested metal (fraction), IR is the weight-specific ingestion rate (g g^{-1} d^{-1}), and k_{ef} is the metal loss rate constant after dietary exposure (d^{-1}). Metals with a high AE, and low k_{ef} have a greater probability of biomagnifying, compared to those with a low AE and high k_{ef}.

AE and k_{ef} for individual fish following sediment and algal intubations were calculated by exponentially regressing metal retention between 48 h and 216 h of depuration. The AE was determined to be the *y*-intercept and the k_{ef} was the slope of the curve. The Cr AEs after sediment intubation were calculated as the percentage remaining after 48 h of depuration, due to near complete elimination of the radioisotope. An average IR value was obtained from the literature (0.07 g g^{-1} d^{-1}; Prinslow et al. 1974).

The biological half-life ($tb_{½}$; defined as the time it takes for 50% of a metal to be excreted from the body) of a metal can be calculated to estimate a metal's residence time in an organism and is calculated as follows:

$$tb_{½} = \ln 2/k_{ef} \qquad \text{(Eq. 2)}$$

RESULTS

Assimilation and retention of metals after sediment intubation

After intubation with radiolabeled sediment AEs were highest for MeHg (10-14%), followed by Hg(II) (1.9-4.1%), As (0.8-1.7%), Cd (0.04-0.3%), and lowest for Cr (0.01-0.03%) (Table 3). The AE values for each field location did not differ significantly from each other for Cr, Hg(II), and MeHg ($p>0.05$), but did differ for As ($p<0.05$; ER vs MI) and Cd ($p<0.01$; BH vs ER).

Metal elimination from killifish followed a biphasic loss pattern; during the first 24 h of depuration the rapid loss corresponded to gut clearance of unassimilated metal, while the slower loss for the remaining 8 d corresponded to the physiological turnover of assimilated metal (Fig. 1). Nearly all metal bound to sediment from the 3 field locations was eliminated during the first 24 h of depuration. At the end of the 9 d depuration, the percentage of original metal retained was 0.5-0.9% As, 0.03-2% Cd, 0.05-0.06% Cr, 1.1-1.9% Hg(II), and 9.1-14% MeHg. Loss rate constants (k_{ef}s) for Hg(II) (0.074-0.113 d^{-1}), As (0.057-0.097 d^{-1}), and Cd (0.074-0.089 d^{-1}) were similar to one another, and significantly higher than for MeHg (0.004-0.020 d^{-1}) (Table 3). k_{ef}s for Cr could not be calculated due to near complete elimination of the radioisotope. The k_{ef} values for each field location did not differ significantly from one another for As, Cd, and Hg(II) ($p>0.05$), but did differ for MeHg ($p<0.01$; ER vs MI).

Assimilation and retention of metals after algal intubation

Following intubation with radiolabeled algae the AEs were highest for MeHg (82%), followed by Hg(II) (18%), As (15%), Cd (10%), and lowest for Cr (0.7%). For all metals, AEs were higher after intubation with algae than with sediment. If the sediment AEs for the three

field locations are averaged then AEs are 12-, 56-, 35-, 5.6-, and 6.8-fold higher for As, Cd, Cr, Hg(II), and MeHg, respectively, after intubation with algae (Table 3).

Metals were eliminated from killifish following the same biphasic loss pattern noted after intubation with sediment (Fig. 2). Within the first 24 h 80% of As, 89% of Cd, 90% of Cr, 74% of Hg(II), and 18% of MeHg had been eliminated. At the end of the 9 d depuration period the percentage of original metal retained was 2.3% As, 7.3% Cd, 0.3% Cr, 8.8% Hg(II), and 76% MeHg. Loss rate constants (k_{ef}s) were highest for As (0.223 d^{-1}), followed by Cr (0.119 d^{-1}), Hg(II) (0.085 d^{-1}), Cd (0.041 d^{-1}), and lowest for MeHg (0.009 d^{-1}) (Table 3). These k_{ef} values were lower than that calculated for Cd and higher than that calculated for As following sediment intubation, whereas the values were comparable for Hg(II) and MeHg.

Tissue distribution and corresponding metal concentrations

Table 4 shows the tissue distribution of As, Cd, Hg(II), and MeHg, as the percentage of total body burden and radioactivity concentration (Bq g^{-1} dry wt) associated with each tissue compartment at the end of depuration after intubation with radiolabeled sediment and algae. Tissue distribution for Cd BH and all Cr experiments could not be determined due to low detection in the fish. After intubation with sediment, As and MeHg were predominantly associated with the body (70-73% and 42-43% respectively), whereas Cd and Hg(II) were associated with the viscera (81-97% and 68-91% respectively). The percentage body burden associated with each tissue compartment did not vary between field locations, except for Hg(II) in the head (4-22%) and viscera (68-91%), and Cd in the viscera (81-97%) and body (1-11%).

Radioactivity concentrations were highest in the viscera for Cd, Hg(II), and MeHg, and nearly evenly concentrated between the viscera and body for As.

Following algal intubation, As and MeHg were predominantly associated with the body (48% and 49% respectively), while Cd and Hg(II) were associated with the viscera (81% and 77% respectively). This is the same distribution pattern as observed after sediment intubation. Radioactivity concentrations were highest in the viscera for all metals. Radioactivity concentrations could not be compared between metals and between sediment and algae due to exposure to different metal concentrations.

Modeling metal bioaccumulation in killifish

The biological half-life ($tb_{½}$) of metals in killifish was highest for MeHg (35-173 d), followed by similar $tb_{½}$ values for As, Cd, Cr, and Hg(II) (7.1-12, 7.8-9.4, and 6.1-9.4 d, respectively) after intubation with radiolabeled sediment (Table 5). Assuming it takes 7 half-lives for all of the assimilated metal to be excreted, MeHg would be retained for 243-1213 d, As for 50-85 d, Cd for 55-66 d, and Hg(II) for 43-66 d. After intubation with radiolabeled algae the $tb_{½}$ was highest for MeHg (77 d), followed by Cd (17 d), Hg(II) (8.2 d), Cr (5.8 d), and lowest for As (3.1 d) (Table 5). This corresponds to a retention time of 539 d for MeHg, 118 d for Cd, 57 d for Hg(II), 41 d for Cr, and 22 d for As.

TTFs were <1 for As, Cd, Hg(II), and MeHg after sediment intubation (except ER MeHg; TTF = 2.0) regardless of field location, indicating that these metals would not be expected to biomagnify from sediments in killifish. TTFs could not be calculated for Cr due to near complete elimination of the radioisotope. After intubation with algae, MeHg was the only metal with a

TTF >1 (TTF = 6.4), indicating that MeHg would be expected to biomagnify, whereas As, Cd, Cr, and Hg(II) had TTFs <1, indicating that these metals would not be expected to biomagnify (Table 5).

DISCUSSION

Assimilation of metals after sediment intubation

To our knowledge this is the first study to investigate the bioavailability of several metals associated with contaminated sediment for fish. As no significant differences were noted in the TTFs of metals among the three sediments (except ER MeHg), it is apparent that sediment geochemistry between the three sediment sites (Baumann & Fisher 2011) did not influence the AE and k_{ef} of each metal in killifish. The AEs calculated for killifish in this study are much lower than those calculated for deposit feeding polychaetes and marine bivalves that ingested radiolabeled sediment. AEs in polychaetes ranged from 43-83% for MeHg, 7-30% for Hg(II), 1.5-59% for Cd, 1.2-12% for As and 0.7-4.6% for Cr (Wang et al. 1998, 1999, Baumann & Fisher 2011). AEs in bivalves ranged from 5-87% (generally >30%) for MeHg, 6-35% for Cd, <1-20% for Cr, and 1-9% for Hg(II) (Gagnon & Fisher 1997, Wang et al. 1997, Griscom et al. 2000, 2002a). No literature values could be found to compare As values in bivalves to those calculated for killifish in this study. For all metals investigated in this study the sediment AEs are lower than the algal AEs; this observation was also noted in another study using the clam *Macoma balthica* (Griscom et al. 2002a). The difference in AEs between sediment and algae could be due in part to what fraction the metal is bound to in the sediment. In algae, the metal is bound to the more labile organic matter, whereas in the sediment little metal is bound to labile

organic matter, and this metal is much less bioavailable. It should also be noted that because the fish were intubated and did not feed naturally, the AEs may be underestimated, as the sediment was forced into the intestine during the intubation procedure.

The lower AEs noted for killifish than for deposit-feeding polychaetes and bivalves could be a result of the difference in gut physiology between these organisms. The pH of gut fluid in worms (pH 6.88 in *Nereis succinea*; Ahrens et al. 2001) and bivalves (pH 5.0 in the clam *Macoma balthica* and 5.6 in the mussel *Mytilus edulis*; Griscom et al. 2002b) is neutral or mildly acidic, whereas the pH of killifish gut fluid is mildly acidic to alkaline. A study by Babkin and Bowie (1928) determined that the intestinal fluid in fasting killifish has a pH between 8.0 to 9.2, and a pH between 8.4 to 9.0 after feeding on clams. More recently, Wood et al. (2010) found a comparable fasting pH (7.7) but determined that the pH of the intestinal fluid was 5.7 in seawater killifish and 6.8 in freshwater killifish 1-3 hours after feeding on fish pellets. The pH can influence the solubility of metal from the sediment fraction to which it is bound; metals bound to the AVS (acid-volatile sulphide) and iron-oxide fractions are extracted in the low pH of the clam and mussel gut fluid, and the proportion of metal extracted is greater in the clam which has a more acidic gut fluid (Griscom et al. 2002b). Baumann & Fisher (2011) calculated that 13-42% of As, 33-50% of Cd, and 75-91% of Cr was bound to the AVS and iron and manganese oxide fractions 2 d after the sediment from the three field locations used in this study were radiolabeled directly. The pH of the killifish intestinal fluid would not be expected to affect the bioavailability of metal bound to the AVS and iron-oxide fractions. Furthermore, the killifish does not have a stomach (Babkin & Bowie 1928); because the stomach secretes gastric acid, the absence of the stomach can provide some explanation for the higher pH of the intestinal fluid. Worms also have a high concentration of amino acids in their digestive fluid which can solubilize metals

from sediment and surfactants which can solubilize PAHs (polycyclic aromatic hydrocarbons) (Mayer et al. 1996, Ahrens et al. 2001). Mayer et al. (1996) investigated the solubility of metals in the gut fluid of the lugworm (*Arenicola marina*) and the sea cucumber (*Parastichopus californicus*), the lugworm solubilized more metal as a result of a much higher dissolved amino acid concentration. This has also been observed in fish; when sturgeon and catfish gut fluid were exposed to sediment labeled with MeHg, the sturgeon solubilized more MeHg in the gut fluid due to a higher concentration of amino acids (Leaner & Mason 2002a).

Assimilation of metals after algal intubation

The wide range of AEs observed after killifish were intubated with radiolabeled algae (0.7 % for Cr to 82% for MeHg) indicates there is large variability in AEs among the metals. The general ranking of AEs (MeHg > Hg(II) > As > Cd > Cr) is identical to the ranking observed after sediment intubation. To our knowledge this is the first study to calculate AEs after fish consumed radiolabeled algae for As, Cd, Cr, and Hg(II). Leaner and Mason (2004) calculated an AE of 90% for the sheepshead minnow (*Cyprinodon variegatus*) fed MeHg radiolabeled pellets of the green alga *Tetraselmis*. Our AE value of 82% could be slightly lower because the sheepshead minnows were fed naturally, whereas the killifish used in this study were intubated. The Cd, Hg(II) and MeHg AE values calculated from this study fall within the range of literature values for freshwater and marine fish fed zooplankton and worm prey (2.7-39%, 8-51% and 56-95% respectively) (Reinfelder & Fisher 1994, Ni et al. 2000, Xu & Wang 2002, Wang & Wong 2003, Pickhardt et al. 2006, Mathews & Fisher 2008, Dutton & Fisher 2010, 2011). Our calculated As AE (15%) is higher than that calculated for killifish fed amphipod prey (9.4%;

Dutton & Fisher 2011), and our calculated Cr AE (0.7%) is at the lower end of the range observed for fish fed amphipod and worm prey (0.2-19%; Ni et al. 2000, Dutton & Fisher 2011).

The large range in AEs from algae observed between the metals could be due to varying cellular distributions of the metals in *D. tertiolecta* cells. Numerous studies have found that AEs of ingested elements in herbivores are related to the cytoplasmic content of these elements in algae, first noted for diatoms by Reinfelder & Fisher (1991), although variations in cellular distributions of elements among different algal taxa have been noted (Ng et al. 2005) and the relationship to herbivore AEs may vary. This relationship between cellular distribution of metals and AEs in herbivores may explain our findings, where the MeHg AE is 4.6-fold higher than that for Hg(II). Pickhardt & Fisher (2007) found that 59-64% of MeHg is associated with the cytoplasm in freshwater phytoplankton, whereas only 9-16% of Hg(II) is associated with cytoplasm, and is therefore less assimilable when ingested. Furthermore, it has been found that >98% of Cr is bound to algal cell surfaces, and when fed to the mussel *Mytilus edulis* AEs ranged between 0.2-1.3% (Wang & Fisher 1996), comparable to our low Cr AE value of 0.7%. This relationship has also been observed when fish were fed zooplankton; AEs were lower in fish when a large proportion of the metal was associated with the zooplankton exoskeleton. For example, 97% of Cd in copepods was bound to the exoskeleton and fish that fed on these copepods assimilated 2.7%, due to the fish not being able to digest the exoskeleton (Reinfelder & Fisher 1994). This was also noted when killifish were fed radiolabeled amphipods and worms, with AEs higher for Cr and Hg(II) after the fish were fed soft-bodied worms (Dutton & Fisher 2011).

Loss of metals after sediment and algal intubations

Loss rate constants (k_{ef}s) after sediment and algal intubations were similar for Hg(II) and MeHg, higher for As after algal intubation, and higher for Cd after sediment intubation. The physiological turnover rate of metals probably reflects the turnover rates of the tissues in which the metals reside. The tissue distributions of Hg(II) and MeHg were similar for sediment and algal diets (Table 4), and therefore their k_{ef}s did not vary among diets. In contrast, more As and less Cd was in the viscera following the algal diet than the sediment diets (Table 4), matching their k_{ef} patterns. This suggests that k_{ef}s were principally related to the loss of metal from the viscera.

The algal As k_{ef} value calculated in this study (0.223 d^{-1}) is similar to that calculated after killifish were fed radiolabeled amphipods (0.287 d^{-1}); whereas the sediment k_{ef}s were approximately 3- times lower (Dutton & Fisher 2011). The calculated algal k_{ef} for Cd (0.041 d^{-1}) falls within the range calculated in other studies where fish were fed worm and zooplankton prey (0.03-0.073 d^{-1}; Xu & Wang 2002, Mathews & Fisher 2008, Dutton & Fisher 2010, 2011). The Cr algal k_{ef} value calculated in this study (0.119 d^{-1}) is 1.9-times higher than after killifish were fed radiolabeled worms (0.064 d^{-1}; Dutton & Fisher 2011). The sediment and algal k_{ef} values calculated in this study for Hg(II) and MeHg (0.074-0.113 and 0.004-0.020 d^{-1} respectively) fall within the range of those calculated in other studies using freshwater and marine fish fed zooplankton and worm prey (0.003-0.194 d^{-1} for Hg(II) and 0.007-0.018 d^{-1} for MeHg; Pickhardt et al. 2006, Mathews & Fisher 2008, Dutton & Fisher 2010, 2011).

Tissue distribution of metals

The tissue distribution of the metals investigated in this study fall within the range of values calculated in other studies when fish were fed radiolabeled prey. Of all the metals examined, Cd shows the greatest variability in literature values. Our study concluded that 81-97% of Cd remains associated with the viscera, which is similar to values calculated in another killifish study (85% associated with the intestine; Dutton & Fisher 2011) and mangrove snapper (*Lutjanus argentimaculatus*, 81%; Xu & Wang 2002), but much higher than the percentage calculated for the Atlantic silverside (*Menidia menidia*, 13-16%, Dutton & Fisher 2010), striped bass (*Morone saxatilis*, 20%; Baines et al. 2002), and killifish (50%, Mathews & Fisher 2008). This range of values indicates that some fish are better protected against the gastrointestinal uptake of Cd than others. Cd shares the same gastrointestinal uptake pathway as Ca, and elevated levels of Ca reduce the uptake of Cd (Franklin et al. 2005).

The different tissue distributions of Hg(II) and MeHg indicate that MeHg can more readily pass across the intestinal wall where it is redistributed around the body via the blood and accumulates in the fillet which is sulfur-rich. A study by Leaner & Mason (2002b) found that MeHg binds to cysteine and crosses the intestine via an amino acid uptake pathway in the channel catfish (*Ictalurus punctatus*). The higher percentage distribution and concentration of MeHg in the body poses a risk to killifish predators, including the blue crab and striped bass (Kneib 1986, Hartman & Brandt 1995), and therefore potentially human consumers. The tissue distribution of Hg(II) and MeHg presented in this study are comparable to other literature values. For Hg(II), Pickhardt et al. (2006) found that 92-96% of Hg(II) was associated with the viscera in mosquitofish (*Gambusia affinis*) and 67.5-73% in redear sunfish (*Lepomis microlophus*), 28-72% was associated with the viscera in the Atlantic silverside (Dutton & Fisher 2010), and 81-84% was associated with the viscera in another killifish study (Dutton & Fisher 2011). For

MeHg, 54% of MeHg was associated with the body in the redear sunfish and 68% in mosquitofish (Pickhardt et al. 2006), 51-57% in the Atlantic silverside (Dutton & Fisher 2010), and 51-58% in two other killifish studies (Mathews & Fisher 2008, Dutton & Fisher 2011), which are comparable, but slightly higher than our values of 42-49%.

Like MeHg, As was also redistributed around the body, where 48% was associated with the body after algal intubation and 70-73% after sediment intubation. The reason for this difference is not apparent to us, although it is noteworthy that very little As was acquired from either diet. Literature values for the tissue distribution of As in fish are limited. One laboratory study found that 62% of As was associated with the body following a 9 d depuration after acquiring As from amphipods (Dutton & Fisher 2011). Another study analyzed field collected herring, cod, and flounder and found that As accumulates in the fillet (Larsen & Francesconi 2003). Arsenate is known to behave as a phosphate analog in phytoplankton, sharing the same uptake pathway (Sanders & Windom 1980) and after arsenate is taken up it is reduced to a variety of organoarsenic species, including arsenobetaine (Neff 1997). A recent study found that the phosphate transporter, NaPi-IIb1, is most likely responsible for arsenate accumulation in zebrafish tissues (Beene et al. 2011). Speciation of As in field collected fish found that 89-100% of As in the muscle tissue was present as arsenobetaine, whereas arsenate accounted for 0%; however 0-38% of the As in the intestine was present as arsenate (Kirby & Maher 2002). We did not speciate As in the fish tissue in this study so cannot conclude if this is the case for killifish. The tissue distribution data from this study and others indicate that once the metal has been solubilized from the ingested prey or substrate, it is remobilized around the body in the same way, regardless of the source.

Biomagnification of metals in killifish

Trophic transfer factors (TTFs) were <1 after sediment intubation for As, Cd, and Hg(II) at all three field locations, and MeHg at BH and MI, indicting these metals are not expected to biomagnify from sediment to killifish. Killifish intubated with sediment from ER had a TTF >1, indicating MeHg will biomagnify in this field location. TTFs could not be calculated for Cr due to elimination of the radioisotope. After intubation with algae, MeHg was the only metal expected to biomagnify (TTF = 6.4) due to a high assimilation and low elimination rate. In comparison, As, Cd, Cr, and Hg(II) had TTFs <1 due to low assimilation and high elimination rates. The IR of sediment and algae is most likely less than the 0.07 g^{-1} g^{-1} d^{-1} value used to calculate the TTFs in this study. Therefore, the TTFs of metals bound to sediment and algae are likely to be even lower than the values calculated here. MeHg associated with ER sediment and algae will not biomagnify at this trophic step if the IR of ER sediment is <0.04 g^{-1} g^{-1} d^{-1} and the IR of algae is <0.011 g^{-1} g^{-1} d^{-1}.

To our knowledge this is the first study to calculate TTFs after exposure to radiolabeled sediment and algae, so no comparisons can be made to other studies. Following exposure to radiolabeled algae, Hg(II) and MeHg TTFs are similar to those calculated for killifish which consumed radiolabeled amphipods and worms, higher for As and Cd, and lower for Cr (Dutton and Fisher 2011). TTFs following exposure to radiolabeled sediment compared to amphipods and worms were lower for Cd at all three locations and MeHg at BH and MI, but similar for As at all three locations and MeHg at ER . Hg(II) TTFs were similar to values calculated after killifish consumed amphipods, but higher than that calculated after killifish consumed worms (Dutton and Fisher 2011).

Sediment and algae as a source of metals to killifish

Metals can accumulate to high concentrations in industrialized coastal regions. This study concludes that metals bound to sediment are not a significant direct source of metals to fish due to their low bioavailability. This reduces the likelihood of health implications to the killifish themselves and their predators. Killifish do not actively consume sediment, but the risk of metal accumulation from incidental uptake while consuming benthic prey is minimal. Algae, however, can be an important food source to killifish, especially in salt marshes (Kneib & Stiven 1978, Kneib 1986), and this can be a significant source of metal, especially MeHg.

FIGURE CAPTIONS

Fig. 1. *Fundulus heteroclitus*. Loss of As, Cd, Cr, Hg(II), and MeHg from killifish over 9 d following intubation with radiolabeled Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) sediment. Values represent means ± 1 SE; n = 5 per location.

Fig. 2. *Fundulus heteroclitus*. Loss of As, Cd, Cr, Hg(II), and MeHg from killifish over 9 d following intubation with radiolabeled algae. Values represent means ± 1 SE; n = 9-10.

Figure 1

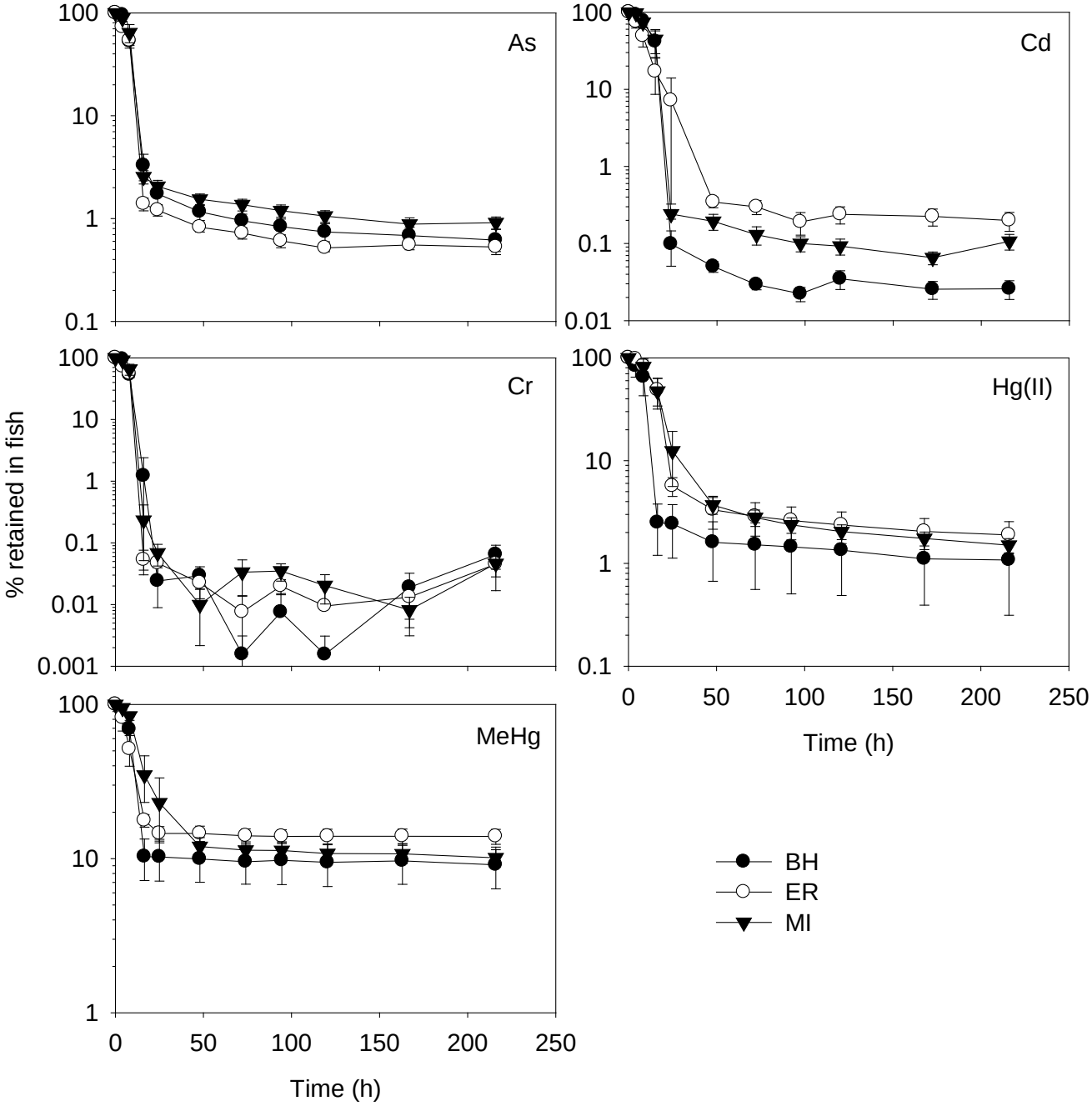

Figure 2

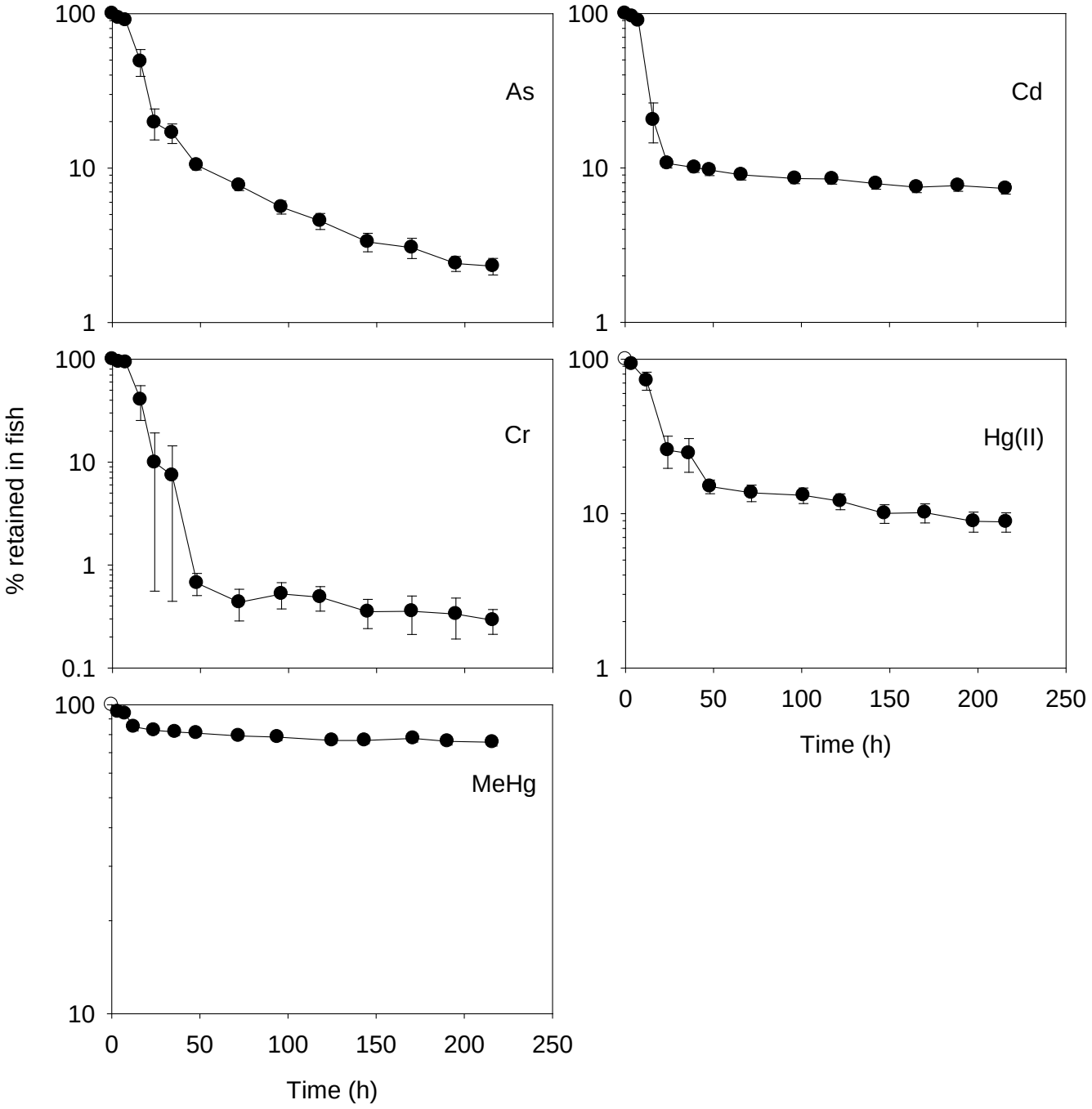
% retained in fish
As
Cd
Cr
Hg(II)
MeHg
100
10
1
0.1
0
50
100
150
200
250
Time (h)

Table 1. Sediment properties. Percentage of sediment >63µm in size (coarse fraction), organic carbon content, and background concentration of metals (As, Cd, Cr, total Hg) for sediment collected from Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI). Values represent means ± 1 SD; n = 3.

	Coarse fraction (%)	Organic carbon content (%)	Background concentration ($mg\ kg^{-1}$)			
			As	Cd	Cr	Hg
BH	8.4 ± 1.3	5.5 ± 0.3	26 ± 0.3	0.5 ± 0.02	116 ± 10	0.2 ± 0.1
ER	12 ± 0.7	7.5 ± 0.6	13 ± 0.5	0.9 ± 0.03	38 ± 2.4	0.2 ± 0.2
MI	9.4 ± 0.5	4.2 ± 0.1	8.8 ± 0.4	0.4 ± 0.05	72 ± 1.7	0.1 ± 0.07

Table 2. Water properties. Salinity, dissolved organic carbon (DOC) concentration, and background metal concentrations (As, Cd, Cr, total Hg) for water collected from Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI). For DOC concentration, the values represent means ± 1 SD; n =3. For background metal concentrations, the units are μg L^{-1} for As, Cd, and Cr, and ng L^{-1} for Hg.

	Salinity (ppt)	DOC concentration (μM)	Background concentration			
			As	Cd	Cr	Hg
BH	7.6	219 ± 12	0.97	0.02	0.19	2.9
ER	19.5	384 ± 1.9	1.38	0.21	0.25	3.4
MI	22	169 ± 6.7	2.20	0.19	0.20	2.7

Table 3. *Fundulus heteroclitus*. Assimilation efficiencies (AE) and loss rate constants (k_{ef}) calculated for killifish after intubation with radiolabeled sediment and algae. n = 5 for sediment and 9-10 for algae. BH: Baltimore Harbor, ER: Elizabeth River, MI: Mare Island, nd: not determined.

		AE (%)			k_{ef} (d^{-1})		
		Mean	SE	Range	Mean	SE	Range
As	BH	1.3	0.2	0.9-1.7	0.097	0.018	0.043-0.136
	ER	0.8	0.1	0.5-1.1	0.057	0.010	0.018-0.071
	MI	1.7	0.2	1.0-2.3	0.081	0.004	0.073-0.091
	Algae	15	1	8.1-21	0.223	0.019	0.109-0.318
Cd	BH	0.04	0.01	0.03-0.06	0.089	0.035	0.006-0.196
	ER	0.3	0.05	0.2-0.5	0.074	0.017	0.029-0.110
	MI	0.2	0.04	0.03-0.3	0.080	0.017	0.024-0.123
	Algae	10	0.8	5.7-12	0.041	0.008	0.013-0.091
Cr	BH	0.03	0.01	0.006-0.06	nd	nd	nd
	ER	0.02	0.01	0.006-0.05	nd	nd	nd
	MI	0.01	0.01	0.002-0.04	nd	nd	nd
	Algae	0.7	0.2	0.1-1.8	0.119	0.023	0.029-0.245
Hg(II)	BH	1.9	1.1	0.5-5.0	0.088	0.025	0.044-0.148
	ER	3.7	1.3	0.7-8.5	0.074	0.007	0.056-0.094
	MI	4.1	0.8	1.4-5.7	0.113	0.013	0.065-0.139
	Algae	18	2	7.2-25	0.085	0.009	0.032-0.125
MeHg	BH	10	3	4.4-18	0.010	0.003	0.004-0.017
	ER	14	2	10-19	0.004	0.002	0.0004-0.011
	MI	12	2	5.3-15	0.020	0.003	0.009-0.025
	Algae	82	2	73-90	0.009	0.001	0.004-0.015

Table 4. *Fundulus heteroclitus*. Tissue distribution of metals in killifish at the end of 9 d depuration after intubation with radiolabeled sediment and algae. Values represent the percentage of total body burden and radioactivity concentrations (Bq g^{-1} dry wt) associated with each tissue compartment (head, viscera, body). Tissue partitioning of Cr and BH Cd could not be determined due to near complete elimination of the radioisotope. Values represent means ± 1 SE; n = 5 for sediment and 9-10 for algae. BH: Baltimore Harbor, ER: Elizabeth River, MI: Mare Island, nd: not determined.

		Head		Viscera		Body	
		%	Bq	%	Bq	%	Bq
As	BH	19 ± 2	11 ± 1	8 ± 2	19 ± 4	73 ± 2	21 ± 2
	ER	21 ± 2	10 ± 1	9 ± 3	18 ± 7	70 ± 1	17 ± 3
	MI	19 ± 2	21 ± 5	10 ± 0.8	40 ± 13	71 ± 2	42 ± 10
	Algae	21 ± 2	15 ± 2	31 ± 5	93 ± 25	48 ± 3	19 ± 3
Cd	BH	nd	nd	nd	nd	nd	nd
	ER	2 ± 1	0.13 ± 0.08	97 ± 1	32 ± 12	1 ± 0.7	0.09 ± 0.07
	MI	7 ± 3	0.13 ± 0.06	91 ± 4	8 ± 3	2 ± 2	0.01 ± 0.01
	Algae	8 ± 1	5.9 ± 0.6	81 ± 2	197 ± 47	11 ± 2	5.1 ± 1.3
Hg(II)	BH	22 ± 8	1.8 ± 0.3	68 ± 5	18 ± 6	10 ± 3	0.8 ± 0.4
	ER	4 ± 1	3.0 ± 0.9	91 ± 3	170 ± 43	5 ± 2	1.6 ± 0.6
	MI	7 ± 1	6.8 ± 1.3	86 ± 3	148 ± 22	7 ± 3	3.9 ± 1.7
	Algae	12 ± 0.8	1.7 ± 0.2	77 ± 1	28 ± 3	11 ± 0.7	0.8 ± 0.1
MeHg	BH	22 ± 1	51 ± 7	36 ± 3	181 ± 39	42 ± 2	50 ± 8
	ER	22 ± 1	107 ± 11	35 ± 3	419 ± 62	43 ± 1	110 ± 11
	MI	21 ± 2	64 ± 11	37 ± 1	240 ± 31	42 ± 0.8	65 ± 9
	Algae	24 ± 0.4	36 ± 3	27 ± 1	78 ± 8	49 ± 1	38 ± 3

Table 5: *Fundulus heteroclitus*. Model predicted biological half-lives ($tb_{½}$) and trophic transfer factors (TTFs) of metals in killifish following intubation with radiolabeled sediment and algae. Values used are in Table 3. BH: Baltimore Harbor, ER: Elizabeth River, MI: Mare Island, nd: not determined.

		$tb_{½}$ (d)	TTF
As	BH	7.1	0.009
	ER	12	0.010
	MI	8.6	0.015
	Algae	3.1	0.047
Cd	BH	7.8	0.0003
	ER	9.4	0.0028
	MI	8.7	0.0018
	Algae	17	0.17
Cr	BH	nd	nd
	ER	nd	nd
	MI	nd	nd
	Algae	5.8	0.004
Hg(II)	BH	7.9	0.015
	ER	9.4	0.035
	MI	6.1	0.025
	Algae	8.2	0.15
MeHg	BH	69	0.7
	ER	173	2.0
	MI	35	0.4
	Algae	77	6.4

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Intraspecific comparisons of metal bioaccumulation in juvenile Atlantic silversides (*Menidia menidia*)

ABSTRACT: We experimentally assessed the uptake, loss and resulting tissue distribution of Am, Cd, Hg(II), MeHg, Se and Zn in two North American populations (Nova Scotia and South Carolina) of juvenile Atlantic silversides (*Menidia menidia*) from aqueous and dietary sources using a radiotracer technique. Northern silversides (Nova Scotia) have a higher rate of ingestion and growth compared to their southern (South Carolina) counterparts to overcome a shorter growing season. Uptake rate constants from the dissolved phase were highest for MeHg and lowest for Cd. Assimilation efficiencies of ingested metals were highest for MeHg (82-89%) and lowest for Am (0.3-1.9%). No pronounced difference in metal uptake was noted between the two populations, except for MeHg after aqueous exposure, and Cd after dietary exposure, where South Carolina fish retained more metal. Elimination rate constants did not vary significantly between populations and exposure routes (except for Hg(II) after a dietary exposure) indicating that both populations process metals at the same rate. The tissue distribution of each metal in the fish varied among metals and exposure routes. Using a metal bioaccumulation model, the calculated steady-state body burden of metal was highest for Zn, and lowest for Am. Calculated body burdens were higher in South Carolina silversides for Am, Cd, and Zn. For all metals except Am, the diet is the dominant exposure route. Of the six metals investigated, MeHg and Zn in both populations and Cd in the South Carolina population are expected to biomagnify at this trophic step.

INTRODUCTION

Understanding metal bioaccumulation in fish is important for evaluating the risk of metal exposure to the fish themselves, their predators, and potentially human consumers of contaminated seafood. Fish, like other aquatic organisms, are exposed to metals via the dissolved phase and their diet. Often, studies using fish only focus on the dissolved (Jeffree et al. 2006) or dietary (Reinfelder & Fisher 1994a, Ni et al. 2000, Baines et al. 2002, Mathews & Fisher 2008a,b) exposure route, and few studies combine the two exposure routes to calculate total body burdens (Xu & Wang 2002, Wang & Wong 2003, Pickhardt et al. 2006). Studies have shown that diet is the dominant exposure pathway for metals in freshwater and marine fish (Xu & Wang 2002, Pickhardt et al. 2006, Mathews & Fisher 2009). Dietary exposure accounts for 40-88% of inorganic Hg (Hg(II)), > 68% of Cd, > 95% of Zn, 97% of Se, and > 98% of methylmercury (MeHg) body burden when fish are fed crustacean prey (Xu & Wang 2002, Pickhardt et al. 2006). While dietary studies have focused on prey choice as an influence on the assimilation efficiency (AE; defined as percentage of ingested metal which crosses the gut lining) of various metals, few have investigated the physiological factors governing metal bioaccumulation in fish, including the effects of ingestion rate and growth rate (Xu & Wang 2002, Zhang & Wang 2006).

Previous studies using marine invertebrates (copepods and mussels) indicate that AE is dependent on ingestion rate; when food is in low supply gut passage time increases, allowing digestive enzymes to solubilize a greater proportion of ingested metal, therefore increasing the AE (Wang & Fisher 1996, Xu & Wang 2001). This relationship has also been noted in fish, with higher Se and Zn AEs at lower ingestion rates in the mangrove snapper, *Lutjanus argentimaculatus* (Xu & Wang 2002), and grunt, *Terapon jarbua* (Zhang & Wang 2006). No

concise relationship has been found for Cd, with higher AEs at lower ingestion rates in *L. argentimaculatus* (Xu & Wang 2002), but no significant relationship between ingestion rate and AE in *T. jarbua* (Zhang & Wang 2006). Prior studies have also shown that growth rate can influence metal concentrations in aquatic animals, with faster growers having lower metal concentrations than slower growers due to somatic growth dilution. *Daphnia pulex* fed high quality algae labeled with MeHg grew 3.5 times faster than those fed poor quality algae, resulting in somatic growth dilution, and lower steady-state body burdens (Karimi et al. 2007). This has also been noted in Atlantic salmon, with faster growing fish having a lower body burden of Hg (Ward et al. 2010).

The Atlantic silverside (*Menidia menidia*) inhabits estuaries, bays, and salt marshes along the east coast of North America, from the Gulf of Newfoundland, Canada, to northern Florida, USA (Johnson 1975), and undergoes an annual lifecycle with < 1% of breeding adults reaching two years old (Conover & Ross 1982). *M. menidia* is a model organism to investigate the role of ingestion rate and growth rate on the body burden of various metals. There are latitudinal differences in ingestion rate and growth rate throughout the species range, with northern populations having a higher rate of ingestion and growth than southern populations (Conover & Present 1990, Present & Conover 1992). This counter-gradient variation evolved to overcome the shorter growing season, and size-selective winter mortality at higher latitudes (Conover & Present 1990). At the end of the growing season the body length of *M. menidia* is the same throughout the species range, indicating the northern fish grow faster throughout the shorter growing season (Conover & Present 1990). However, there are trade-offs associated with higher rates of physiological processes, with northern fish experiencing reduced swimming performance, and therefore increased predation pressure (Billerbeck et al. 2001, Lankford et al.

2001). Rates of metal uptake and loss from water and diet, and the resulting body burden, could be significantly different between the two endpoints of this species range, due to the documented differences in physiological rates in juvenile *M. menidia*.

To assess the effects of different ingestion and growth rates on metal bioaccumulation, we conducted a series of controlled laboratory pulse-chase experiments to investigate the rate of metal (Am, Cd, Hg(II), MeHg, Se, and Zn) uptake and loss after aqueous and dietary exposure, and resulting tissue distribution, in two populations of the Atlantic silverside (Nova Scotia and South Carolina; representing populations near the northern and southern limit of the species range) using a radiotracer technique. For dietary exposures, radiolabeled diatoms (*Thalassiosira pseudonana*) were fed to invertebrate prey (brine shrimp nauplii, *Artemia franciscana*), which were fed to juvenile Atlantic silversides. Calculated kinetic parameters describing metal bioaccumulation in these fish were entered into a biokinetic model (Wang et al. 1996) to calculate the steady-state metal concentration in both populations, the primary uptake route for each metal, and the potential for each metal to biomagnify at this trophic step.

Metals were chosen based on their chemical characteristics and environmental concern. Se and Zn are biologically essential, whereas Am, Cd, Hg(II), and MeHg are non-essential metals. We chose metals with varying binding preferences for sulfur, nitrogen, and oxygen, which influences their protein association. Am is a Class A (oxygen-seeking) metal, Hg a Class B (sulfur-seeking) metal, and Cd and Zn are borderline metals (Nieboer & Richardson 1980). These metals have a wide range of AEs in herbivores (Reinfelder & Fisher 1991, Wang & Fisher 1998), which has been correlated with the cytoplasmic distribution of metals in phytoplankton food (Reinfelder & Fisher 1991, 1994b), and this, in turn, can affect the assimilation efficiency of metals in fish when fed crustacean prey (Reinfelder & Fisher 1994a). Cd, Hg(II), MeHg, Se

and Zn are found at elevated levels in coastal water (Kennish 1997). MeHg is of particular concern due to the associated health risks in humans (Guallar et al. 2002, Chang et al. 2008), resulting in seafood consumption advisories. Am is a synthetic actinide, produced from the decay of ^{241}Pu, and has been released as nuclear waste into coastal water and from nuclear weapons fallout (Park et al. 1983). Am is a particle-reactive trivalent cation, which remains sorbed to the cell surface and not transported into the cell (Reinfelder & Fisher 1991). It is expected that this element behaves similarly to many other particle-reactive nonessential trivalent metals such as many of the rare earth elements.

MATERIALS AND METHODS

Experimental conditions

All experiments were carried out in 0.2 µm sterile filtered (Millipak 200, Millipore, Bedford, MA, USA) Southampton (SHSW) seawater, collected five miles offshore of Southampton (Long Island, NY, USA; salinity = 34, pH 7.9, dissolved organic carbon = 115 ± 19 µM C [mean ± SD, n = 3]). Background metal concentrations were analyzed by ICP-MS at the Trace Element Analysis Core Facility, Dartmouth College (Hanover, NH, USA; total Hg), and Rutgers Inorganic Analytical Laboratory, Rutgers University (New Brunswick, NJ, USA; Cd, Se, Zn). Phytoplankton and invertebrate prey were held at 18 ± 0.5°C, and fish at 21 ± 0.5°C. All experimental organisms were held on a 14:10 h light:dark cycle.

Fish maintenance and acclimation

Juvenile F1 and F2 generation lab reared Atlantic silversides (*Menidia menidia*) from Nova Scotia (44°N) and South Carolina (33°N) were used in this study. Both populations were of the same age (approximately 2 months); Nova Scotia fish were 59 mm ± 5 SD long, with a mean wet weight of 0.9 g ± 0.18 SD, while South Carolina fish were 38 mm ± 5 SD long, with a mean wet weight of 0.32 g ± 0.1 SD. Fish were spawned and raised at the Flax Pond Marine Lab (Oldfield, NY, USA) from field collected fish following a protocol described elsewhere (Conover & Present 1990, Present & Conover 1992), and transferred to our lab at least 3 wk before the start of experiments, acclimated to SHSW, and fed a daily diet of brine shrimp nauplii (*Artemia franciscana*). Brine shrimp nauplii was the chosen prey species because it is representative of crustacean zooplankton, and studies have shown Atlantic silversides survive best when fed this prey in the lab (Beck & Poston 1980). At least 4 d prior to each experiment 8-10 fish from each population were transferred to individual containers with 600 ml SHSW and an airstone to acclimate to experimental conditions. Fish were starved for 24 h prior to experiments to allow for total gut clearance. The experimental temperature was set at 21°C because both populations experience it during the spawning season in the wild (Conover & Present 1990), and fish are held at this temperature at the Flax Pond Marine Lab.

Aqueous metal exposure to fish

Radioisotopes were added to SHSW and allowed to equilibrate for at least 6 h. Atlantic silversides from each population (n = 8-10) were exposed to 250 ml of radiolabeled SHSW in individual containers. Radioisotope additions per fish were 5.9 kBq ^{241}Am, 5.4 kBq ^{109}Cd, 1.6 kBq ^{203}Hg(II), 0.6 kBq MeHg, and 13 kBq ^{65}Zn; this corresponds to the following metal concentrations: 0.98 nM ^{241}Am, 0.27 nM ^{109}Cd, 0.23 nM ^{203}Hg(II), 0.25 nM MeHg, and 3.00 nM

^{65}Zn. Exposure time was 12 h for Am and MeHg, and 36 h for Cd, Hg(II), and Zn. Exposure period varied between metals to allow an exposure time sufficient to radiolabel the fish while minimizing the likelihood of metal loss from the fish during this uptake period. ^{75}Se uptake from the dissolved phase was not investigated because aqueous selenite shows little reactivity for fish (Besser et al. 1993). Fish were not fed during the labeling period, so dissolved metal was the only source for the fish. At the end of the exposure period fish received two 30 sec rinses in filtered unlabeled SHSW to remove excess radioisotope, and were immediately radioassayed. They were then returned to individual containers containing 600 ml unlabeled SHSW to depurate for 6 d, during which they were fed unlabeled brine shrimp nauplii. Radioactivity in the fish was analyzed at regular intervals throughout the first 24 h, and then daily for another 5 d. Fish were fed brine shrimp nauplii daily and underwent regular water changes throughout the depuration period. After 6 d, the fish were euthanized in 450 ppm MS222 (tricaine methane sulphonate), and dissected into three body compartments: head (including gills), viscera, and body (skeleton, fillet, fins, and skin). Tissue samples were immediately radioassayed, dried in an oven at 60°C for 24 h, and the dry weights recorded. Not all fish initially exposed to the radiolabeled SHSW survived throughout depuration, therefore only data from fish which survived the whole experiment are shown in the results (n = 4-9 per population). No toxic effects of metal exposure (death, excess mucus production, abnormal swimming behavior) were observed during the metal uptake period. Juvenile Atlantic silversides are very sensitive to handling (Present and Conover 1992) which this mortality is attributed to.

Dietary metal exposure to fish

The marine diatom *Thalassiosira pseudonana* was uniformly radiolabeled for 4 d with radioisotopes in 1 L SHSW, amended with nutrients at f/2 concentrations (Guillard & Ryther 1962), but modified so no Cu, Zn, and EDTA were added. Radioisotope additions were 79 kBq ^{241}Am, 216 kBq ^{109}Cd, 71 kBq ^{203}Hg(II), 61 kBq MeHg, 55 kBq ^{75}Se, and 183 kBq ^{65}Zn; this corresponds to 3.50 nM ^{241}Am, 0.66 nM ^{109}Cd, 3.00 nM ^{203}Hg(II), 2.00 nM MeHg, 3.96 nM ^{75}Se, and 3.32 nM ^{65}Zn. At the end of uptake algal cells were filtered through a 3 µm polycarbonate membrane, rinsed with unlabeled SHSW to remove excess radioisotope, resuspended in 500 ml non-radiolabeled SHSW to attain a cell density of 1 x 10^6, and allowed to equilibrate for 4 h. Approximately 2000 brine shrimp nauplii (*Artemia franciscana*) were added to the algal suspension and allowed to feed for 20 h. Nauplii were then filtered through a 10 µm polycarbonate membrane, rinsed to remove excess radioactivity from the carapace, and added to 50 ml unlabeled SHSW to make a brine shrimp slurry. 1 ml of slurry (approximately 60 brine shrimp nauplii) was presented to 8 fish from each population, and allowed to feed for 45 min. No feces were produced during the feeding period. After feeding, fish were removed from their container, and immediately radioassayed. Fish were then returned to individual containers with 600 ml unlabeled SHSW and fed unlabeled brine shrimp nauplii to purge their guts of unassimilated radiolabeled food. At each time point feces were collected and radioassayed to monitor the gut passage time of unassimilated radiolabeled food. The 6 d depuration, euthanization, and dissection procedure followed that described previously. Only data from fish that survived the entire experimental period are shown (n = 5-8 per population).

Radioisotopes and radioanalysis

^{109}Cd (t½ = 463.3 d) and ^{65}Zn (t½ = 244.1 d), both dissolved in 0.1 N HCl, were purchased from Los Alamos National Laboratory (Los Alamos, NM, USA), ^{241}Am (t½ = 432.7 y, dissolved in 3 $NHNO_3$) was purchased from Amersham, ^{75}Se (t½ = 119.8 d, as selenite, dissolved in 0.1 N HCl) was purchased from Isotope Products (Valencia, CA, USA), and ^{203}Hg(II) (t½ = 46.6 d, dissolved in 1 N HCl) was obtained from Georgia State University (Atlanta, GA, USA). MeHg ($CH_3{}^{203}$Hg(II)), held in deionized water, was synthesized in our lab from Hg(II) using a method described in Imura et al. (1971), Rouleau and Block (1997), and Bancon-Montigny et al. (2004). Experimental radioisotope additions were in microliter quantities, and equimolar volumes of sodium hydroxide (NaOH) were added to neutralize the acid added during radiolabeling. The pH was monitored and unaffected by the radioisotope addition. ^{241}Am and ^{75}Se, and ^{109}Cd and ^{65}Zn were double-labeled (two isotopes in the same experimental container), while ^{203}Hg(II) and MeHg were single-labeled.

Radioactivity in the fish was assayed non-invasively using a Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector, allowing the same fish to be analyzed throughout depuration, therefore reducing biological variability. Counting times did not exceed 5 min to reduce stress on the fish, yet obtain propagated counting errors typically ≤ 5%. However, propagated counting errors could reach 25% by the end of depuration if most of the radioactivity was lost and counts were close to background level. Water, feces, and dissected fish tissue samples were radioassayed using an inter-calibrated LKB Pharmacia-Wallac 1282 CompuGamma CS gamma counter (Turku, Finland) for either 5 min (^{241}Am, ^{109}Cd, ^{65}Zn), or 10 min (^{203}Hg, ^{75}Se). All sample counts were adjusted for background radioactivity and radioactive decay. The γ-emissions of ^{109}Cd, ^{241}Am, ^{203}Hg, ^{75}Se, and ^{65}Zn were detected at 22, 59.5, 279, 400, and 1115 keV, respectively.

Modeling metal bioaccumulation in fish

The bioaccumulation of metals in fish, like other aquatic organisms, can be defined as a balance between metal uptake and loss from aqueous and dietary sources. This was originally described in Thomann (1981), and then modified in Wang et al. (1996) and Reinfelder et al. (1998). This model has been successfully tested using marine invertebrates (Fisher et al. 1996, 2000, Wang & Fisher 1998), and more recently freshwater and marine fish (Wang & Wong 2003, Pickhardt et al. 2006). Kinetic parameters derived from laboratory experiments can be used to model predicted values in the field. Under steady-state conditions the equation is as follows:

$$C_{ss} = (k_u \cdot C_w)/(g + k_{ew}) + (\mathrm{AE} \cdot \mathrm{IR} \cdot C_f)/(g + k_{ef}) \qquad \text{Eq. 1}$$

where C_{ss} is the steady-state concentration of metal in an organism (µg g^{-1}), k_u is the uptake rate constant from the dissolved phase (ml g^{-1} d^{-1}), C_w is the dissolved metal concentration in water (µg ml^{-1}), g is the growth rate constant (d^{-1}), k_{ew} is the loss rate constant after dissolved exposure (d^{-1}), AE is the assimilation efficiency of ingested metal (%), IR is the ingestion rate (g g^{-1} d^{-1}), C_f is the metal concentration in food (µg g^{-1}), and k_{ef} is the loss rate constant after dietary exposure (d^{-1}).

Radioactivity in fish at the end of uptake was used to calculate the k_u, by dividing radioactivity in individual fish (g^{-1} dry weight) by radioactivity in ml^{-1} water and by time (d). AE and k_es were calculated by fitting an exponential regression between the 48 h and 144 h depuration time points. The AE was determined to be the y-intercept, and k_e was the slope of the

curve. The biological half-life ($tb_{½}$) of a metal in the fish after aqueous and dietary exposure is defined as the time it takes for 50% of the metal to be excreted, and is calculated as:

$$tb_{½} = \ln 2/k_e \qquad \text{Eq. 2}$$

We used food consumption data from Billerbeck et al. (2000) to calculate the weight-specific ingestion rate (food consumption divided by dry wt of fish), resulting in an ingestion rate of 0.58 g g^{-1} d^{-1} for Nova Scotia silversides and 0.37 g g^{-1} d^{-1} for South Carolina silversides. Growth rate constants were calculated using the following allometric relationship between length and weight:

$$W = a \cdot L^b \qquad \text{Eq. 3}$$

where W is the fish weight (g), L is the fish length (cm), and a and b are allometric coefficients (a = 0.006, b = 3.023; Jessop 1983). L values are the average length of fish used in this study (Nova Scotia 5.9 cm, South Carolina 3.8 cm). The growth rate relationship can be calculated from the derivative of the allometric equation with respect to time, described as follows:

$$dW/dt = a \cdot b \cdot L^{b-1} \cdot (dL/dt) \qquad \text{Eq. 4}$$

For dL/dt we applied 0.082 cm d^{-1} for Nova Scotia silversides (Jessop 1983), and 0.027 cm d^{-1} for South Carolina silversides (Sosebee 1991). The calculated daily growth rate (0.051 g d^{-1} Nova Scotia, 0.007 g d^{-1} South Carolina) was then divided by the average wet weight of the fish used in this study (Nova Scotia 0.9 g, South Carolina 0.32 g) to calculate a growth rate constant of 0.057 d^{-1} for Nova Scotia silversides and 0.022 d^{-1} for South Carolina silversides.

The C_w values used were background metal concentrations in SHSW (Cd, Hg, Se, Zn) or literature values for Am (Cochran et al. 1987), and MeHg (Hammerschmidt & Fitzgerald 2006),

and are as follows: 1.9 x 10^{-15} µg ml^{-1} Am, 0.17 ng ml^{-1} Cd, 3.3 pg ml^{-1} Hg, 0.03 pg ml^{-1} MeHg, 0.03 ng ml^{-1} Se, and 8.7 ng ml^{-1} Zn. C_f values for zooplankton (dry weight) were obtained from the literature, and are as follows: 2.5 µg g^{-1} Cd, 1.3 µg g^{-1} Se, and 167 µg g^{-1} Zn (Fisher et al. 2000). We used a mean total Hg value of 0.22 µg g^{-1} (IAEA 2004); MeHg accounts for 75% of total Hg in crustacean zooplankton (Francesconi & Lenanton 1992), so we applied a C_f value of 0.165 µg g^{-1} for MeHg, and 0.055 µg g^{-1} for Hg(II). The Am C_f was calculated by multiplying the C_w by the concentration factor of Am in zooplankton (4000, IAEA 2004) to obtain a value of 7.6 x 10^{-12} µg g^{-1}.

Eq. 1 can be rearranged to calculate the relative importance of dissolved versus dietary exposure routes to the steady-state body burden of metal using the following equation:

$$R = [(\mathrm{AE} \cdot \mathrm{IR} \cdot C_f)/(g + k_{ef})]/C_{ss} \cdot 100 \qquad \text{Eq. 5}$$

where R is the percentage attributed to dietary exposure. Eq. 1 can also be rearranged to calculate the trophic transfer factor (TTF), which indicates the potential for a metal to biomagnify at this trophic step based upon the ratio of metal in the predator to metal in the prey. TTF is calculated as follows:

$$\mathrm{TTF} = (\mathrm{AE} \cdot \mathrm{IR})/(g + k_{ef}) \qquad \text{Eq. 6}$$

A TTF > 1 indicates that a metal is likely to biomagnify at this trophic step, while a TTF < 1 indicates that biomagnification is unlikely (Reinfelder et al. 1998).

RESULTS

Uptake of metals from the dissolved phase

For all metals examined, most of the added metal remained in the dissolved phase (that is, not taken up by the fish) throughout the exposure period. The percentage of added metal associated with fish at the end of exposure was 0.06-0.17% for Am, 0.04-0.09% for Cd, 1.1-2.1% for Hg(II), 31-33% for MeHg, and 0.17-0.22% for Zn. The calculated uptake rate constants from the dissolved phase (k_u) were consistently higher in the South Carolina population (Table 1). k_us (ml g^{-1} d^{-1}) were highest for MeHg (1155 and 4375), followed by Hg(II) (15 and 17), Am (11 and 13), Zn (1.9 and 5), and Cd (0.8 and 1.3). k_u values were 1.2, 1.6, 1.1, 3.8, and 2.6 times higher in South Carolina silversides for Am, Cd, Hg(II), MeHg, and Zn respectively. Am and Hg(II) k_u values showed no significant differences in the rate of metal uptake between the two populations (t-test, $p > 0.05$), while there was a significant difference for Cd, MeHg and Zn ($p < 0.01$). Both populations had higher k_u values for MeHg than Hg(II). Calculated dry-weight concentration factors (CFs, defined as dpm g^{-1} fish divided by dpm ml^{-1} dissolved in seawater) show that all metals were more enriched in fish than the surrounding water (CF > 1), and South Carolina silversides had consistently higher CF values for all metals. South Carolina CF values (mean ± 1 SE) were 6.4 ± 0.4 for Am, 2.0 ± 0.2 for Cd, 25 ± 2.7 for Hg(II), 2155 ± 247 for MeHg, and 7.4 ± 1.2 for Zn. Nova Scotia CF values were 5.7 ± 0.7 for Am, 1.2 ± 0.1 for Cd, 22 ± 2.7 for Hg(II), 569 ± 142 for MeHg, and 2.8 ± 0.1 for Zn. Dry wt CFs can be converted to wet wt CFs by dividing the former by 4.5 (dry wt is 22% of the wet wt value for *M. menidia*).

After aqueous exposure in SHSW depuration curves indicate a rapid elimination of metal within the first 24 h, and then slower physiological turnover for the following 5 d (Fig. 1). After 24 h of depuration the percentage of metal eliminated was as follows: 34% Am, 23% Cd, 24% Hg(II), 5% MeHg, and 20% Zn from Nova Scotia silversides, and 46% Am, 39% Cd, 26% Hg(II), 13% MeHg, and 40% Zn from South Carolina silversides. South Carolina silversides

consistently retained a lower percentage of the initial metal body burden throughout depuration. At the end of 6 d depuration the percentage of initial metal remaining was 46% Am, 61% Cd, 51% Hg(II), 92% MeHg and 59% Zn in Nova Scotia silversides, and 44% Am, 43% Cd, 47% Hg(II), 79% MeHg, and 38% Zn in South Carolina silversides (Fig. 1). Efflux rate constants after aqueous exposure (k_{ew}) are given in Table 1. For Nova Scotia silversides the efflux rate constants (d^{-1}) for Hg(II) (0.071) > Zn (0.061) > Cd (0.052) > Am (0.038) > MeHg (0.006). For South Carolina silversides Cd (0.076) = Hg(II) (0.075) > Zn (0.056) > Am (0.023) > MeHg (0.014). No significant difference between k_{ew} for the two populations was noted except for MeHg ($p < 0.05$).

Uptake of metals from diet

In both silverside populations AEs for MeHg greatly exceeded those for all other metals. For Nova Scotia silversides AEs were highest for MeHg (89%), followed by Zn (24%), Cd (15%), Se (10%), Hg(II) (8%), and lowest for Am (0.3%). For South Carolina silversides AEs were highest for MeHg (82%), followed by Cd (39%), Zn (29%), Hg(II) (15%), Se (13%), and lowest for Am (1.9%) (Table 2). The AEs for Am were given as percentage remaining after 48 h of metal loss; exponential regressions could not be fitted to the data due to near complete elimination of the radioisotope. The AEs for MeHg, Se, and Zn are similar for the two populations, but the slight difference in MeHg AEs was found to be statistically significant (t-test, $p < 0.05$). South Carolina silversides assimilated 6.3-times more Am, 2.6-times more Cd (both significant, $p < 0.05$), and 1.9-times more Hg(II) (not significant, $p > 0.05$) than Nova Scotia silversides. When comparing the two Hg species, Nova Scotia and South Carolina silversides assimilated 11–times and 5.5–times more MeHg than Hg(II), respectively.

After feeding, all metals were eliminated from both silverside populations following a two-compartment loss pattern: the rapid loss during the first several hours corresponded to defecation of unassimilated metal, and the slower loss over the remaining depuration period corresponded to physiological turnover of metal (Fig. 2). Within the first 24 h, 99.5% Am, 85% Cd, 88% Hg(II), 10% MeHg, 87% Se, and 76% Zn was lost from the Nova Scotia population, and 98.6% Am, 63% Cd, 86% Hg(II), 18% MeHg, 83% Se, and 70% Zn was lost from the South Carolina population. At the end of 6 d depuration the percentage of initial metal remaining was 0.3% Am, 11% Cd, 5% Hg(II), 84% MeHg, 6% Se, and 17% Zn for Nova Scotia fish, and 1.2% Am, 30% Cd, 4% Hg(II), 76% MeHg, 7% Se, and 22% Zn for South Carolina fish (Fig. 2). Efflux rate constants after dietary exposure (k_{ef}) varied by metal, and except for Hg(II), did not vary between the two populations (Table 2). For the Nova Scotia population the efflux rate (d^{-1}) of Se (0.109) > Hg(II) (0.086) > Cd (0.073) > Zn (0.058) > MeHg (0.011), and for the South Carolina population Hg(II) (0.194) > Se (0.105) > Cd (0.052) = Zn (0.051) > MeHg (0.013).

Tissue distributions and corresponding metal concentrations

Fig. 3 shows the tissue distribution (as percentage of total dpm in whole fish) of Am, Cd, Hg(II), MeHg, and Zn in Atlantic silversides at the end of depuration after aqueous exposure in SHSW. Tissue distribution varied by metal, but apart for Cd, did not vary between the two silverside populations for each metal. Am and Hg(II) were mainly associated with the head (64-69% Am, 49-55% Hg(II)), whereas Zn was distributed between the head (37-40%) and body (44%). Am was the only metal to show no association with the viscera (0-1%). MeHg was predominantly associated with the body (70-72%). Cd had a higher association with the viscera

in Nova Scotia silversides (47%) than in South Carolina silversides (32%), whereas South Carolina fish had a greater percentage associated with the body (42% versus 30%).

Fig. 4 shows the tissue distribution (as percentage of total dpm in whole fish) of Cd, Hg(II), MeHg, Se, and Zn in Atlantic silversides at the end of depuration after feeding on radiolabeled brine shrimp nauplii. Tissue distribution for Am could not be determined due to near complete elimination of the radioisotope. Tissue partitioning varied by metal, and except for Hg(II) there was no difference in the body partitioning of each metal between the two populations. Cd and MeHg were predominantly associated with the body (80-84% Cd, 51-57% MeHg), whereas Zn was mainly associated with the head (49-52%). Se was mainly distributed between the head (44-45%) and body (41-44%). The tissue distribution of Hg(II) varied significantly between the two populations; the percentage associated with the body was higher in Nova Scotia fish (67%) than in South Carolina fish (15%), while the percentage associated with the viscera was higher in South Carolina fish (72% versus 28%).

The body distribution of Cd, Hg(II), MeHg, and Zn varied with exposure route (Figs. 3, 4). At the end of depuration after aqueous exposure, Cd was distributed throughout the three tissue compartments, whereas after dietary exposure Cd was mainly associated with the body. Hg(II) had a higher percentage associated with the head after aqueous exposure, but after dietary exposure it was predominantly associated with the viscera (South Carolina population) or body (Nova Scotia population). MeHg had a higher percentage associated with the body, and lower percentages associated with the head and viscera after an aqueous exposure. Zn was predominantly associated with the head after a dietary exposure, whereas it was nearly evenly distributed between the head and body after an aqueous exposure.

Table 3 shows the weight-normalized radioactivity concentration (Bq g^{-1}) of each metal in tissue compartments at the end of depuration after aqueous and dietary exposures. Comparisons can be made between populations after aqueous exposure to each individual metal as all fish were exposed to the same dissolved metal concentration, whereas no comparison can be made between populations after dietary exposure to each metal as fish consumed different amounts of food. After aqueous exposure, radioactivity concentrations of Cd, Hg(II), and Zn were highest in the viscera, and South Carolina silversides had higher tissue burdens; whereas the Am concentration was highest in the head for both populations, and Nova Scotia silversides had a higher tissue burden. For MeHg South Carolina fish had a higher body burden in all tissue compartments, but the tissue burden was highest in the viscera for South Carolina fish, and distributed between the head and body in Nova Scotia fish. After dietary exposure Hg(II), MeHg, and Se tissue concentrations were highest in the viscera for both populations, whereas Cd was most concentrated in the body. Zn was most concentrated in the viscera in Nova Scotia fish, compared to the head in South Carolina fish.

Modeling metal bioaccumulation in Menidia menidia

The biological half-life ($tb_{½}$) of metals in both silverside populations are shown in Table 4. For metals in which aqueous and dietary exposure routes could be compared, the $tb_{½}$'s were highest for MeHg (50-116 d), followed by Am (17-30 d), Zn (11-14 d), Cd (9-13 d), and lowest for Hg(II) (4-10 d). The $tb_{½}$ for Se could only be calculated after a dietary exposure (6-7 d). For Cd, Hg(II), and Zn there was little difference in $tb_{½}$ between populations and exposure routes. There was little difference between the $tb_{½}$'s of MeHg among exposure routes for the South Carolina silversides, but a large difference for Nova Scotia silversides; the $tb_{½}$ was also 2.3-times

greater in the Nova Scotia population than in the South Carolina population after aqueous exposure. The $tb_{½}$ of Am is 1.7 times higher in South Carolina silversides after an aqueous exposure; we could not determine the k_{ef} after dietary exposure due to near complete elimination of the radioisotope, so for our model we applied a value of 0.04 d^{-1} calculated for juvenile striped bass (Baines et al. 2002). Assuming it takes seven $tb_{½}$'s for all radioisotopes to be eliminated from an organism, the residence time of each metal can be calculated. After aqueous exposure the calculated residence times (d) of each metal in Nova Scotia and South Carolina silversides were as follows: Am (126, 210), Cd (91, 63), Hg(II) (70, 63), MeHg (812, 350), and Zn (77, 84), and after dietary exposure are as follows: Am (153, 153), Cd (63, 91), Hg(II) (56, 28), MeHg (441, 371), Se (42, 49), and Zn (84, 98).

Model-predicted body burdens of metals in both silverside populations at steady-state (C_{ss}) were highest for Zn (202 and 246 µg g^{-1} for Nova Scotia and South Carolina silversides, respectively), followed by Cd (1.7 and 4.9 µg g^{-1}), MeHg (1.3 and 1.4 µg g^{-1}), Se (0.45 and 0.49 µg g^{-1}), Hg(II) (18 and 15 ng g^{-1}), and lowest for Am (3.6×10^{-10} and 1.4×10^{-9} ng g^{-1}) (Table 5). The calculated body burden was 4, 2.9, 1.1, 1.1, and 1.2 times higher for Am, Cd, MeHg, Se, and Zn, respectively, in South Carolina silversides, and 1.2 times higher for Hg(II) in Nova Scotia silversides. Diet was the dominant source for Cd, Hg(II), MeHg, and Zn, accounting for > 96% of accumulated metal. Diet is assumed to account for 100% of accumulated metal for Se (Stewart et al. 2010). Am was the only metal for which the aqueous phase accounted for a significant percentage of accumulated metal (38.3-61.1%) (Table 5). TTFs were < 1 for Am, Hg(II), and Se, indicating that these metals would not be expected to biomagnify in juvenile *M. menidia*, whereas TTFs were ~ 1 for Zn and >> 1 for MeHg in both populations, and > 1 for Cd in South

Carolina silversides (Table 5), indicating that biomagnification is likeliest for MeHg at this trophic step.

DISCUSSION

Aqueous uptake of metals

Our results show that the rate of metal uptake from the dissolved phase varied by metal. For all metals and both populations, concentration factors (CFs) were greater than one, indicating fish are more enriched in metal than the surrounding water. CFs and uptake rate constants (k_us) were higher in fish from South Carolina than Nova Scotia, indicating that body size may influence the rate of metal uptake, as observed in Zhang & Wang (2007). We attribute this difference to the larger surface area to volume ratio in South Carolina silversides, which were smaller. Differences in respiration rate between the two populations could also account for some of the difference, but respiration rates were not measured during this study.

The MeHg k_u values in this study (1155 and 4375 ml g^{-1} d^{-1}) are similar to those noted in the marine sweetlips, (*Plectorhinchus gibbosus*, 4515 ml g^{-1} d^{-1}, Wang & Wong 2003), and the freshwater redear sunfish (*Lepomis microlophus*, 1280 ml g^{-1} d^{-1}, Pickhardt et al. 2006), but higher than observed in freshwater mosquitofish, *Gambusia affinis*, (185-338 ml g^{-1} d^{-1}, Pickhardt et al. 2006). Our Hg(II) k_u values (15 and 17 ml g^{-1} d^{-1}) are slightly lower than those observed in freshwater fish (38-78 ml g^{-1} d^{-1}, Pickhardt et al. 2006), and 10-fold lower than what has been observed in marine fish (195 ml g^{-1} d^{-1}, Wang & Wong 2003). Our values could be lower than observed in other studies due to Hg(II) binding to dissolved organic matter. The higher MeHg k_u compared to Hg(II) k_u (77-times higher than Hg(II) in Nova Scotia silversides, and 257-times higher in South Carolina silversides) is consistent with previous reports for other

fish (Wang & Wong 2003, Pickhardt et al. 2006), indicating that Hg(II) is absorbed less efficiently than MeHg. Hg(II) uptake is considered to be a more passive process due to Hg forming uncharged, inorganic complexes in water (Mason et al. 1996), whereas MeHg uptake across the gills is thought to be an energy mediated process (Andres et al. 2002).

Our uptake rate constants for Am (11 and 13 ml g^{-1} d^{-1}) are consistent with observations in juvenile sea bream (*Sparus auratus*, 10 ml g^{-1} d^{-1}, Mathews et al. 2008), and Cd uptake (0.8 and 1.3 ml g^{-1} d^{-1}) is similar to that observed in other marine species, including sea bream (5 ml g^{-1} d^{-1}), and mangrove snapper, *Lutjanus argentimaculatus*, (5.1 ml g^{-1} d^{-1}, Xu & Wang 2002). Am is highly particle-reactive (Fisher & Reinfelder 1995), accounting for its higher uptake rate than the other metals examined (except Hg(II) and MeHg). Cd uptake rates here are lower than what would be expected for freshwater fish, due to complexation of Cd^{2+} with Cl^{-} in saline water resulting in reduced bioavailability. A prior study using the tidewater silverside, *Menidia beryllina*, showed that fish accumulate less Cd via the dissolved phase at higher salinities (Jackson et al. 2003). Our Zn uptake rates (1.9 and 5.0 ml g^{-1} d^{-1}) are similar to that noted in the sea bream (4 ml g^{-1} d^{-1}, Mathews et al. 2008), but lower than observed in the mangrove snapper (10 ml g^{-1} d^{-1}, Xu & Wang 2002).

Assimilation efficiencies

The wide range of assimilation efficiencies (0.3% for Am to 89% for MeHg) observed in our study indicates that AEs are highly variable among metals. The AE ranking we noted is consistent with the observations of Reinfelder & Fisher (1991) in copepods, which noted that AEs in these animals are related to the percentage of metal associated with the cytoplasm in

phytoplankton food. Apart from Cd, no pronounced difference was noted in metal AEs between the two populations.

Our MeHg AE values (89% for Nova Scotia, 82% for South Carolina) are slightly lower, but comparable to other literature values for freshwater, estuarine, and marine fish fed zooplankton prey (89-95%; Wang & Wong 2003, Pickhardt et al. 2006, Mathews & Fisher 2008a). For both populations MeHg assimilation greatly exceeded Hg(II) assimilation, being 11-fold higher in Nova Scotia silversides, and 5.5-fold higher in South Carolina silversides. This difference in assimilation could be a result of fish gut chemistry, with MeHg being more readily desorbed from ingested food, and passively and actively transported across the intestinal wall (Leaner & Mason 2002). Furthermore, it has been noted that MeHg AEs are higher than Hg(II) AEs in fish, because a larger percentage of MeHg in zooplankton is associated with the soft tissue (Lawson & Mason 1998); this higher assimilation of MeHg in zooplankton is attributed to the accumulation of MeHg in algal cytoplasm, while inorganic Hg remains largely bound to cell surfaces (Mason et al. 1995). The calculated Hg(II) AEs in this study (8% for Nova Scotia, 15% for South Carolina) are similar to those observed in other freshwater and marine fish (8.5-27%; Wang & Wong 2003, Pickhardt et al. 2006), but much lower than that observed in the freshwater mosquitofish (42-51%, Pickhardt et al. 2006). However, those authors noted that after 2 d depuration some mosquitofish were still producing radioactive feces, suggesting a longer gut passage time than for the Atlantic silverside (< 8 h), resulting in a higher AE due to more time to solubilize the metal in the gut.

Cd assimilation showed the greatest variation between the two silverside populations. The AEs observed (15% for Nova Scotia, 39% for South Carolina) are within the range noted for other estuarine and marine fish fed zooplankton prey (21-28%; Baines et al. 2002, Mathews &

Fisher 2008b). However, our values were higher than those observed for killifish (*Fundulus heteroclitus*, 7%, Mathews & Fisher 2008a), grunt (*Terapon jarbua*, 6.3%, Zhang & Wang 2006), and mangrove snapper (9.8%, Xu & Wang 2002) fed zooplankton prey. These differences in AEs could be due to the variation in Cd bioavailability among different zooplankton prey items. Ni et al. (2000) noted that when the mudskipper (*Periophthalmus cantonensis*) and glassy (*Ambassis urotaenia*) were fed brine shrimp nauplii the AEs resembled our values (26-32%), but when the fish were fed copepods the AEs decreased to 10-14%. This was also noted in another study in which uniformly radiolabeled copepods were fed to silversides (*M. menidia*, *M. beryllina*), resulting in an AE of 3% (Reinfelder & Fisher 1994a). This pronounced difference in Cd AE between the two populations cannot be attributed to differences in feeding behavior; Cd was double-labeled with Zn, and there is no significant difference in Zn AEs between silverside populations.

Am had the lowest AEs of all the investigated metals. Our Am AE values (0.3-1.9%) are lower than other values noted in fish (4-6%; Baines et al. 2002, Mathews & Fisher 2008b) fed zooplankton prey. Nearly all Am had been lost from both silverside populations within the first 8 h of depuration, and if the fish were radioassayed more frequently during this time Am could be used as a tracer to calculate an accurate gut passage time, as noted in copepods (Fisher & Reinfelder 1991). Low Am AEs in fish can be attributed to a low Am AE in zooplankton, which in turn is attributed to the low percentage of Am associated with cytoplasm in phytoplankton (Reinfelder & Fisher 1991, 1994b). Also, Am is not known to share cellular uptake channels with other elements, unlike Cd, another biologically non-essential element which can be taken up through Ca and Zn uptake channels (Brzóska & Moniuszko-Jakoniuk 2001, Franklin et al. 2005).

Zn and Se, are both biologically essential, regulated metals found in proteins, and used as cofactors in enzymes (Eisler 1985, 1993). Both metals showed little variation in assimilation between the two populations. The Zn AEs reported in this study (24% in Nova Scotia, 29% in South Carolina) are comparable to that noted in the grunt (23%), striped bass (23-40%), and mudskipper (21%) fed zooplankton prey (Ni et al. 2000, Baines et al. 2002, Zhang & Wang 2006). Our values are higher than those noted in the glassy (5-17%, Ni et al. 2000), mangrove snapper (14.5%, Xu & Wang 2002), sea bream (14%, Mathews & Fisher 2008b), and another study using silversides (6%, Reinfelder & Fisher 1994a). This difference could be attributed to the different partitioning of Zn between the exoskeleton and soft body tissue of the zooplankton prey. Our Se AEs (10% for Nova Scotia, 13% for South Carolina) are lower than AEs calculated for other fish fed zooplankton prey (29-77%; Reinfelder & Fisher 1994a, Baines et al. 2002, Xu & Wang 2002, Zhang & Wang 2006, Mathews & Fisher 2008b), for reasons not apparent.

For Am, Cd, and Hg(II) South Carolina fish assimilated more metal than Nova Scotia fish, but the difference was only statistically significant for Am and Cd (t-test, $p < 0.05$), and Cd was the only metal where a pronounced difference was noted. Although Am showed the largest difference between the two populations nearly all of the radioisotope was lost, so radioactive counts in the fish were not high above background level, resulting in a large propagated counting error which could influence our values. The higher metal AE in South Carolina fish could possibly be explained by an estimated 1.6-times lower ingestion rate. Xu & Wang (2002) noted that as the ingestion rate of the mangrove snapper increased from 0.05 to 0.57 g g^{-1} d^{-1} the AE of Cd, Se, and Zn decreased substantially (from 24-7%, 69-54%, and 43-17% respectively), indicating that metal assimilation from the diet is dependent on ingestion rate, a parameter that is commonly ignored in dietary studies. This relationship suggests that a higher ingestion rate

allows less time for food-bound metal to have contact with the gut, resulting in less metal becoming solubilized and bioavailable. We did not measure gut passage time in our study, but observed that for both populations the most radioactive feces were produced within the first 4-8 h after feeding. However, Xu & Wang (2002) noted that at high ingestion rates (as noted in both our silverside populations (0.37 and 0.58 g g^{-1} d^{-1})) there is no difference in Cd, Se, and Zn AE, indicating that ingestion rate only has an influence on AE when food is in low supply. This is consistent with our findings for Se, and Zn, but not for Cd.

Nova Scotia silversides have a 1.8-2.2 times higher growth efficiency (defined as percentage of consumed energy used to create biomass), as well as a higher ingestion rate than South Carolina silversides (Present & Conover 1992). Nova Scotia silversides also accumulate lipids at a faster rate than South Carolina fish, presumably to reduce the risk of size-selective winter mortality (Schultz & Conover 1997). MeHg was the only metal where Nova Scotia fish assimilated more metal than South Carolina fish; although the AE difference between the two populations was 7%, it was found to be statistically significant ($p < 0.05$), and the increased lipid content may enable Nova Scotia fish to accumulate more MeHg from their diet, resulting in a higher AE.

Efflux rates after aqueous and dietary exposure

The similar efflux rates between the two population and exposure routes (except for South Carolina silversides after Hg(II) exposure where the efflux rate is 2.6 times higher after a dietary exposure) could be attributed to a similar routine metabolic rate at the experimental temperature we used. Billerbeck et al. (2000) found that the metabolic rate (measured as O_2

consumption) was comparable for the two populations at 17°C and 22°C (our experiments were conducted at 21°C), but was significantly higher in Nova Scotia silversides at 28°C. Therefore, the physiological turnover of metal within tissues would be expected to be similar, consistent with the comparable efflux rates for metals in both populations. The similarity in metabolic rate could also explain why the uptake rate of Am and Hg(II) were similar between the two populations. The high efflux rates for all metals except MeHg after dietary exposure indicates digestive processes aid in the rapid removal of metal; this was particularly evident for Hg(II) in South Carolina silversides which is primarily localized in the gut, and had the highest efflux rate of 19% d^{-1}. The high efflux rates after aqueous exposure indicate that different pools of metals are rapidly turned over in the fish and excreted, and unlike for dietary exposure egestion does not play a role.

Our k_{ew} values are similar to the findings of other studies for Am, Cd, Hg(II), MeHg, and Zn (Wang & Wong 2003, Pickhardt et al. 2006, Mathews et al. 2008). However, our values are higher than the turbot for Am, Cd, and Zn (Jeffree et al. 2006); that study used larger more mature fish with lower metabolic rates than the juveniles we used. Our Cd and Zn efflux rates were higher than observed by Xu & Wang (2002); they used fish of a similar size to ours, but silversides may have a higher metabolic rate than mangrove snappers due to rapid growth which would account for this difference. Our Hg(II) k_{ew} values are at least 1.7 times higher than that noted in freshwater fish (Pickhardt et al. 2006). This difference could be due to osmoregulatory differences between freshwater and marine fish; marine fish actively drink water to replace what is lost from tissues and salt removal across gills, and if Hg(II) is excreted unselectively through the same ion-transport channels that is used for salt removal the efflux rate will be higher.

Our k_{ef} values for MeHg are consistent with other studies using fish fed zooplankton prey (Wang & Wong 2003, Pickhardt et al. 2006, Mathews & Fisher 2008a). Our k_{ef} values for Cd, Se, and Zn are within the range noted in other studies where fish were fed zooplankton prey (Xu & Wang 2002, Mathews & Fisher 2008a,b), but unlike for MeHg there is greater variability in these efflux rates between studies. Our Hg(II) k_{ef} values were higher than noted in freshwater fish (Pickhardt et al. 2006), but the Nova Scotia silverside k_{ef} was consistent with the marine sweetlips (Wang & Wong 2003), whereas the South Carolina silverside k_{ef} was 2.0 times higher. At the end of 6 d depuration Hg(II) was primarily associated with the gut in South Carolina silversides, accounting for the high k_{ef}, suggesting that metal loss should be monitored for a longer period of time. Wang & Wong (2003) noted that during the first 7 d of metal loss in fish Hg(II) efflux rates were higher after both dietary and aqueous exposures, and decreased significantly between 9-28 d of metal loss (k_{ef} decreased from 0.096 to 0.055 d^{-1}, k_{ew} decreased from 0.072 to 0.029 d^{-1}). Our Cd and Zn efflux rates were higher than that noted for the larger, more mature sea bream and turbot fed fish prey (Mathews et al. 2008), suggesting that size influences the loss of assimilated metal. This is consistent with the findings of Baines et al. (2002) who found lower efflux rates of assimilated Cd and Zn in 88 d old striped bass (0.03 d^{-1} for both metals) than in 43 d old fish (0.07 d^{-1} Cd, 0.013 d^{-1} Zn), consistent with the inverse relationship of metabolic rate and age.

Distribution of metals in fish tissue

Tissue partitioning varied by metal and exposure route. The gills are considered to be the primary uptake site for metals during an aqueous exposure for most metals, and the digestive

tract after a dietary exposure. Except for Hg(II) after dietary exposure, and Cd after aqueous exposure, the tissue partitioning of each metal did not vary between the two populations after either aqueous or dietary exposure, indicating that both populations physiologically processed metal in the same way. After aqueous exposure Am was mainly associated with the head (64-69%), consistent with what has been observed in other studies (Jeffree et al. 2006, Mathews et al. 2008). Am is a Class A metal (Nieboer & Richardson 1980), with a strong binding preference to the mineral phase within organisms. The head could have the highest percentage because it is predominantly bone. Tissue partitioning after dietary exposure could not be determined due to near complete elimination of the radioisotope. After dietary exposure, Se crossed over the gut lining and was transported around the body, consistent with the findings of Baines et al. (2002) and Xu and Wang (2002). Zn tissue partitioning was split mainly between the head and body after an aqueous exposure, and primarily associated with the head after a dietary exposure. Our tissue distribution data is consistent with other studies looking at either aqueous or dietary uptake of Zn (Baines et al. 2002, Mathews et al. 2008). Zn is a borderline metal (Nieboer & Richardson 1980) with some association for both the mineral and protein phase, and therefore could be expected to bind to both bone in the head region and protein within body tissue.

Other studies describing Cd behavior in fish after dietary exposure indicate that tissue distribution varies by species. Both silverside populations used in our study had the highest percentage of Cd associated with the body (80-84%), consistent with observations in striped bass (Baines et al. 2002). Other studies have shown that Cd remains associated with the gut (Xu & Wang 2002, Mathews & Fisher 2008a). Why some fish are better protected from Cd uptake across the intestine is not fully understood. Studies using rainbow trout, *Oncorhynchus mykiss*, have shown that Cd shares the same uptake pathway as Ca^{2+} in the gills and intestinal tract, and a

diet enriched in Ca^{2+} can inhibit Cd uptake at these exposure sites by downregulating the Ca^{2+} uptake pathway in the gills and intestine (Franklin et al. 2005, Wood et al. 2006). However, we did not measure Ca^{2+} levels in our prey item to conclude whether this could have an influence in our study. Alternatively, due to their high growth rate silversides could rapidly assimilate Ca^{2+} to form their skeleton, allowing Cd to be taken up across the gut lining. However, tissue concentrations of Cd are highest in the body, followed by the viscera. The high concentration of Cd in the viscera combined with the high efflux rate indicates that some of the assimilated Cd is detoxified and excreted by the silversides after dietary exposure. After aqueous exposure, the highest percentage of Cd is associated with the viscera in Nova Scotia silversides, and the body in South Carolina silversides, but the highest Cd concentration is in the viscera for both populations. Other studies also noted a high percentage of Cd associated with the viscera in marine fish (Jeffree et al. 2006, Mathews et al. 2008), but this percentage decreases throughout depuration indicating a redistribution of Cd over time. Marine fish actively drink seawater to osmoregulate, and a study has shown that even though aqueous Cd accumulation decreases with an increase in salinity, the percentage body burden of Cd associated with the viscera increases with salinity, suggesting drinking is an uptake mechanism for Cd in marine fish (Dutton & Fisher, unpublished data). A higher percentage of Cd could be associated with the viscera in Nova Scotia silversides if they have a higher drinking rate, but this was not measured in our study.

Our results show that the two species of mercury we examined behaved very differently. At the end of depuration after aqueous exposure 49-55% of Hg(II) remained associated with the head, consistent with findings in mosquitofish and redear sunfish (Pickhardt et al. 2006), whereas 70-72% of MeHg was associated with the body, which is higher than noted previously (~55%,

Pickhardt et al. 2006). After dietary exposure 51-57% of MeHg body burden was associated with the body, consistent with that found in killifish (58%, Mathews & Fisher 2008a) and redear sunfish (55%, Pickhardt et al. 2006). MeHg has been shown to solubilize more readily than Hg(II) during digestion and be transported across the intestine wall through an amino acid transport pathway, where it is redistributed around the body via the blood (Leaner & Mason 2002, Leaner & Mason 2004), and bound to protein due to its strong affinity for sulfur. After dietary exposure, a greater percentage of Hg(II) was associated with the viscera in South Carolina silversides, whereas Nova Scotia silversides had a higher percentage associated with the body; reasons for this difference are not apparent. Pickhardt et al. (2006) also noted a difference in the percentage of Hg(II) associated with the viscera between different freshwater fish species. The long retention time of Hg(II) in the gut as noted in the South Carolina silverside population and other fish studies (Pickhardt et al. 2006) may allow some Hg(II) to become methylated by gut bacteria as seen in freshwater piscivorous fish (Rudd et al. 1980). This was not observed in our study, and the high South Carolina silverside Hg(II) k_{ef} (19% d^{-1}) suggests this is unlikely.

Metal bioaccumulation and biomagnification in Menidia menidia

When the calculated kinetic parameters from this study are entered into the biokinetic model, the predicted steady-state body burden (C_{ss}) is higher in the South Carolina population for Am, Cd, and Zn. The difference in predicted C_{ss} values for Am, Cd, and to a lesser extent Zn between the two populations can in part be attributed to higher AEs in South Carolina silversides. In Nova Scotia silversides, rapid growth to overcome the shorter growing season and size-selective winter mortality is attributed to a higher ingestion rate and growth efficiency

compared to South Carolina silversides (Conover & Present 1990, Present & Conover 1992). The higher growth rate may result in significant somatic growth dilution of accumulated metals, possibly reducing the metal concentration in the tissues. A field study focusing on the growth rate of Atlantic salmon has shown that Hg burdens are lower in faster growing fish, and this is attributed to somatic growth dilution (Ward et al. 2010). Many bioaccumulation studies do not consider growth rate in the biokinetic model as the value is usually small compared to the efflux rate (Luoma & Rainbow 2005). However, for juvenile Atlantic silversides the rapid growth rate must be included to predict accurate body burdens. For MeHg and Se the higher ingestion rate in Nova Scotia silversides is offset by the higher growth rate, resulting in comparable C_{ss} values to those in South Carolina silversides. Nova Scotia silversides have a higher Hg(II) body burden, which is attributed to a higher Hg(II) k_{ef} in the South Carolina population. Our C_{ss} values for each metal are comparable to the findings of other studies (Baines et al. 2002, Pickhardt et al. 2006, Mathews & Fisher 2009).

For Cd, Hg(II), MeHg, and Zn the diet contributed to > 96 % of the metal body burden. This is consistent with the results of other studies using fish fed zooplankton and fish prey (Xu & Wang 2002, Pickhardt et al. 2006, Mathews & Fisher 2009) for Cd, MeHg, and Zn. However, Pickhardt et al. (2006) noted that diet contributed 73-88% of the Hg(II) burden in mosquitofish, and 40-55% in redear sunfish, significantly lower than our calculated values. Differences between the two studies can be traced to the much higher k_u values for Hg(II) observed for mosquitofish and redear sunfish than we found for Atlantic silversides. Due to the low particle reactivity of selenite in the dissolved phase, we predict 100% of Se is accumulated from the diet, consistent with other findings (Xu & Wang 2002, Stewart et al. 2010). Am was the only metal where the aqueous phase had a large influence on steady-state body burden due to its very low

AE, as also noted for other fish species (Mathews & Fisher 2009). Water quality criteria need to recognize that dietary uptake is the dominant exposure route for most metals in marine fish.

The Atlantic silverside is not a commercially harvested fish, but it represents an important link between lower trophic levels and its predators. Studies have shown that juvenile silversides primarily feed on copepods (Adams 1976, Gilmurray & Daborn 1981), and are preyed upon by piscivorous fish and sharks (Hartman & Brandt 1995, Rountree & Able 1996, Buckel et al. 1999). Of the six metals examined in both silverside populations, Am, Hg(II), and Se are not expected to biomagnify (TTFs < 1) due to relatively low assimilation and high elimination rates (resulting in a short biological half-life), consistent with the findings of Xu & Wang 2002, Wang & Wong 2003, Mathews & Fisher 2008a,b, Mathews et al. 2008). MeHg is clearly expected to biomagnify (TTF > 1), due to high assimilation and low elimination, as noted in other estuarine and marine fish (Wang & Wong 2003, Pickhardt et al. 2006, Mathews & Fisher 2008a). Zn is shown to biomagnify slightly in both populations, and Cd in the smaller South Carolina fish. Some studies have shown that Zn is not expected to biomagnify (Mathews & Fisher 2008b, Mathews et al. 2008), while others have predicted Zn will if the ingestion rate and assimilation efficiency are high enough (Xu & Wang 2002, Mathews et al. 2008). TTF could also be a function of body size; Zhang & Wang (2007) noted that as body size increased the TTF of Zn decreased. Cd has been found to biomagnify in freshwater fish (Croteau et al. 2005), but not in estuarine and marine fish (Xu & Wang 2003, Mathews & Fisher 2008a,b). Apart from Hg(II), TTFs are slightly higher in South Carolina silversides, an observation we attribute to higher AEs in South Carolina silversides (except MeHg) and a higher growth rate in Nova Scotia silversides resulting in somatic growth dilution in juvenile *M. menidia*.

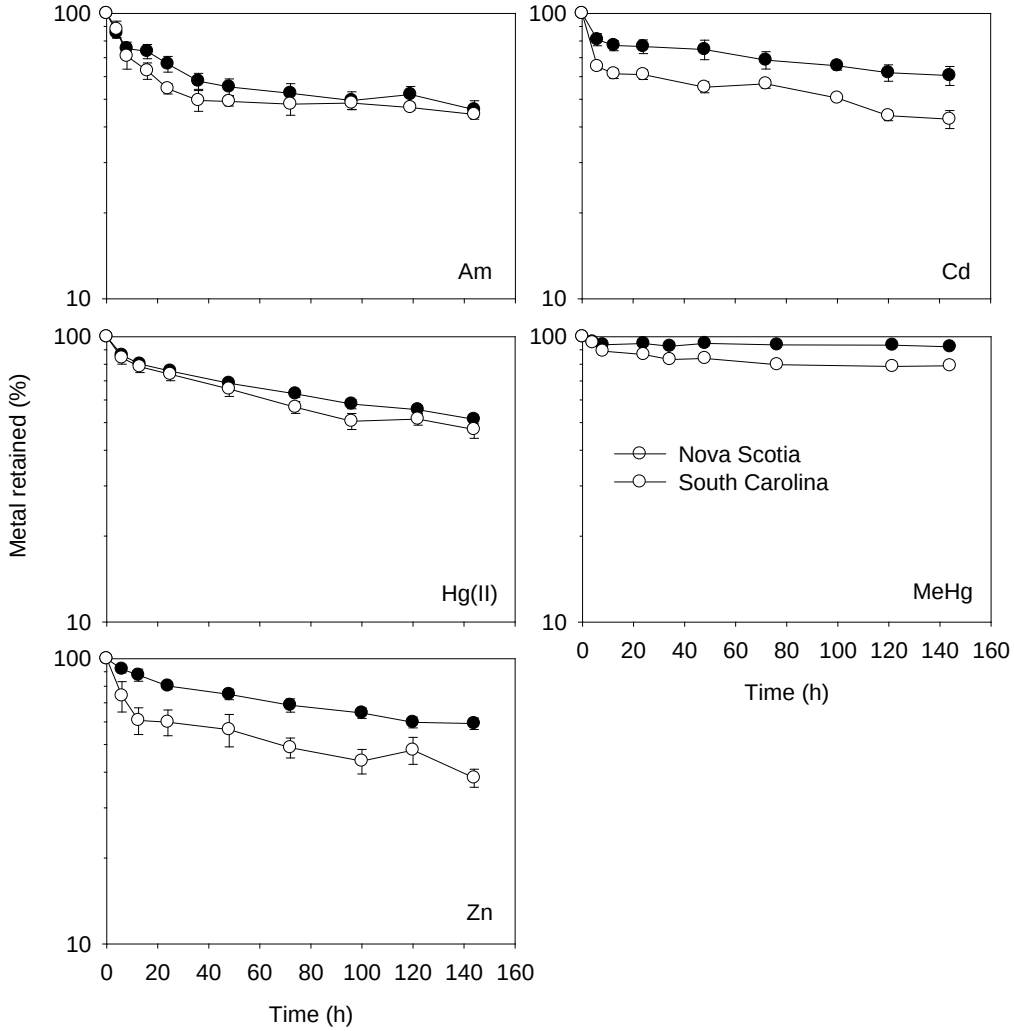

Fig. 1. Metal retention in Atlantic silversides (*Menidia menidia*) from Nova Scotia and South Carolina after aqueous exposure in Southampton seawater (n = 4-9 per population). Retention is expressed as percent of initial body burden after exposure. Values represent means ± 1 SE.

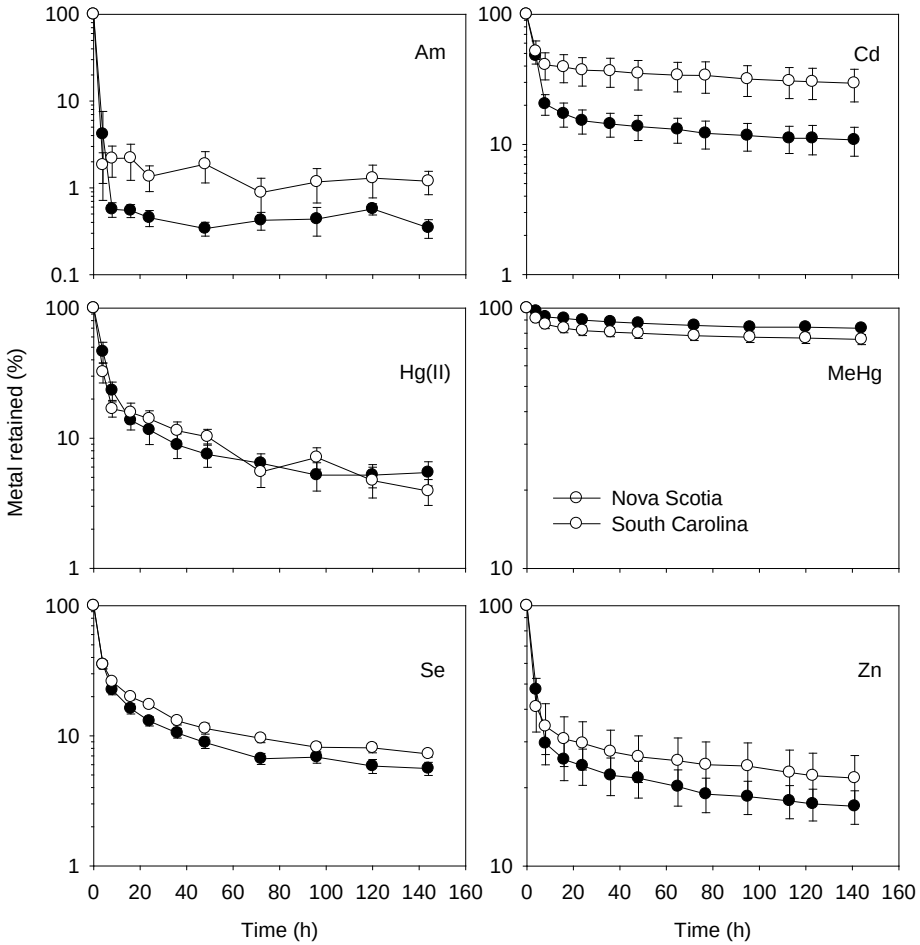

Fig. 2. Metal retention in Atlantic silversides (*Menidia menidia*) from Nova Scotia and South Carolina after pulse feeding on brine shrimp (*Artemia franciscana*) nauplii (n = 5-8 per population). Retention is expressed as percent of initial body burden after exposure. Values represent means ± 1 SE.

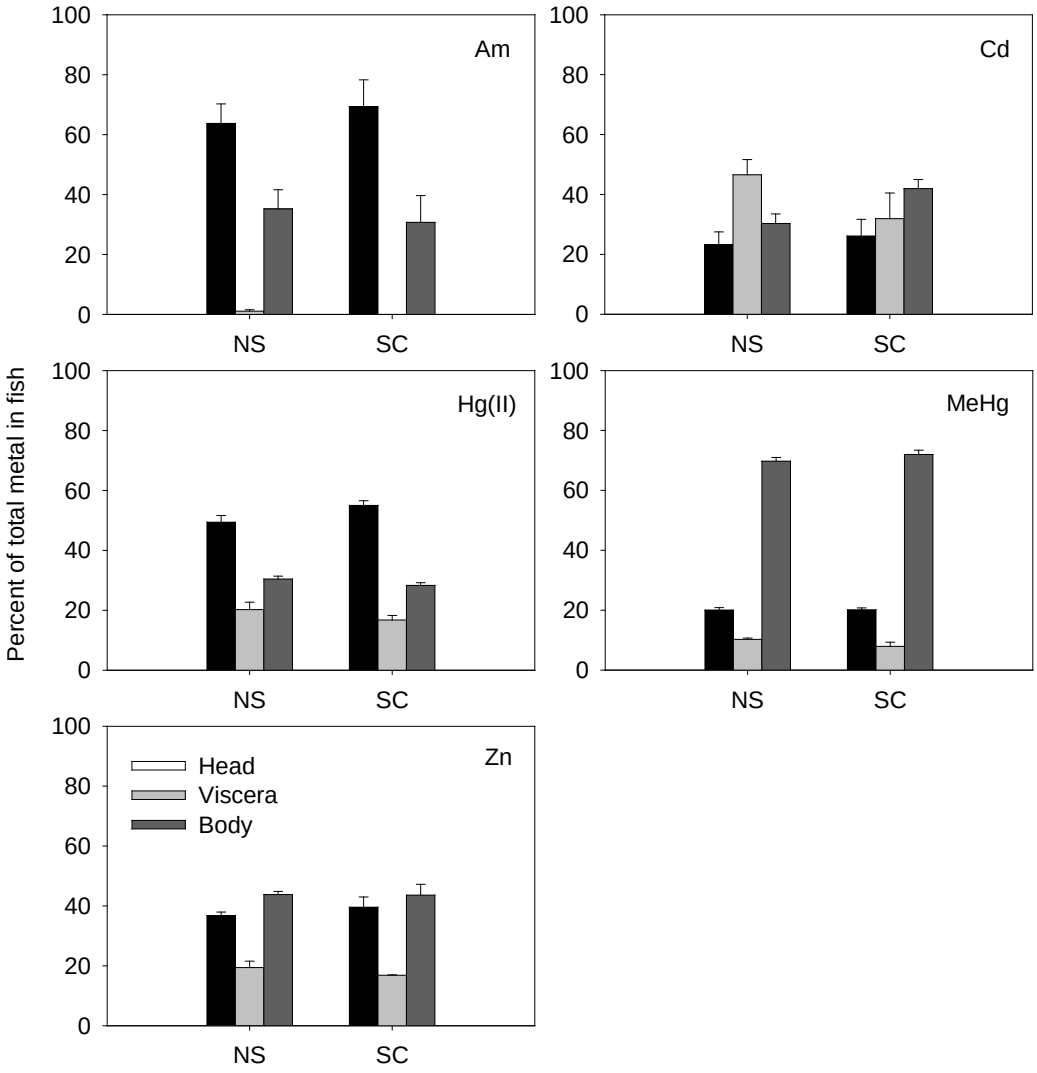

Fig. 3. Metal partitioning in Atlantic silversides (*Menidia menidia*) from Nova Scotia (NS) and South Carolina (SC) at the end of depuration after aqueous exposure in Southampton seawater. Bars represent the percentage of total body burden associated with each tissue compartment (head, viscera, body). Values represent means ± 1 SE; n = 4-9.

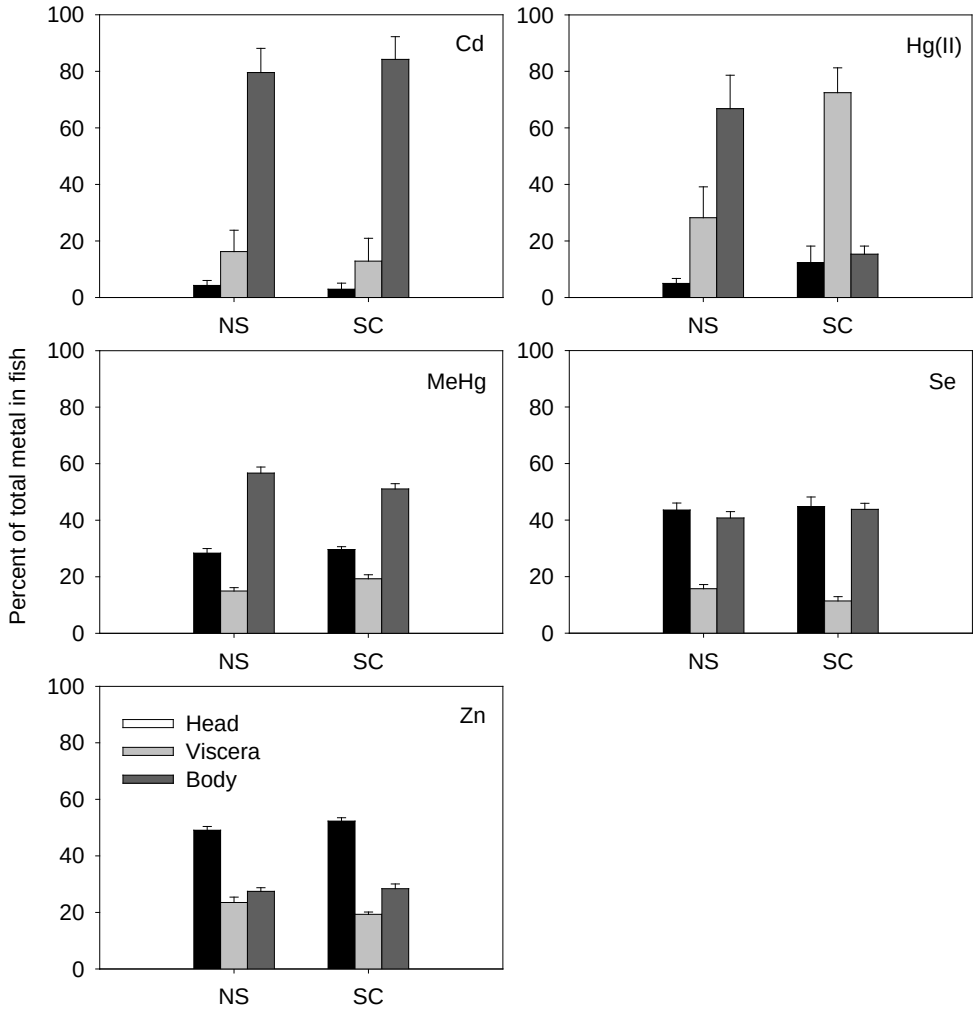

Fig. 4. Metal partitioning in Atlantic silversides (*Menidia menidia*) from Nova Scotia (NS) and South Carolina (SC) at the end of depuration after dietary exposure. Bars represent the percentage of total body burden associated with each tissue compartment (head, viscera, body). Values represent means ± 1 SE; n = 5-8. Tissue distribution for Am could not be determined because its concentration was below detection at the end of depuration.

Table 1. Uptake rate constants (k_u) and efflux rate constants (k_{ew}) in Atlantic silversides (*Menidia menidia*) from Nova Scotia (NS) and South Carolina (SC) after aqueous exposure in Southampton seawater. Statistically significant differences (by t-test) between each kinetic parameter and the two populations are represented by * ($p < 0.05$) and ** ($p < 0.01$). n = 4-9 per population; nd: not determined.

		k_u (ml g^{-1} d^{-1})			k_{ew} (d^{-1})		
	Population	Mean	SE	Range	Mean	SE	Range
Am	NS	11	1	7-17	0.038	0.008	0.016-0.065
	SC	13	1	11-15	0.023	0.013	0.003-0.060
Cd	NS	0.8**	0.04	0.6-0.9	0.052	0.004	0.039-0.063
	SC	1.3**	0.1	1.0-1.8	0.076	0.015	0.034-0.124
Hg(II)	NS	15	2	7-25	0.071	0.004	0.056-0.094
	SC	17	2	14-22	0.075	0.009	0.057-0.099
MeHg	NS	1155**	289	361-2631	0.006*	0.002	0.002-0.014
	SC	4375**	501	2653-5761	0.014*	0.002	0.005-0.018
Se	NS	nd	nd	nd	nd	nd	nd
	SC	nd	nd	nd	nd	nd	nd
Zn	NS	1.9**	0.1	1.6-2.1	0.061	0.005	0.047-0.071
	SC	5.0**	0.8	3.2-7.1	0.056	0.015	0.018-0.101

Table 2. Assimilation efficiencies (AE) and efflux rate constants (k_{ef}) in Atlantic silversides (*Menidia menidia*) from Nova Scotia (NS) and South Carolina (SC) after feeding on brine shrimp nauplii (*Artemia franciscana*). Statistically significant differences (by t-test) between kinetic parameters and the two populations are represented by * ($p < 0.05$). n = 5-8 per population; nd: not determined

		AE (%)			k_{ef} (d^{-1})		
	Population	Mean	SE	Range	Mean	SE	Range
Am	NS	0.3*	0.06	0.13-0.7	nd	nd	nd
	SC	1.9*	0.70	0.21-5.0	nd	nd	nd
Cd	NS	15*	3	5-28	0.073	0.017	0.043-0.155
	SC	39*	10	7-54	0.052	0.009	0.026-0.092
Hg(II)	NS	8	2	3.2-13	0.086	0.014	0.040-0.139
	SC	15	3	7.6-26	0.194	0.055	0.038-0.455
MeHg	NS	89*	0.8	86-92	0.011	0.001	0.007-0.013
	SC	82*	4	68-94	0.013	0.002	0.006-0.017
Se	NS	10	0.8	8-14	0.109	0.008	0.081-0.140
	SC	13	1.5	9-18	0.105	0.017	0.064-0.164
Zn	NS	24	4	11-39	0.058	0.008	0.041-0.083
	SC	29	6	15-61	0.051	0.003	0.039-0.062

Table 3. Radioactivity concentration (Bq g^{-1}) of metals in fish tissues (head, viscera, and body) after aqueous and dietary exposure. NS = Nova Scotia, SC = South Carolina. Values represent means ± 1 SE; nd: not determined. n = 4-9 per population for aqueous exposure and 5-8 per population for dietary exposure.

		Aqueous exposure			Dietary exposure		
	Population	Head	Viscera	Body	Head	Viscera	Body
Am	NS	144 ± 12	17 ± 8	37 ± 10	nd	nd	nd
	SC	114 ± 22	0 ± 0	23 ± 7	nd	nd	nd
Cd	NS	33 ± 7	128 ± 24	12 ± 1	116 ± 39	1372 ± 601	2537 ± 640
	SC	75 ± 47	342 ± 211	22 ± 6	303 ± 124	9842 ± 6856	14338 ± 3812
Hg(II)	NS	113 ± 8	144 ± 16	26 ± 3	8 ± 3	97 ± 48	89 ± 17
	SC	113 ± 9	174 ± 25	22 ± 1	8 ± 3	146 ± 80	8 ± 1
MeHg	NS	948 ± 173	817 ± 152	941 ± 204	3184 ± 920	6169 ± 1701	4799 ± 1528
	SC	2803 ± 280	4733 ± 1217	3318 ± 306	4173 ± 637	9728 ± 4217	4982 ± 829
Se	NS	nd	nd	nd	936 ± 78	2192 ± 478	474 ± 51
	SC	nd	nd	nd	1030 ± 75	1470 ± 336	554 ± 52
Zn	NS	219 ± 26	219 ± 30	73 ± 4	4303 ± 451	5112 ± 1266	1892 ± 291
	SC	612 ± 58	1718 ± 1201	189 ± 35	8255 ± 1665	6986 ± 1189	3006 ± 394

Table 4. Biological half-lives (tb½; d) of metals in Nova Scotia (NS) and South Carolina (SC) populations of the Atlantic silverside (*Menidia menidia*) after aqueous and dietary exposure. k_e values used are in Tables 1 and 2. nd: not determined.

	Population	Aqueous tb½	Dietary tb½
Am	NS	18	17[a]
	SC	30	17[a]
Cd	NS	13	9
	SC	9	13
Hg(II)	NS	10	8
	SC	9	4
MeHg	NS	116	63
	SC	50	53
Se	NS	nd	6
	SC	nd	7
Zn	NS	11	12
	SC	12	14

[a] we used a k_{ef} of 0.04 d^{-1}, calculated for juvenile striped bass (Baines et al. 2002), due to near complete elimination of the radioisotope in our experiments.

Table 5. Model-predicted metal body burdens at steady-state (C_{ss}), percent of body burden attributed to dietary exposure, and trophic transfer factors (TTFs) for Nova Scotia (NS) and South Carolina (SC) Atlantic silverside (*Menidia menidia*) populations. Kinetic parameters used are in Tables 1 and 2. C_{ss} units are µg g^{-1} (Cd, MeHg, Se, Zn) and ng g^{-1} (Am, Hg(II)).

	Population	Css	% attributed to diet	TTF
Am	NS	3.6×10^{-10}	38.3	0.02[a]
	SC	1.4×10^{-9}	61.1	0.11[a]
Cd	NS	1.7	> 99.9	0.67
	SC	4.9	> 99.9	1.95
Hg(II)	NS	18	97.9	0.32
	SC	15	96.1	0.26
MeHg	NS	1.3	> 99.9	7.6
	SC	1.4	> 99.7	8.7
Se	NS	0.45	100	0.35
	SC	0.49	100	0.38
Zn	NS	202	> 99.9	1.2
	SC	246	> 99.8	1.5

[a] we used a k_{ef} of 0.04 d^{-1}, calculated for juvenile striped bass (Baines et al. 2002), due to near complete elimination of the radioisotope in our experiments.

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Metal (As, Cd, Hg, and CH_3Hg) Bioaccumulation from Water and Food by the Benthic Amphipod *Leptocheirus plumulosus*

Abstract–Benthic invertebrates may be exposed to metals in porewater, overlying water, ingested sediments, and other food particles. Rates and routes of metal exposure have important implications for predicting toxicity and interpreting toxicity test results. For the standard test amphipod *Leptocheirus plumulosus*, radiotracer techniques were used to quantify rates of Cd, As, Hg(II), and CH_3Hg bioaccumulation from water and from suspension-feeding on labeled microalgae. Measured parameters were incorporated into a bioaccumulation model to predict steady-state metal concentrations in *L. plumulosus* and to evaluate the relative importance of aqueous and dietary uptake pathways across a range of ingested particle types and ingestion rates. Results indicate that ingested particles contribute strongly to metal bioaccumulation, and that feeding plasticity could strongly influence metal exposure. As *L. plumulosus* switches from suspension-feeding to deposit feeding or selectively feeds on particles for which it has a high assimilation efficiency, metal exposure and body burden will increase. At ingestion rates previously reported for deposit feeding (3 g/g/d), dietary metal sources dominate metal bioaccumulation and can be responsible for greater than 90% of metal bioaccumulated, regardless of metal partitioning or ingested particle type. Theses results suggest that more research on *L. plumulosus* feeding behavior is needed to produce a more complete mechanistic understanding of metal bioaccumulation.

INTRODUCTION

Estuarine benthic invertebrates can accumulate toxic metals from dissolved and particulate sources [1]. Dissolved phase exposure occurs through direct adsorption of metals dissolved in porewater, burrow water or overlying water, whereas particulate phase exposure occurs through metals associated with ingested food or sediments. The contribution of each exposure route to bioaccumulation may be affected by sediment-porewater metal partitioning [2], metal bioavailability [2], food quality [3], and feeding behavior [2,4]. For organisms used in standard sediment toxicity and bioaccumulation tests, delineating exposure pathways and their relative importance is critical for predicting metal bioaccumulation [5] and interpreting toxicity test results [2]. Quantifying how factors such as metal partitioning and feeding behavior affect rates and routes of metal exposure can help provide a mechanistic understanding of metal bioaccumulation in test organisms.

The estuarine benthic amphipod *Leptocheirus plumulosus* is a standard test animal used in acute [6] and chronic [7] sediment toxicity tests. In addition, *L. plumulosus* is widely distributed throughout the eastern United States [8], can reach densities greater than 2.5×10^4 individuals/m^2 [8-9] and may be an important seasonal prey item for benthic-feeding organisms. Variation in exposure route and feeding behavior may be especially important to *L. plumulosus* metal bioaccumulation. *Leptocheirus plumulosus* selectively feeds on high quality food items such as algae [4]. Moreover, in whole-sediment toxicity tests, feeding amphipods a combination of *Isochrysis galbana* and TetraMin® (Tetrawerke, Melle, Germany) flakes, rather than TetraMin flakes alone, increases the probability of classifying test sediment as non-toxic [10]. In estuaries, *L. plumulosus* secondary production is correlated with sedimenting chlorophyll a concentrations [11], suggesting *L. plumulosus* can feed on pelagic food sources that may not be

in equilibrium with sediments. In addition, *L. plumulosus* can ingest food through either suspension feeding or deposit feeding. Ingestion rates for deposit feeding have been reported at 3 g/g/d [12], but ingestion rates for suspension feeding have not previously been reported. If ingestion rates vary substantially between feeding modes, then metal exposure, bioaccumulation, and toxicity may vary as well.

The present study investigates bioaccumulation of four non-essential metals which are common in contaminated estuarine sediments, but have different biogeochemistries and affinities for protein [13]: Cd, As(V), Hg(II), and CH_3Hg. As(V), the dominant form of As in estuarine systems [14] is anionic, whereas Cd, Hg(II) , and CH_3Hg are particle reactive, complex with chloride in seawater, and are cationic [15-16]. For Hg(II) and especially CH_3Hg dietary bioaccumulation is important and can affect metal biomagnification [4]. Radiotracer techniques are used to quantify *L. plumulosus* uptake and efflux rates for dissolved Cd, As, Hg(II) and CH_3Hg, and metal assimilation efficiencies and efflux rates for suspension-feeding on metal-enriched algae. Measured parameters are then used in a biokinetic model to predict routes of metal exposure and metal body burden for each metal across a realistic range of water concentrations, particle types, ingestion rates, and water-particle K_d values. Results are discussed in terms of implications for *L. plumulosus* metal bioaccumulation in toxicity tests and in nature.

METHODS

General

Radiotracer methods were used to describe *L. plumulosus* metal bioaccumulation from water and from suspension-feeding on the prymnesiophyte *Isochrysis galbana*. Separate experiments were conducted for each metal (Cd, As, Hg(II), and CH_3Hg) and uptake pathway. Suspension feeding

on *I. galbana* was evaluated because this approach has been used successfully previously [3], and because preliminary experiments where *L. plumulosus* were allowed to burrow into sediment containing algae proved infeasible. Adult *L. plumulosus* (>3 mm) were obtained from a commercial supplier and were held in 20 ppt Instant Ocean® (Aquarium Systems, Mentor, OH) without food overnight prior to use in experiments. Each exposure used five replicates (n=5) and followed the same general protocol. In each exposure replicate, ten *L. plumulosus* were placed in 100 ml of radiolabeled filtered water for a 13 (Cd), 24 (Hg(II), CH_3Hg), or 48 h (As) uptake period or placed in an algal suspension for a 45 min uptake period. After uptake, all ten amphipods were pipetted out of their radiolabeled liquid, thoroughly rinsed with unlabeled water, pipetted into a plastic container with 30 ml of unlabeled water, and counted for radioactivity. The amphipods were then pipetted into clean, unlabeled water for a 72 to 96 h depuration period, during which they were placed in an 18 ºC incubation chamber with a 14:10 light:dark cycle. For each replicate, all ten amphipods were periodically removed from their depuration water, pipetted into a plastic container together with 30 ml of unlabeled water, and assayed for radioactivity. During uptake and depuration, amphipods were held in glass containers for Hg(II) and CH_3Hg experiments, and in polycarbonate containers for Cd and As experiments. Past research, has indicated that mercury, unlike Cd and As, absorbs into plastic, but sorption to glass containers is typically minimal. At the end of each exposure, amphipods were dried at 60ºC for at least 12 h to obtain dry weights for each replicate. Dry weight comparisons indicated amphipods used in the CH_3Hg dietary uptake exposure weighed significantly more than amphipods used in the Cd aqueous exposure (p<0.05) but no other significant differences were evident.

Sterile 0.2 μm filtered 20 ppt seawater collected from 1m depth in the Elizabeth River (Norfolk, VA USA) (384 μM dissolved organic carbon (DOC)) was used during radioisotope uptake and depuration (hereafter: experimental water). Stocks of ^{109}Cd ($t_{1/2}$ = 462.3 d) and ^{73}As (As (V), $t_{1/2}$ = 80.3 d) were obtained from the U.S. Department of Energy (Los Alamos National Laboratory, Los Alamos, NM). ^{203}Hg(II) ($t_{1/2}$ = 46.6 d) was obtained from Georgia State University (Atlanta, GA, USA) and was methylated [17-19] for use in CH_3Hg bioaccumulation experiments. Because all radioisotope stocks were in HCl, a small volume of 1M sodium hydroxide was added along with stock to prevent changes in experimental water pH during radiolabeling.

Uptake from the dissolved phase

For aqueous exposures, experimental water was injected with radioisotope and allowed to equilibrate for 12 h. Specific activities of radioisotope stocks were 0.52 kBq/μg (^{109}Cd), 1.72 kBq/μg (^{73}As), 0.03 kBq/μg (^{203}Hg(II)) and 0.09 kBq/μg ($CH_3{}^{203}Hg$). Metal concentrations and radioisotope activities in experimental water were: 1 nM and 77.74 kBq/L (^{109}Cd), 0.5 nM and 86.30 kBq/L (^{73}As), 1.69 nM and 14.06 kBq/L (^{203}Hg(II)), and 0.54 nM and 3.195 kBq/L ($CH_3{}^{203}Hg$). During uptake, amphipods were placed in radiolabeled water for 13 (Cd), 24 (Hg(II), CH_3Hg), or 48 h (As). These time periods were chosen in order to produce animals sufficiently well-labeled for depuration measurements while minimizing the potential for efflux during the uptake period. One ml samples of water from each replicate were taken at the beginning and end of uptake and assayed for radioactivity. During depuration, amphipods were assayed for radioactivity at 0, 4, 12, 24, 48, and 72 h, and $3x10^6$ unlabeled *I. galbana* cells were added to each depuration container each day to prevent starvation.

Uptake from Isochrysis galbana

To generate radiolabeled algae, *I. galbana* from a culture in late exponential growth phase was added to radiolabeled culture media to create 500 ml of algae at roughly $5x10^5$ cells/ml. For ^{109}Cd, $^{203}Hg(II)$, and $CH_3{}^{203}Hg$, culture media was f/2 [20] without Zn, Cu, or ethylenediaminetetraacetic acid (EDTA). For ^{73}As, culture media also contained f/200 phosphate to avoid preferential uptake of phosphate over ^{73}As [21]. Specific activities of radioisotope stocks used for spiking were 0.46 kBq/μg (^{109}Cd), 0.878 kBq/μg (^{73}As), 0.07 kBq/μg ($^{203}Hg(II)$) and 0.09 kBq/μg ($CH_3{}^{203}Hg$). After combining algae and radiolabeled water, metal concentrations and activities in the media were: 0.54 nM and 37.31 kBq/L (^{109}Cd), 1.71 nM and 148.80 kBq/L and (^{73}As), 0.73 nM and 14.05 kBq/L ($^{203}Hg(II)$), and 0.68 nM and 16.61 kBq/L ($CH_3{}^{203}Hg$). After a 4 d incubation period, algae were collected on a 0.2 μm polycarbonate filter and resuspended into 500 ml of 20 ppt seawater. *Isochrysis galbana* cell densities (cells/ml) after incubation and resuspension were 6.64 $x10^5$ for Hg(II) and Cd, 5.02 $x10^5$ for As, and 8.65 $x10^5$ for CH_3Hg. The fraction of added radioactive metal associated with filtered algae was 22% for Cd, 17% for As, 72% for Hg(II) , and 65% for CH_3Hg.

Amphipods used in experiments were starved overnight prior to use in pulse feeding. Based on observations of a 65 to 90 min gut passage time for *L. plumulosus* feeding on *I. galbana* at 10^5 cells/ml [22], a 45 min feeding time was used. After uptake, amphipods were pipetted into 100 ml of unlabeled *I. galbana* at the same density for depuration. Depuration algae were changed every 24 h to keep the density of *I. galbana* approximately constant. During depuration, amphipod radioactivity was assayed at 0, 45 min, 2 h, 4 h, 12 h, 24 h, 48 h, 72 h, and 96 h. Amphipod feces were collected by pipette at each depuration time point and counted for radioactivity separately from amphipods.

Modeling

To evaluate the relative importance of aqueous and dietary uptake pathways for metal bioaccumulation, parameters measured during radiotracer experiments were incorporated into a biokinetic model [1,5]. Steady-state metal concentration in an organism (C_{ss}) can be calculated using the first-order equation:

$$C_{ss} = (k_u \cdot C_w) / (k_{ew} + g) + (AE \cdot IR \cdot C_f) /(k_{ef} + g) \qquad (1)$$

where k_u is the metal uptake rate constant from the dissolved phase (L/g /d), C_w is the metal concentration in water (μg/L), k_{ew} is the metal efflux rate constant following uptake from the dissolved phase (1/d), g is the animal's growth rate constant (1/d), AE is the metal assimilation efficiency from ingested particles, IR is the animal's ingestion rate (mg/g/d), C_f is the metal concentration in ingested particles (μg/mg), calculated as $k_{dalgae} \cdot C_w$, and k_{ef} is the metal efflux rate constant following uptake from food (1/d). The fraction of metal uptake from food (R_f) and water (R_w) can be calculated as:

$$R_f = [(AE \cdot IR \cdot C_f) /(k_{ef} + g)] / C_{ss} \qquad (2)$$

$$R_w = [(k_u \cdot C_w) / (k_{ew} + g)] / C_{ss} \qquad (3)$$

The k_{ew} values were calculated from metal retention data in amphipods between 12 to 72 h of the depuration period. To calculate AE and k_{ef}, the % metal retained was plotted against depuration time during the second compartment of depuration (24-96 h) and an exponential regression line was fitted to the data. Assimilation efficiency was calculated as the *y*-intercept and k_{ef} was calculated as the regression line slope [23]. Measured growth rate constants for *L. plumulosus* range from 0.04 to 0.1 /d [24], so a growth rate constant of 0.07/d was used here.

Ingestion rates were calculated as IR = I_f / (AE · C_f), where I_f is the metal influx, calculated using the g of metal in amphipods after 45 min feeding and C_f is the concentration of metal in spiked, filtered and resuspended algae. To calculate C_f, water concentrations were multiplied by k_{dalgae} that was calculated as:

($P_{radioactivity}$) / (*I. galbana* cells/mL · *I. galbana* g /cell) (4)

where $P_{radioactivity}$ is the proportion of radioactivity in filtered cells / the proportion of radioactivity in water after filtering, and *I. galbana* weighs 16×10^{-12} g/cell [25].

Using equations 1 to 3, the effect of variation in four different parameters on R_f and C_{ss} was modeled. All simulations conducted here assume metal bioavailability from water is the same as that observed for experimental water used here. The R_f may be greater than predicted here in estuarine waters with higher salinities (for Cd) or DOC concentrations (for Hg(II) and CH_3Hg), where dissolved metals are less bioavailable.

Variation in AE and IR. The effect of variation in AE and IR was examined by parameterizing Equation 1 one using a range of AE values, and either the suspension feeding IR value measured here or the 3/g/g/d IR reported by Schlekat et al. [12] for deposit feeding, and then calculating R_f values. For Cd, AE was parameterized using measurements available in the literature for a range of algal and sediment particle types (2.9-35.8%) [3,12,22]. For As, Hg(II) and CH_3Hg, it was assumed that variation in AE is similar to that reported for Cd. Specifically, it was assumed that AE varied up to 10% above and 20% below the value measured here. Variation in Cd AE may not be representative of other metals, but to our knowledge no other As, Hg(II), or CH_3Hg AE measurements are available for marine amphipods. For modeling C_w values were assumed to be the same as used in experiments here for Cd (1 nM) and As (0.5 nM). These concentrations

represent the low end of realistic porewater concentrations. For Hg(II) and especially CH_3Hg, the water concentrations used in experiments (Hg(II) : 1.69 nM , CH_3Hg: 0.5 nM) are higher than in most natural waters due to the specific activity of the radioisotopes available. Therefore, modeling used maximum porewater concentrations measured in a New England estuary (Kittery, ME, USA; Chen et al., unpublished data): 0.2 nM (Hg(II)) and 0.046 nM (CH_3Hg). The K_d values measured here for *I. galbana* were assumed constant across variation in AE associated with particle type. As a result, C_f values ($C_w \cdot k_d$) were assumed to be 3.19 μg/g (Cd), 0.79 μg/g (As), 9.68 μg/g (Hg(II)), and 1.34 μg/g (CH_3Hg) for all particle types. Thus, this modeling exercise estimates the effect of realistic variation in *L. plumulosus* AE and IR on exposure pathways assuming that all available particle types have the same metal concentration and seawater has a 20 ppt salinity.

Variation in particle K_d. To examine effects of variation in food concentration or sediment-porewater metal partitioning (when sediment is the primary food source), R_f values were calculated for a range of K_d values (10^4-10^6). The same C_w values as in the AE and IR simulation were used, and it was assumed that other parameter values in Table 1 are unaffected by variation in K_d. Across variation in K_d, R_f values were calculated using the IR measured here for suspension feeding and the 3 g/g/d previously reported for deposit feeding [12].

Radioactivity measurements and calculations

A Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector was used to quantify radioactivity in live amphipods (5 min counts to obtain a propagated counting error ≤ 5%). A LKB Pharmacia-Wallac 1282 CompuGamma CS gamma counter (Turku, Finland) was used to quantify radioactivity in water as well as in amphipod feces. γ emissions were measured at 22

keV for ^{109}Cd (X-rays from ^{109}Cd's daughter ^{109}Ag), 53 keV for ^{73}As, and 279 keV for ^{203}Hg(II) and $CH_3{}^{203}$Hg. All radioactivity measurements were blank adjusted and decay corrected.

RESULTS

Uptake from the dissolved phase

Biokinetic parameters measured for *L. plumulosus* are presented in Table 1 and depuration curves are presented in Figure 1. Mean metal influx rate constants from the dissolved phase (L/g/d) were 0.012 (Cd), 0.028 (As), 1.14 (Hg(II)), and 1.59 (CH_3Hg). During depuration, amphipods showed an initial rapid loss within the first 4 h, followed by a slower loss rate through the remaining 68 h for all metals (Fig. 1). Mean metal efflux rate constants (1/d) were 3.0×10^{-5} (CH_3Hg), 0.0053 (Hg(II)), 0.021 (Cd), and 0.091 (As), (Table 1).

Uptake from I. galbana

When suspension-feeding on *I. galbana*, the mean AE (%) in *L. plumulosus* was 6 (Hg(II)), 11 (As), 24 (Cd), and 80 (CH_3Hg) (Table 1). Efflux followed a two-compartment pattern, an initial rapid loss (0-24 h), due primarily to egestion of unassimilated metals in feces, followed by a slower loss rate (24-96 h) reflecting physiological turnover of assimilated metal (Fig. 1). After 24 h of depuration, the radioactivity of collected feces could not be detected for all metals. Measured efflux rate constants (1/d) were 0.052 (CH_3Hg) 0.089 (Hg(II)) 0.31 (Cd) and 0.58 (As). Mean calculated ingestion rates (mg/g/d) were 49.4 (Cd), 104 (Hg(II)), 140 (As), and 151 (CH_3Hg), respectively, somewhat lower than the range (150-250 mg/g/d) reported for the estuarine amphipod *Melita plumulosa* [26]. Variation in algal cell densities and amphipod weights between exposures were small and did not appear related to differences in ingestion rates

between metals. The distribution of metal in algal cells may differ for each metal, which may affect *L. plumulosus* AE and therefore IR calculations.

Modeling

Calculated k_{dalgae} values were 2.65×10^4 (Cd), 2.65×10^4 (As), 2.42×10^5 (Hg(II)), and 1.35×10^5 (CH_3Hg). Using water concentrations and biokinetic parameter values measured experimentally, predicted R_f values were 0.86 (Cd), 0.77 (As), 0.39 (Hg(II)), and 0.85 (CH_3Hg) for suspension feeding, and were 0.99 (Cd), 0.99 (As), 0.94 (Hg(II)), and 0.99 (CH_3Hg) at a 3 g/g/d IR.

Variation in AE and IR. For Cd, calculated R_f values associated with suspension feeding ranged from 0.43 to 0.90 across the simulated range of AE values. At a 3 g/g/d IR [12], variation in AE was overwhelmed by high ingestion rates and the dietary bioaccumulation pathway dominates, with R_f values ranging from 0.989 to 0.998 (Fig. 2). For As and Hg(II) , a similar pattern was observed; change in R_f associated with variation in AE was damped for deposit feeding compared to suspension feeding (Fig. 3). In contrast, for CH_3Hg, R_f was >80% over the simulated AE range for both IR values. At suspension feeding ingestion rates, predicted C_{ss} (μg/g) values were 0.03 to 0.163 (Cd), 0.007 to 0.039 (As), 0.669 to 1.69 (Hg(II)) and 1.00 to 1.72 (CH_3Hg). At deposit feeding ingestion rates, predicted C_{ss} (μg/g) values were 1.3 to 6.7 (Cd), 0.04 to 0.70 (As), 2.43 to 29.82 (Hg(II)) and 19.99 to 29.88 (CH_3Hg).

Variation in K_d. For Cd, R_f varied between 0.7 and 1 at the suspension feeding IR, but remained fixed above 0.99 at a 3 g/g/d IR when K_d varied between 10^4 and 10^6. A similar pattern was observed for As (Fig. 2) but for Hg(II) R_f variation was greater. For CH_3Hg, R_f showed substantial variation at suspension feeding rates, but R_f values were consistently above 0.89 at deposit feeding ingestion rates, due to the much higher AE and low K_{ef} measured for CH_3Hg.

The C_{ss} values increased linearly with K_d. At suspension-feeding ingestion rates, C_{ss} (μg/g) varied from 0.05-3.50 (Cd), 0.015-0.85 (As), 0.62-2.18 (Hg(II)), and 0.32-10.19 (CH_3Hg). At deposit feeding ingestion rates, C_{ss} (μg/g) varied from 2.12 to 211.2 (Cd), 0.18 to 18.21 (As), 1.06 to 46.19 (Hg(II)), and 2.20 to 198 (CH_3Hg).

DISCUSSION

In test sediments and in situ, *L. plumulosus* metal exposure and bioaccumulation will be affected by several important factors: metal partitioning between aqueous and particulate phases, which affects metal concentrations in water and in ingested particles, biogeochemistry affecting dissolved metal bioavailability (e.g. porewater salinity and dissolved organic carbon, sediment total organic carbon and sulfides), variation in *L. plumulosus* AE associated with different ingested particle types, and variation in *L. plumulosus* IR associated with different feeding modes. These factors will also affect the relative importance of aqueous and dietary bioaccumulation pathways. Here, R_f values calculated across a realistic range of assimilation efficiencies and particle K_d values suggest that diet contributes substantially to and, under some conditions, dominates metal bioaccumulation (Figs. 2,3). In addition, these results highlight the importance of *L. plumulosus* feeding behavior. At the 3 g/g/d IR reported for deposit feeding [13], ingested particles were responsible for over 80% of predicted metal bioaccumulation for all metals across all simulated conditions, except for Hg(II), where ingested particles contributed at least 40% (Figs. 2,3). This suggests variation in feeding mode could substantially affect the relative importance of exposure pathways and metal body burdens.

For Cd, similar patterns have been observed for the estuarine amphipod *Melitia plumulosa* [26]. Biokinetic modeling suggested that at 32 ppt salinity, aqueous Cd may only

account for up to 30% of Cd bioaccumulated by *M. plumulosa* [26]. Similarly, a previous *L. plumulosus* bioaccumulation model developed using experimentally-measured bioaccumulation factors indicated that ingested particles may be responsible for 40 to 100% of CH_3Hg bioaccumulated under conditions tested, depending on ingested particle type and metal concentration [4]. *Leptocheirus plumulosus* Cd AE has been measured previously for sediment and algal particles (2.9-35.8%) [3,12,22], and the Cd AE measured here is within this range. Cadmium assimilation efficiencies for suspension feeding on algae range from 2.9 to 35.8% [3,12] and increase as N content increases within an algal species [3]. For sediments, measured AE values are 9.8% for sediments from a diatom-encrusted mudflat, 18.9% for 63-µm washed, sieved, and autoclaved sediment, and 21.1% for sediments from within a stand of *Spartina alterniflora* [12]. Yu and Fleeger [3] measured Cd uptake and efflux rates for *L. plumulosus* suspension-feeding on *I. galbana* with differing N content using similar methods. Measured assimilation efficiencies were 10.0, 15.3, and 16.2 % for *I. galbana* with N enrichment of 0, 60, and 180 µmol/L, respectively [3]. Corresponding k_{ef} values (1/d) were 0.480, 0.504, and 0.384 [3]. Measured Cd AE (24%) and k_{ef} (0.31/d) values in the present study do differ slightly, although ~~we used~~ larger animals were used here (>3 mm) than by Yu and Fleeger [3] (1-2.0 mm). Larger animals have a longer gut, and generally longer gut transit times, which may increase assimilation efficiency and reduce efflux rates. ~~Our~~ Ingestion rate measurements for suspension feeding in the present study (0.049-0.151 g/g/d) are similar to those reported for the estuarine amphipod *Melita plumulosa* (0.07-0.25 g/g/d), although *M. plumulosa'*s feeding mode was not specified [26]. In contrast, citing unpublished data (unpublished data in [12]), Schlekat et al. [12] reported that *L. plumulosus* may have ingestion rates of up to 3 g/g/d when deposit feeding.

Available published body burden data suggest steady-state metal concentrations predicted here fall within a realistic range for Cd and As. When exposed to uncontaminated Chesapeake Bay sediments for 42 d, *L. plumulosus* had body burdens of 0.085 μg/g (Cd) and 3.23 μg/g (As); when exposed to contaminated sediments, body burdens were 0.237 μg/g (Cd) and 5.23 μg/g (As), respectively, and Hg(II) body burdens were below detection limit [27]. These Cd body burdens overlap with the range of C_{ss} values predicted here across variation in AE and K_d for suspension feeding, and the As body burdens overlap with the range of C_{ss} values predicted here across variation in K_d for deposit feeding (Table 2). The CH_3Hg body burdens measured in experiments where *L. plumulosus* fed on different combinations of enriched or unenriched *I. galbana* and sediments ranged from 0 to 5 μg/g wet weight, which corresponds to 0-0.75 μg/g (assuming dry wt is 15% of wet wt) [4], which overlaps wtih body burdens predicted here for suspension feeding (Table 2). In general, amphipod metal body burdens measured in the field are in the low to mid ppm range [2,5,28], which correspond to model predictions.

Prior to the present study, *L. plumulosus* AEs had been quantified for Cd, Ag, and Zn for a range of algal and sediment particle types [3,12, 22]. However, K_{ef} values were only available for Cd [3], and aqueous uptake or efflux parameters were not available for any metal. Aqueous Cd uptake and efflux rates measured for the estuarine amphipod *M. plumulosa* were 2 to 10 times higher than those measured here for *L. plumulosus*, suggesting that biokinetic parameters are species-specific for estuarine amphipods [26]. To our knowledge, comparable amphipod aqueous uptake rate measurements are not available for As Hg(II) or CH_3Hg. In copepods, 15% or more of metal uptake in aqueous exposures may result from metal sorption to copepod exoskeletons [29] (Wang and Fisher 1998). Amphipods were thoroughly rinsed with unlabeled water prior to radioassaying animals, but did not quantify potential sorption effects. The length

of depuration periods used to calculate aqueous efflux rates vary widely for marine crustaceans, from 24 h [26] to 7 d [29]. Calculated efflux values based on longer depuration periods may more accurately reflect efflux from slowly exchanging metal pools.

Porewater is frequently assumed to be the primary route of exposure of Cd to benthic animals, but results presented here indicate ingested particles could be responsible for at least 40% of metal bioaccumulated at the salinity and dissolved metal concentrations tested here. As water concentrations increase, the model used here assumes that aqueous uptake rates increase linearly [1,5]. However, because bioavailability of Cd in porewater and Cd partitioning to organic matter decrease as salinity increases [22], diet may contribute more to metal bioaccumulation at lower salinities. Results for CH_3Hg indicate that diet is the primary route of exposure, which corresponds with previous observations for *L. plumulosus* [4]. However, Hg(II) and CH_3Hg metal bioavailability also decreases as DOC in water and organic carbon in sediments increases [4]. For As, no mechanistic information relevant to bioaccumulation for marine amphipods was previously available. In general, marine organisms have a limited ability to bioaccumulate inorganic arsenic from seawater but can bioaccumulate organic arsenic compounds from ingested particles [14]. Here, As(V) influx and efflux rates from water were higher than that observed for Cd, but results predict that relative importance of ingested particles is similar for both metals. Cd bioavailability from water decreases with increasing salinity [22], which may partly explain why k_u values for As were higher than for Cd.

Results indicate that variation in *L. plumulosus* feeding mode and feeding selectivity greatly influences metal uptake. As *L. plumulosus* switches from suspension-feeding to deposit feeding or switches from feeding on sediments to a particle such as pelagic algae which results in a higher AE of ingested metal, modeling predicts that metal exposure and body burden will

increase. When *L. plumulosus* deposit feeds, ingested particles dominate metal bioaccumulation, regardless of *L. plumulosus* AE or particle metal concentration, except for inorganic Hg(II) (Figs. 2,3). In addition, predicted C_{ss} increased 9 to 450 times during deposit feeding, depending on metal, simulated AE, and K_d (Table 2). Similarly, when *L. plumulosus* suspension-feeds, ingested particle type can cause R_f to increase by up to 60% (Figs. 2,3) and C_{ss} to increase 10 to 120 times, depending on metal (Table 2). Ingesting sediments after suspension-feeding on algae can cause *L. plumulosus* algal Cd AE to decrease by up to seven times by reducing algal gut residence time [3]. However, model results predict that ingested particles could still be responsible for 10 to 80% of metal bioaccumulation at the lowest AE values simulated here, and suggests switching from suspension feeding to deposit-feeding on sediments would rapidly increase metal bioaccumulation.

Variation in feeding behavior also has important implications for toxicity test methods. In the 10 d acute sediment toxicity test, test sediments are the only food source [5], and during the 28-d chronic test, amphipods are typically fed 20 to 40 mg of TetraMin® flakes 3 d a week [6]. Results here suggest that whether *L. plumulosus* selectively feeds on benthic algae, other highly organic material associated with sediments, or TetraMin® flakes will affect *L. plumulosus* AE and the amount of metal bioaccumulated. Further, if *L. plumulosus* primarily ingests recently deposited or suspended algae in estuaries, standardized toxicity tests may not accurately reflect *L. plumulosus* food sources or feeding behavior. More research on variation in *L. plumulosus* ingestion rate, feeding mode, and feeding selectivity is needed. This was the first study to quantify suspension feeding ingestion rates, but only used one algal type. The one deposit-feeding ingestion rate estimate available was originally cited by Schlekat et al. [12] as unpublished data, and is much higher than that measured for most other amphipods, although

relatively few measurements exist. As a result, ingestion rates used in modeling here may represent an upper limit for *L. plumulosus* when deposit feeding. In addition, factors which cause *L. plumulosus* to switch between feeding modes or to feed selectively on certain particles are not known. Results presented here suggest feeding modes can have a large effect on metal bioaccumulation and may potentially affect toxicity. A more complete mechanistic understanding of metal bioaccumulation will require a more detailed knowledge of *L. plumulosus* feeding behavior.

Table 1. Mean ± standard error measured biokinetic parameters and predicted C_{ss} and R_f for *Leptocheirus plumulosus* AE=assimilation efficiency.

	Aqueous		Dietary	
	K_u (L g^{-1} d^{-1})	K_{ew} (d^{-1})	AE(%)	K_{ef} (d^{-1})
Cd	0.012 ± 0.002	0.021 ± 0.009	24 ± 4	0.31 ± 0.0003
As	0.028 ± 0.003	0.091 ± 0.006	11 ± 2	0.58 ± 0.005
Hg	1.14 ± 0.10	0.005 ± 0.06	6 ± 1	0.089 ± 0.0003
CH_3Hg	1.59 ± 0.1	$3.0 \times 10^{-5} \pm 0.8 \times 10^{-5}$	80 ± 1	0.052 ± 0.001

Table 2. Predicted C_{ss} (µg/g dry wt) values for *Leptocheirus plumulosus* associated with variation in assimilation efficiency (AE), K_d and ingestion rate (IR). Simulations assume 20 psu salinity, C_w (nM) = 1 (Cd), 0.5 (As), 0.2 (Hg), 0.046 (CH_3Hg), suspension feeding IR is that measured here, and deposit feeding IR is 3 g/g/d. In simulations varying AE, K_d was assumed to be the same as measured for *Isochrysis galbana.*

	Variation in AE		Variation in K_d	
	Suspension feeding	Deposit feeding	Suspension feeding	Deposit feeding
Cd	0.03-0.163	1.3-6.74	0.05-3.50	2.12-211.2
As	0.007-0.039	0.04-0.70	0.015-0.85	0.18-18.21
Hg	0.669-1.69	2.43-29.82	0.62-2.18	1.06-46.19
CH_3Hg	0.995-1.72	19.99-29.88	0.32-10.19	2.20-198

Figures

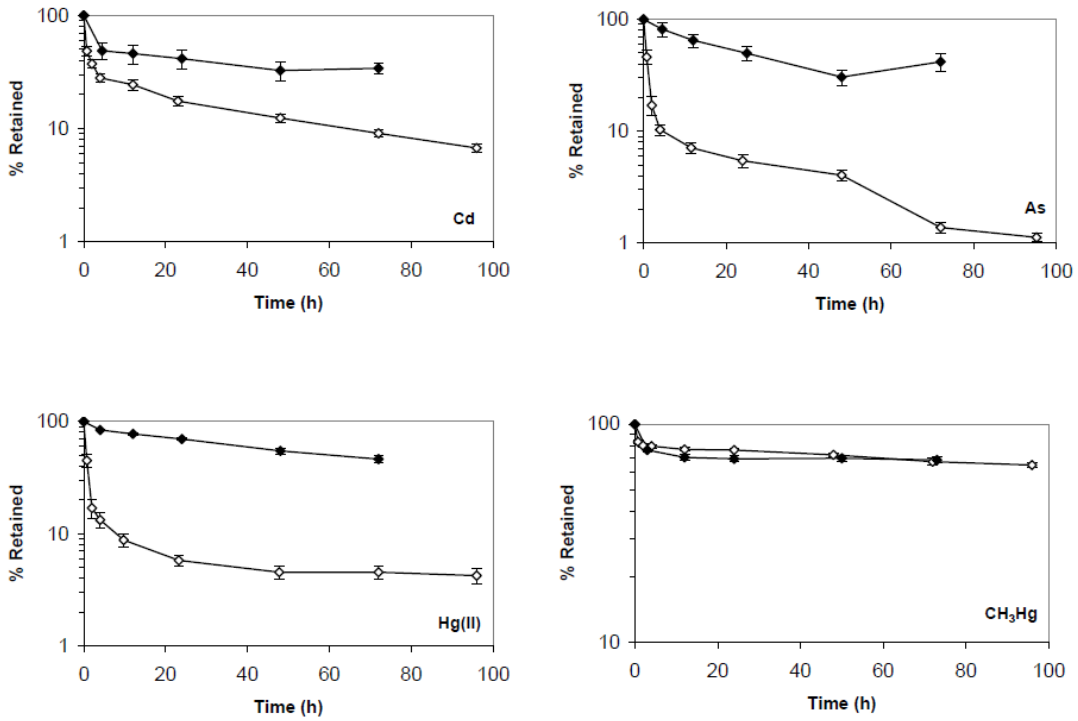

Figure 1. *Leptocheirus plumulosus* depuration after uptake from food (*Isochrysis galbana*) (◇) and water (◆) for Cd, As, inorganic Hg(II) and CH_3Hg. Error bars indicate standard error.

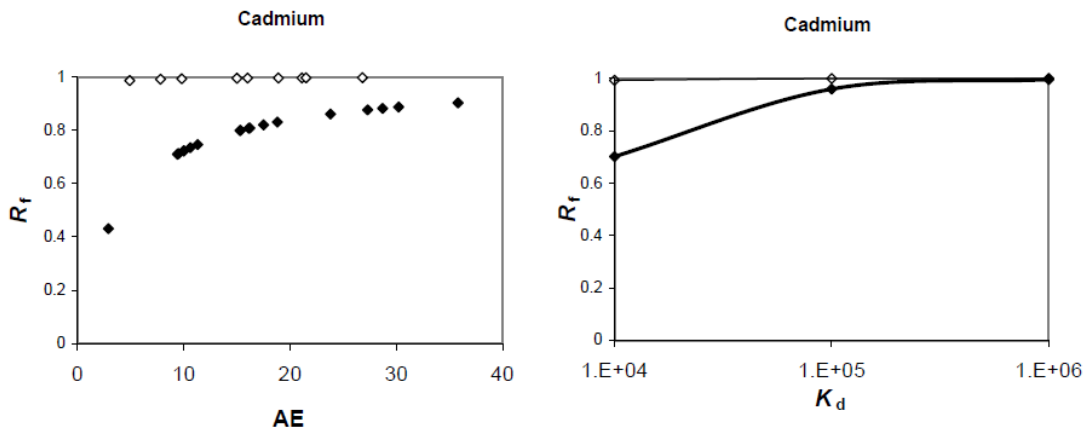

Figure 2. The relative contribution of ingested particles (R_f) to Cd bioaccumulation resulting from variation in assimilation efficiency, K_d, and ingestion rate for *Leptocheirus plumulosus.* Variation in assimilation efficiency for Cd is derived from literature values; variation in K_d is assumed to vary around the value (2.65×10^4) measured for *Isochrysis galbana.* Open diamonds indicate deposit feeding and black diamonds indicate suspension feeding.

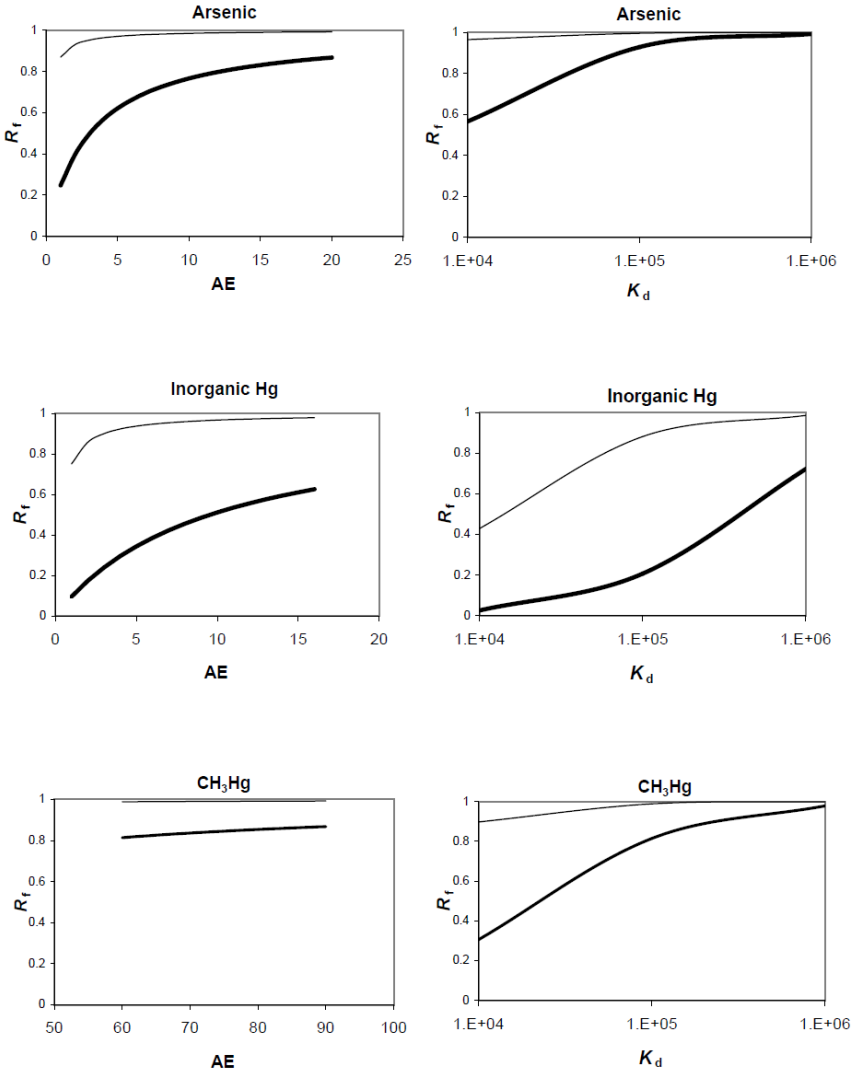

Figure 3. The relative contribution of ingested particles (R_f) to metal (As, Hg, CH_3Hg) bioaccumulation resulting from variation in assimilation efficiency, K_d, and ingestion rate for *Leptocheirus plumulosus.* Variation in assimilation efficiency AE for As, Hg, and CH_3Hg is assumed to follow a similar pattern as observed for Cd. Variation in K_d is assumed to vary around values measured here for *Isochrysis galbana.* Thin lines indicate deposit feeding and heavy lines indicate suspension feeding.

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Multielement stoichiometry in aquatic invertebrates: when growth dilution matters

Abstract

Element concentrations in organisms can be variable, often causing deviations from otherwise consistent, taxon-specific multielement stoichiometries. Such variation can have considerable ecological consequences, yet physiological mechanisms remain unclear. We tested the influence of somatic growth dilution (SGD) on multiple element concentrations under different bioenergetic conditions. SGD occurs when rapid individual growth causes a disproportional gain in biomass relative to gain of a specific element. SGD can strongly affect elements in various organisms, but we lack a general framework to unify results across studies and assess its overall importance. We derived the general conditions that trigger SGD from an element accumulation model. We parameterized the model with bioenergetic and element-specific rates summarized from the literature to compare SGD effects on 15 elements (nonessential metals, essential trace elements, macronutrients) in three aquatic invertebrate taxa. For all taxa, we found that SGD: (1) occurs to some degree for all 15 elements over realistic ranges of growth and ingestion rates, and (2) has the greatest effect on elements with low efflux (excretion) rates, including certain nonessential metals (e.g., MeHg, Po), essential trace elements and macronutrients (e.g., N, Fe). Thus, SGD can strongly affect concentrations of a spectrum of elements under natural conditions. These results provide a framework for predicting variation in elemental composition of animals.

The chemical composition, or stoichiometry, of organisms has well-known implications for the nutritional value of resources (Cross et al., 2003; Elser et al., 2000; Schade et al., 2003; Sterner and Hessen, 1994), element trophic transfer and cycling (Elser and Urabe, 1999; Vanni, 2002). Organisms typically have consistent stoichiometric signatures that are unique to their taxonomic group (Andersen and Hessen, 1991; Elser et al., 2000; Frost et al., 2003) and include trace elements as well as macronutrients (Ho et al., 2003; Karimi and Folt, 2006; Martin and Knauer, 1973; Morel and Hudson, 1985). However, closer scrutiny shows that elemental signatures often vary *in situ*. Certain elements are consistently more variable than others (Andersen and Hessen, 1991; Chen et al., 2000a; Downing, 1997; Karimi and Folt, 2006; Twining et al., 2004) and concentrations of many elements vary with body size and/or growth status (Langston and Spence, 1995b; Villar-Argaiz et al., 2002). Using data on element uptake and loss kinetics from 65 published studies of aquatic invertebrates, we evaluated the influence of growth on body concentrations of 15 elements with different physiological functions. Our overall aim is to provide the basis for developing a framework for explaining the influence of physiological processes such as growth and metabolism on variation in multielement signatures in animals.

Our central hypothesis is that somatic growth dilution (SGD) (Karimi et al., 2007) plays a pronounced, yet largely overlooked role in modifying the multi-element stoichiometry of organisms. SGD is a reduction in the somatic concentration of an element during rapid growth. In general, SGD of an ingested element occurs when total biomass gain outpaces element gain from food, thereby diluting mass-specific element concentration in the body. Element concentration does not change when the ratio of biomass gain to element gain remains constant with growth but will change if the new growth effectively dilutes the whole body concentration of the element. If key factors such as high food quality or low metabolic rate cause growth efficiency (i.e., the ability to convert food into tissue) to outpace element incorporation into tissue, SGD will occur. Since natural populations experience a wide range of growth efficiencies (Morgan et al., 2001; Straile, 1997), it is likely that SGD could strongly influence multiple element concentrations.

Support for the importance of SGD in affecting stoichiometric signatures of aquatic organisms comes from many studies documenting correlations between rapid growth and reduced concentrations of (1) mercury and other nonessential metals in fast growing consumers (Essington and Houser, 2003; Schindler et al., 1995; Simoneau et al., 2005; Stafford and Haines, 2001; Trudel and Rasmussen, 2006; Ward et al., 2010a; Ward et al., 2010b), (2) macronutrients (e.g. N, P) in bacteria (Chrzanowski and Grover, 2008), and (3) essential trace elements (e.g. Zn, Se) and nonessential metals in a diverse range of organisms, including periphyton (Hill and Larsen, 2005), bivalves (Amiard et al., 1986; Amiard and Berthet, 1996; Geffard et al., 2002), and fish (Ciardullo et al., 2008; Ward et al., 2010b). However, the relative sensitivity of specific elements to SGD is unknown and results are inconsistent across studies (e.g. SeeDutton, 1997; Stafford and Haines, 2001; Ward et al., 2010a). Also, exceptions have been noted for nutrients strongly coupled to growth. For example, P content typically increases with growth rate due to an increasing allocation to P-rich rRNA (Elser et al., 2003; Elser et al., 1996). Thus, the influence of SGD on the overall chemical composition of organisms remains unclear.

Element accumulation models provide a theoretical basis for the probable effect of rapid growth on stoichiometry of certain elements. These models predict that rapid growth is most effective at diluting concentrations of elements with low efflux rates (metabolic loss rates after assimilation from the gut into the body) (Reinfelder et al., 1998). Elements with low efflux rates tend to remain in the body long after exposure, so their concentrations are most likely to become diluted and uncoupled from element intake when there are rapid increases in weight gain. This prediction is consistent with results of a previous experimental study showing SGD of methylmercury (a well-known low-efflux molecule) in *Daphnia pulex* (Karimi et al., 2007). However, studies have not adequately assessed the overall importance of SGD by identifying and comparing growth-sensitive elements in multiple taxa.

Our study examines somatic growth dilution as a driver of variation in element concentration under reasonable bioenergetic conditions, across multiple elements with a broad range of biological functions, including nonessential, potentially toxic elements and essential elements in three aquatic invertebrate taxa. Our specific goals were to (1)

define the range of ingestion, growth and efflux rates that trigger SGD, (2) use that information to identify elements for which SGD will have the strongest influence, and 3) compare the influence of SGD on particular elements among taxa. We expected that efflux rates would be low for any element that is retained in the body. Specifically, we expected efflux would be lower for nonessential metals that are known to bioaccumulate (e.g., MeHg, Po, Cd) compared to essential metals and macronutrients that can be stored in the body to a lesser extent.

Element Accumulation Model

We derived the general physiological conditions that trigger SGD of dietary elements from an element accumulation model and parameterized the model with bioenergetic and element-specific kinetic rates summarized from the literature. The kinetic model of element accumulation (Reinfelder et al., 1998; Wang and Fisher, 1999c) describes the steady state element concentration in tissues (C_{ss}, $\mu g \cdot g^{-1}$) resulting from dietary intake as

$$C_{ss} = \frac{AIC_f}{K_e + g} \qquad (1)$$

where A is element assimilation efficiency (proportion of ingested element that crosses the gut lining, %), I is specific ingestion rate ($g \cdot g^{-1} \cdot d^{-1}$), C_f is element concentration in food ($\mu g \cdot g^{-1}$), K_e is efflux rate (loss of assimilated element, d^{-1}), and g is specific growth rate of the animal ($g \cdot g^{-1} \cdot d^{-1}$).

Aquatic animals obtain macronutrients from their diet and trace elements from food and water (Wang and Fisher, 1999c). For certain trace elements, the relative importance of food and water sources can vary. However, diet generally is the primary source of many of the trace elements in this study for aquatic animals (Fisher and Reinfelder, 1995b; Griscom et al., 2002b; Luoma et al., 1992a; Mathews and Fisher, 2009; Roditi et al., 2000a; Wang et al., 1999b). Thus, for simplicity, this model focuses on the effects of SGD on elements acquired through diet.

Predictions of C_{ss} of numerous elements using this model and laboratory-derived kinetic rate constants have closely matched independent field measurements for a variety of organisms and ecosystems (Fisher et al., 2000; Luoma and Rainbow, 2005b; Roditi et al., 2000a). This match suggests that this approach accounts for the major factors governing element concentrations in aquatic animals in natural waters and that the lab-derived parameters are applicable to natural ecosystems.

Model Sensitivity Analysis

SGD of food-borne elements depends on growth efficiency, or the relative rates of growth and ingestion. Therefore, we derived a sensitivity inflection line for each element, indicating the threshold between conditions under which C_{ss} is driven by growth or ingestion. We defined the sensitivity inflection line by calculating partial derivatives of C_{ss} relative to g and I and setting them equal to each other. The sensitivity of C_{ss} to ingestion ($\delta C_{ss}/\delta I$) is defined as

$$\frac{\partial C_{ss}}{\partial I} = \frac{AC_f}{K_e + g} \tag{2}$$

The sensitivity of C_{ss} to growth rate $\delta C_{ss}/\delta g$ is defined as

$$\frac{\partial C_{ss}}{\partial g} = \frac{-AIC_f}{\left(K_e + g\right)^2} \tag{3}$$

The sensitivity inflection line occurs when the absolute value of *Eq. 2* is equivalent to the absolute value of *Eq. 3*, or when

$$I = K_e + g \tag{4}$$

Similarly, solving for when C_{ss} is more sensitive to ingestion than growth, or $\delta C_{ss}/\delta I > \delta C_{ss}/\delta g$, yields the equation

$$I < K_e + g \tag{5}$$

Hence, a greater sensitivity of C_{ss} to ingestion can only occur at relatively high K_e, since as K_e approaches 0, growth alone would have to exceed ingestion (growth efficiency >1). Similarly, C_{ss} is more sensitive to growth when $\delta C_{ss}/\delta g > \delta C_{ss}/\delta I$, or

$$I > K_e + g \tag{6}$$

Thus, C_{ss} is more likely to be sensitive to growth when K_e is relatively low.

Empirical Data Sources

To compare effects of SGD among elements in aquatic invertebrates, we estimated mass-specific element concentrations by parameterizing the element accumulation model with growth, ingestion, element assimilation and efflux rates from published studies of *Daphnia*, marine copepods (primarily *Acartia tonsa*) and marine mytilid mussels (primarily *Mytilus edulis*) (Appendix A). Each taxa is ubiquitous and plays an important ecological role. *Daphnia* and copepods are important zooplankton prey for fish in freshwater and marine food webs, respectively. Mytilid mussels filter large amounts of organic matter in coastal waters, are harvested for human consumption, and are commonly used as bioindicators for coastal pollution (Goldberg et al., 1978). These taxa also play major roles in element fluxes through the production of egested and excreted waste (Fowler and Knauer, 1986). We included macronutrients (e.g., N, P), essential trace elements (e.g., Zn, Se, potentially toxic at high doses) and nonessential metals (e.g., Hg, Pb; no known biological benefit and toxic at elevated concentrations).

For each element, we calculated C_{ss} for each ingestion-growth scenario and generated the C_{ss} response surface and sensitivity inflection line position using MATLAB (2006b, The MathWorks, Natick, MA). We allowed growth and ingestion rates to range from 0 to the maximum value found from the survey of published studies for each animal (Table 1 and Appendix A, Table A1). We constrained the analysis to only include scenarios for which growth rate ≤ ingestion rate (i.e. growth efficiency ≤1). For each element, assimilation efficiency, efflux rate (Table 2 and Appendix A, Table A2) and element concentration in food were held constant. Assimilation and efflux values used in the analysis were the mean of average values from each published study. Efflux rate constants were capped at 1 d^{-1} in all model analyses (Table 2). For each element, values

of element concentration in food were within the range found in phytoplankton in the environment (Chen et al., 2000a; Martin and Knauer, 1973; Watras et al., 1998).

Growth rates, assimilation efficiencies and efflux rate constants were estimated from digitized figures when values were not reported. For *Daphnia*, we included published values for congeners (*Daphnia pulex*, *D. magna*, *D. rosea*, *D. obtusa*) and *Daphnia*-dominated zooplankton assemblages. For mussels, we primarily included studies on *Mytilus edulis* and three studies on the related species *Mytilus galloprovincialis* (Fisher et al., 1996b), *Perna viridis* (Wang et al., 2004) and *Musculista senhousia* (Magni et al., 2000). Copepod data primarily included studies on *Acartia tonsa* and other marine calanoid species.

Element-specific assimilation and efflux values were taken primarily from experimental studies that used a pulse-chase method in which animals were fed phytoplankton uniformly labeled with a radioisotope and allowed to depurate while feeding on unlabeled food and purging their guts of unassimilated radioactive material. Studies that exposed animals to multiple pulse-chase feeding regimes before measuring depuration were excluded because values from these studies indicate efflux from long-term storage pools within the animal rather than efflux from recently assimilated element. Studies that exposed organisms simultaneously to metals dissolved in water and in food, or in water only, also were excluded. Experimental feeding periods were generally less than the gut passage time for the animals in most studies, with a few exceptions (Chang and Reinfelder, 2000, 2002; Fisher and Teyssie, 1986; Fisher et al., 1996b; Liu et al., 2006).

Assimilation efficiency of the ingested element was calculated as the y-intercept, and efflux as the slope, of the regression between the percent element concentration retained in the whole animal and time of depuration for the slowly-exchanging pool (i.e., after all of the label had been assimilated from the gut), assuming a log-linear function (Wang and Fisher, 1999c). When not reported, we calculated efflux values from digitized data using only data collected after complete digestion of the radiolabeled food, as observed by the authors or as clearly visible as a second compartmental loss during depuration. Element efflux was calculated from whole, live organisms. Ingested elements

that are associated with mussel shells or with chitinous exoskeletons typically represent a small fraction of the body burden in the whole animal (Wang et al., 1995). Thus, studies that estimated element retention and loss from soft tissues were included.

For published studies on macronutrient (N and P) assimilation and efflux in *Daphnia* and mussels that did not use the pulse-chase radiotracer method, we calculated efflux rates from excretion rates of dissolved N and P. Excretion rates are comparable to efflux loss rates, as they both only include loss of assimilated element. To convert N and P excretion rate constants for *Daphnia* (e.g., mg N mg *Daphnia*$^{-1}$ d^{-1}) to efflux rate constants (d^{-1}), we assumed that *Daphnia* are 50% C, 9% N and 1.4% P by weight (Andersen and Hessen, 1991). We found no study that reported N assimilation efficiencies for *Daphnia.* Therefore, we used N assimilation efficiencies of freshwater copepods (Table 2 and Appendix A, Table A2) in our analyses. To convert macronutrient excretion rates for mussels, we assumed that mussels are 36.6% C, 7.8% N and 0.5% P (Smaal and Vonck, 1997). Macronutrient composition of natural invertebrate populations can vary (Karimi and Folt, 2006), but this variation has a negligible effect on our calculated efflux values compared to the broad range of excretion values we found across studies (Table 2) and does not affect our results.

Results

Our model results show that rapid, efficient growth reduces tissue concentrations (C_{ss}) of all elements included in our study. However, SGD most strongly reduces C_{ss} when element efflux rate is low (Fig. 1). At low efflux rates, the highest growth efficiency results in the lowest C_{ss}. The position of the sensitivity inflection line indicates that when element efflux rate is low, SGD occurs over a broader range of ingestion and growth rates for all three animals, thus is more likely to occur *in situ* (Fig. 2). Of particular interest is that for the lowest efflux elements (MeHg in *Daphnia*, Po, Fe, Cu, N in copepods, and S, MeHg and Pb in mussels), there are few points below the inflection line, indicating that these elements are usually highly sensitive to growth (Fig. 2A). In contrast, some metals (Am, Ag, inorganic Hg) have relatively high efflux rates (Fig. 2B), indicating that bioaccumulation of these elements is unlikely in these organisms.

Finally, consistent patterns in element efflux and assimilation efficiencies among taxa emerged, despite basic biological differences and despite the variability in efflux rates and assimilation efficiencies within taxa (Table 2 and Appendix A, Table A2). Specifically, efflux, growth and ingestion rates are generally higher for zooplankton than mussels, as might be expected given their relative sizes (Tables 1, 2). Finally, MeHg assimilation efficiency was among the highest, and efflux rate among the lowest in both *Daphnia* and mussels (no MeHg efflux data were available for copepods).

Discussion

Our findings show that SGD has the potential to significantly influence concentrations of many elements with low-efflux rates, including macronutrients, essential trace elements and nonessential metals under environmentally relevant conditions. No matter what the conditions under which low efflux rates occur, or their physiological impact (e.g. sequestration of nutrients or bioaccumulation of nonessential metals), rapid growth can reduce the mass-specific concentration of low-efflux elements and alter the overall elemental composition of organisms.

Of the nonessential metals included in our study, MeHg, Po and Pb had the lowest efflux rates, consistent with studies that empirically have shown SGD for MeHg (Karimi et al., 2007) and Pb (Ward et al., 2010b). SGD may explain why Po concentrations in marine zooplankton are higher in oligotrophic waters that have fewer nutrients to support growth (Jeffree et al., 1997). We lack sufficient data on efflux rates to assess the universality of a strong SGD influence on these elements across taxa. However, there is strong evidence that MeHg has consistently low efflux rates for the organisms included in our study as well as humans (ATSDR, 1999) and fish (Mathews and Fisher, 2008; Pickhardt et al., 2006). Low MeHg efflux is associated with its observed propensity to persist in the body and biomagnify in aquatic food chains (Mason et al., 1996; Watras et al., 1998). In contrast, other nonessential metals (Ag, Am) have higher efflux rates and are not known to biomagnify. Due to its relatively invariant, low efflux rate compared to other elements, MeHg is likely an endmember representing the greatest sensitivity to

SGD in diverse organisms. SGD likely reduces concentrations of other organic, biomagnifying contaminants with low efflux rates, including PCBs (Fisk et al., 1998) and DDT (Wang and Wang, 2005). Overall, SGD may be an effective means for reducing concentrations of biomagnifying, nonessential metals and preventing toxicity, particularly when efflux and storage-detoxification mechanisms are limited.

SGD also appears capable of reducing concentrations of essential trace elements and macronutrients. N, Cu and Fe in marine copepods and S in marine mussels had relatively low efflux rates and were highly sensitive to SGD. Studies have found that marine copepod egg production can be limited by N (Kiorboe, 1989) and Fe (X. Chen, S.B. Baines and N.S. Fisher, unpublished manuscript). Low efflux may enable copepods to sequester N and Fe and avoid nutrient deficiencies. While N storage has not been well-documented, copepods contain ferritin in diapausing stages (Tarrant et al., 2008), suggesting the ability to store iron. Similarly, Cu can be stored in metallothionein (Kagi and Kojima, 1987). In general, most organisms can store many different metals in metallothionein, which is thought to play a critical role in modulating metabolism involving metal-containing enzymes and in preventing metal toxicity (Karin, 1985; Roesijadi, 1996). Efflux rates for elements that are bound in these long-term storage pools generally are lower than short-term efflux rates with few exceptions (Tsui and Wang, 2004b; Wang and Fisher, 1997; Wang et al., 1996c; Yu and Wang, 2002, 2004), thus are more sensitive to growth dilution. Finally, bivalves may have low S efflux because, in addition to its presence in proteins, S is an important component of the shell (Rosenberg and Hughes, 1991) and byssal threads (Swann et al., 1998) which may have low, tissue-specific S loss rates.

Our finding that essential nutrients can undergo SGD is consistent with central tenets of stoichiometric theory, provided the element in question is not limiting growth at the same time. For example, the Growth Rate Hypothesis states that rapid growth is known to be strongly, positively correlated with increasing P content due to a greater demand for P-rich rRNA for protein synthesis (Elser et al., 1996). We would not expect SGD of P whenever an increase in P concentration is necessary for growth to occur. However, the growth rate-P coupling has been shown to weaken when P is not limiting,

such as during food scarcity (Elser et al., 2003; Makino and Cotner, 2004). Similarly, threshold element ratio (TER) models demonstrate that when food abundance is low and carbon limits growth, carbon gain (termed gross growth efficiency of C) outpaces gains of other nutrients (gross growth efficiency of P or N) (Sterner and Elser, 2002; Sterner, 1997; Urabe and Watanabe, 1992). Once a nutrient is diluted to a threshold, limiting concentration we expect growth to decrease, preventing further dilution. Thus, there is likely a negative feedback between growth rate and the concentration of essential elements that are tightly coupled to growth. TER models assume that the stoichiometric composition of animals is homeostatic. However, growth limitation is imposed by different essential elements under different conditions and differences among elements in their allocation to new growth depend on what is limiting for a specific physiological state (Sterner, 1997). Collectively then, stoichiometry theory and SGD suggest that homeostatic elemental ratios are flexible and should reflect differences in physiological state as well as factors such as body size.

One major implication of our study is that the strength of SGD may ontogenetically vary and may help explain allometric relationships observed in growth-sensitive elements. Relationships between body size and element concentration are variable (Brix and Lyngby, 1985) and cannot be described by a single allometric constant for the same element even among conspecifics (Wang and Fisher, 1997). Previous studies suggest that growth and ingestion rates are important drivers of metal allometry (Pan and Wang, 2008; Wang and Fisher, 1997). A strong SGD effect may explain observed decreases in metal concentrations at larger body sizes when all else is equal (e.g. age, ingestion rate) (Karimi et al., 2007; Ward et al., 2010b). However, even when element concentrations increase with body size, SGD could result in younger, fast growing organisms having lower concentrations than slower growing organisms of the same size. Moreover, SGD effects may be particularly strong at smaller body sizes when growth rate (Von Bertalanffy, 1957) and growth efficiency (Jorgensen, 1976) are high. This hypothesis is consistent with studies that have directly attributed high growth to enhanced reduction of metal concentrations within juveniles compared to other life stages in molluscs (Geffard et al., 2002; Leung et al., 2001; Ringwood, 1991) and marine fish (Zhang and Wang, 2007). Finally, metal allometry may be explained partly by observed

relationships between body size and element assimilation or efflux, depending on the element (Wang and Fisher, 1997; Zhang and Wang, 2007). The combined effects of each of these factors throughout development are difficult to predict, but ultimately determine changes in element content in a growing organism.

Ultimately, the overall effect of SGD on stoichiometry depends on element efflux rates and growth efficiency (growth relative to ingestion). A host of factors can influence these processes and SGD. For example, food quality (e.g. digestibility), food availability, activity level and temperature influence ingestion and growth in predictable ways. In contrast, effects of these factors on element biokinetics often are element-specific and difficult to generalize. For example, increasing temperature has no effect on MeHg efflux, but lowers inorganic Hg efflux rate in *Daphnia* (Tsui and Wang, 2004a). In contrast, higher temperatures significantly increase Cd and Co efflux in blue mussels (Baines et al., 2005), likely reducing the sensitivity of these elements to SGD.

To take this argument to a deeper mechanistic level requires understanding the factors that drive element efflux. Yet, even as one of the most fundamental processes regulating internal element concentrations (Mertz, 1981) little is known about the regulation of efflux. Efflux and assimilation can vary, even within the same species (Table 2 and Appendix A, Table A2). For instance, P efflux rates in *Daphnia* range ten-fold (DeMott et al., 1998; Lehman, 1980; Urabe, 1993) and can be as low as MeHg efflux rates (Table 2). Whether P and other nutrients have low efflux rates under non-limiting conditions determines their sensitivity to SGD. The weight of evidence suggests that P efflux is low under P-limited conditions in *Daphnia* (DeMott et al., 1998; He and Wang, 2007). However, other studies suggest that P can be stored in the body under non-limiting conditions (Becker and Boersma, 2005; Frost and Elser, 2002; Lee et al., 2006; Sterner and Schwalbach, 2001; Woods et al., 2002). Macronutrient storage and efflux under non-limiting conditions are less well-documented compared to metals. Finally, individual tissues often have distinct biological half-lives for a given element (Fisher et al., 1996b), thereby further complicating our general understanding of efflux patterns among elements and species.

Lastly, SGD appears likely to occur frequently under natural conditions, which has important ecological implications. SGD-driven changes in stoichiometry provide a basis for predicting variation in the nutritional value of prey and effects on element trophic transfer and cycling under different growth conditions. For example, SGD has the largest influence on low-efflux elements that, when assimilated, tend to be bound to tissue and cycled through food webs. During periods of rapid growth, SGD should decrease somatic concentrations and the trophic transfer of these growth-sensitive elements. In contrast, SGD has minor effects on elements that are either unassimilated and egested as fecal pellets, or assimilated and rapidly effluxed in dissolved forms. The geochemical fates of elements lost in these two forms clearly differ, with unassimilated element transport via fecal pellets leading to shorter residence times in the water column than elements that are released into the dissolved phase and are biologically recycled (Fisher and Reinfelder, 1995b). Overall, efflux and growth are fundamental, complementary processes by which organisms modify element concentrations. Our findings provide an initial framework with which to examine the influence of growth and efflux on elements in other organisms and with alternative exposure routes.

Appendix A: Empirical Data Sources

Table A1a. *Daphnia* ingestion and growth rates reported in published studies.

Minimum Ingestion Rate ($g\ g^{-1}\ d^{-1}$)	Maximum Ingestion Rate ($g\ g^{-1}\ d^{-1}$)	Minimum Growth Rate ($g\ g^{-1}\ d^{-1}$)	Maximum Growth Rate ($g\ g^{-1}\ d^{-1}$)	Organism	Reference
0.744	3.072	N/A	N/A	*D. obtusa*	(Sterner et al., 1993a)
N/A	N/A	0.2	0.52	*D. galeata x hyalina*	(Boersma et al., 2001)
N/A	N/A	0.075	0.22	*Daphnia sp.*	(Burns, 2000)
N/A	N/A	0.22	0.33	*Daphnia sp.*	(Becker et al., 2004)
N/A	N/A	0.01	0.4	*Daphnia dentifera*	(Urabe et al., 2002)
N/A	N/A	0.03	0.55	*Daphnia magna*	(Muller-Navarra et al., 2000)
N/A	N/A	0.01	0.6	*Daphnia sp.*	(DeMott et al., 2004)

Table A1b. Copepod ingestion and growth rates reported in published studies.

Minimum Ingestion Rate ($g\ g^{-1}\ d^{-1}$)	Maximum Ingestion Rate ($g\ g^{-1}\ d^{-1}$)	Mean Ingestion rate ($g\ g^{-1}\ d^{-1}$)	Mean Growth Rate ($g\ g^{-1}\ d^{-1}$)	Organism	Reference

N/A	N/A		0.42		0.09	*T. longicornus*	(Dam and Peterson, 1991; Lonsdale et al., 1996; Wang and Fisher, 1998)
N/A	N/A		0.44		0.02	*Acartia sp.*	(Chang and Reinfelder, 2002; Lonsdale et al., 1996)*
N/A	N/A		0.33		0.03	*A. tonsa*	(Stewart and Fisher, 2003)
N/A	N/A		0.24	N/A		*A. tonsa*	(Houde and Roman, 1987)**
N/A	N/A	N/A			1.044	*A. tonsa*	(Huntley and Lopez, 1992)
0.188	1.2		0.478	N/A		*A. tonsa*	(Kiorboe et al., 1985)***

* calculated in Chang and Reinfelder, 2002

**calculations made assuming copepods are 50% C

***calculations made assuming that food (dry weight) is 50% C

Table A1c. Mussel ingestion and growth rates reported in published studies.

Minimum Ingestion Rate ($g\ g^{-1}\ d^{-1}$)	**Maximum Ingestion Rate ($g\ g^{-1}\ d^{-1}$)**	**Minimum Growth Rate ($g\ g^{-1}\ d^{-1}$)**	**Maximum Growth Rate ($g\ g^{-1}\ d^{-1}$)**	**Organism**	**Reference**
0.015	0.553	0.009	0.12	*M. edulis*	(Baines and Fisher, 2008)*
		0.011	0.097	*M. edulis*	(Clausen and Riisgard, 1996)
		0.01	0.1	*M. edulis*	(Jorgensen, 1996)

0.00375	0.0391	*M. edulis*	(Riisgard and Poulsen, 1981)	

*growth rates from Scope for Growth estimates

Table A2a. *Daphnia* assimilation efficiencies and efflux rates reported in published studies.

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Min Efflux Rate (d^{-1})	Max Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
Ag	1	62.6	31.8	0.26	0.75	0.505	*Daphnia magna*	(Lam and Wang, 2006)
Cd	8.8	50.9	29.85	0.17	1.13	0.65	*Daphnia magna*	(Guan and Wang, 2004)
Cd	17.3	32.5	24.9	0.068	0.153	0.1105	*Daphnia magna*	(Yu and Wang, 2004)
Cd	29.5	76.7	53.1	0.11	0.27	0.19	*Daphnia magna*	(Yu and Wang, 2002)
Cr-III	8.3	43.7	26	0.12	2.32	1.22	*Daphnia magna*	(Yu and Wang, 2002)
Hg-II	14.1	15.1	14.6	0.55	1.697	1.1235	*Daphnia magna*	(Tsui and Wang, 2004a)
Hg-II	9.1	47	28.05	0.73	2.28	1.505	*Daphnia magna*	(Tsui and Wang, 2004b)
MeHg	94.75	96.02	95.385	0.0413	0.0415	0.0414	*Daphnia pulex*	(Karimi et al., 2007)
MeHg	90.6	93.2	91.9	0.076	0.193	0.1345	*Daphnia magna*	(Tsui and Wang, 2004a)
MeHg	64.2	97.4	80.8	0	0.22	0.11	*Daphnia magna*	(Tsui and Wang, 2004b)
N	N/A	N/A	N/A	0.08	0.35	0.215	*Daphnia pulex*	(Lehman, 1980)
N	N/A	N/A	N/A	0.4	0.76	0.58	*Daphnia sp.*	(Perez-Martinez and Gulati, 1998)
N	N/A	N/A	N/A	1.87	3.222	2.546	*Daphnia obtusa*	(Sterner and Smith, 1993b)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Min Efflux Rate (d^{-1})	Max Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
N	N/A	N/A	N/A	0.07	0.28	0.17	bulk zooplankton (*Daphnia*)	(Urabe, 1993)
P	69	98	83.5	0.075	0.651	0.363	*Daphnia magna*	(DeMott et al., 1998)
P	34	93	63.5	N/A	N/A	N/A	*Daphnia sp.*	(Ferrao et al., 2005)
P	N/A	N/A	N/A	0.09	0.9	0.495	*Daphnia pulex*	(Lehman, 1980)
P	N/A	N/A	N/A	0.86	2.03	1.445	*Daphnia sp.*	(Perez-Martinez and Gulati, 1998)
P	N/A	N/A	N/A	0.103	0.230	0.166	*Daphnia rosea*	(Peters and Lean, 1973)
P	N/A	N/A	N/A	0.527	3.422	1.974	*Daphnia obtusa*	(Sterner and Smith, 1993b)
P	N/A	N/A	N/A	0.077	0.51	0.26	bulk zooplankton (*Daphnia*)	(Urabe, 1993)
Se	21.1	64.6	42.85	0.41	0.82	0.615	*Daphnia magna*	(Guan and Wang, 2004)
Se	15.6	32.4	24	0.267	0.519	0.393	*Daphnia magna*	(Yu and Wang, 2004)
Se	24.5	57.9	41.2	0.16	0.55	0.355	*Daphnia magna*	(Yu and Wang, 2002)
Zn	3.7	7.1	5.4	0.49	1.45	0.97	*Daphnia magna*	(Guan and Wang, 2004)
Zn	1.4	3.5	2.45	0.376	1.068	0.722	*Daphnia magna*	(Yu and Wang, 2004)
Zn	7.6	66.5	37.05	0.3	0.92	0.61	*Daphnia magna*	(Yu and Wang, 2002)

Table A2b. Copepod assimilation efficiencies and efflux rates reported in published studies.

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Maximum Efflux Rate for model (<1.0 d^{-1})	Mean Efflux Rate (d^{-1})	Mean Efflux Rate for model (<1.0 d^{-1})	Organism	Reference
Ag	N/A	N/A	15	N/A	N/A	N/A	N/A	N/A	*A. tonsa* and *A. hudsonica*	(Hook and Fisher, 2002)
Ag	N/A	N/A	17.4	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *Temora longicornis*	(Reinfelder and Fisher, 1991)
Ag	8	19	10	0.344	0.424	0.424	0.384	0.384	*T. longicornus*	(Wang and Fisher, 1998)
Ag	2.8	21.9	12.35	0.44	1	1	0.72	0.72	*A. spinicauda*	(Xu and Wang, 2004)
Am	0.35	1.36	0.855	N/A	N/A	N/A	N/A	N/A	*Acartia tonsa*	(Fisher and Reinfelder, 1991a)
Am	2.6	8.1	5.35	N/A	N/A	N/A	N/A	N/A	*Anomalocera patersoni*	(Fisher et al., 1991b)
Am	N/A	N/A	0.9	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *Temora longicornis*	(Reinfelder and Fisher, 1991)
Am	3	9	6	0.77	0.93	0.93	0.85	0.85	*A. tonsa* (80%) and *T. longicornis* (20%)	(Wang and Fisher, 1996a)
Cd	14.1	41.8	27.95	N/A	N/A	N/A	N/A	N/A	*Anomalocera patersoni*	(Fisher et al., 1991b)
Cd	N/A	N/A	62	N/A	N/A	N/A	N/A	N/A	*A. tonsa* and *A. hudsonica*	(Hook and Fisher, 2002)
Cd	N/A	N/A	30.4	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *Temora longicornis*	(Reinfelder and Fisher, 1991)
Cd	13.2	68.4	40.8	N/A	N/A	N/A	N/A	N/A	*Pseudodiaptomus coronatus*	(Sick and Baptist, 1979)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Maximum Efflux Rate for model (<1.0 d^{-1})	Mean Efflux Rate (d^{-1})	Mean Efflux Rate for model (<1.0 d^{-1})	Organism	Reference
Cd	33	53.3	40	0.408	0.685	0.685	0.5465	0.5465	*T. longicornus*	(Wang and Fisher, 1998)
Cd	85	96	90.5	0.31	0.48	0.48	0.395	0.395	*A. tonsa* (80%) and *T. longicornis* (20%)	(Wang and Fisher, 1996a)
Cd	16.2	79.3	47.75	0.39	0.72	0.72	0.555	0.555	*Calanus sinicus*	(Xu and Wang, 2001a)
Cd	83.8	94.1	88.95	0.56	0.57	0.57	0.565	0.565	*A. spinicauda*	(Xu and Wang, 2002)
Cd	21.1	88.5	54.8	0.52	1.28	1	0.9	0.76	Calanoid sp.	(Xu et al., 2001b)
Co	14.2	20.5	15	0.375	0.954	0.954	0.6645	0.6645	*T. longicornus*	(Wang and Fisher, 1998)
Co	25	48	36.5	0.78	0.86	0.86	0.82	0.82	*A. tonsa* (80%) and *T. longicornis* (20%)	(Wang and Fisher, 1996a)
Cu	29.9	61.1	40.3	N/A	N/A	N/A	N/A	N/A	*Acartia* (90%) and *Temora* (10%)	(Chang and Reinfelder, 2000)
Cu	N/A	N/A	N/A	0.056	0.076	0.076	0.066	0.066	*Acartia sp.*	(Chang and Reinfelder, 2002)
Fe	7	18	12.5	N/A	N/A	N/A	N/A	N/A	*A. tonsa*	(Hutchins and Bruland, 1994)
Fe	5	35	20	N/A	N/A	N/A	N/A	N/A	*A. tonsa*	(Hutchins et al., 1995)
Fe	10.1	17.3	13.7	0.036	0.0816	0.0816	0.0588	0.0588	*Acartia sp.*	(Schmidt et al., 1999)
Hg	12	39.7	25.85	N/A	N/A	N/A	N/A	N/A	*Anomalocera patersoni*	(Fisher et al., 1991b)
Hg	N/A	N/A	14	N/A	N/A	N/A	N/A	N/A	*A. tonsa* and *A. hudsonica*	(Hook and Fisher, 2002)
Hg	N/A	N/A	15	N/A	N/A	N/A	N/A	N/A	*A. tonsa, Temora longicornis, Centropages*	(Mason et al., 1996)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Maximum Efflux Rate for model (<1.0 d^{-1})	Mean Efflux Rate (d^{-1})	Mean Efflux Rate for model (<1.0 d^{-1})	Organism	Reference
									sp.	
MeHg	N/A	N/A	62	N/A	N/A	N/A	N/A	N/A	*A. tonsa, Temora longicornis, Centropages sp.*	(Mason et al., 1996)
Mn	15	30	22.5	N/A	N/A	N/A	N/A	N/A	*A. tonsa* and *A. hudsonica*	(Hook and Fisher, 2002)
Mn	3	10	6.5	N/A	N/A	N/A	N/A	N/A	*A. tonsa*	(Hutchins and Bruland, 1994)
N	N/A	N/A	N/A	0.06	0.25	0.25	0.155	0.155	*A. tonsa*	(Kiorboe et al., 1985)
N	13.6	99.1	56.35	N/A	N/A	N/A	N/A	N/A	tropical zooplankton	(Leborgne, 1982)
N	N/A	N/A	N/A	0.06	0.1	0.1	0.08	0.08	*Undinula vulgaris* (adult)	(Verity, 1985)
N	N/A	N/A	N/A	0.05	0.21	0.21	0.13	0.13	*A. tonsa*	(Checkley et al., 1992)
N (NH4)	N/A	N/A	N/A	0.049	0.43	0.43	0.141	0.141	mesozooplankton	(Alcaraz et al., 1994)
P	N/A	N/A	N/A	0.064	0.16	0.16	0.1	0.1	*Calanus finmarchus*	(Conover, 1961)
P	48.1	99.2	73.65	N/A	N/A	N/A	N/A	N/A	tropical zooplankton	(Leborgne, 1982)
P	19.3	78.1	48.7	0.3	0.48	0.48	0.39	0.39	*Acartia erythraea*	(Liu et al., 2006)
P	N/A	N/A	71.9	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *T. longicornis*	(Reinfelder and Fisher, 1991)
Po	19.6	55.1	37.35	0.01	0.07	0.07	0.03	0.03	*A. tonsa*	(Stewart and Fisher, 2003)
Pu	0.6	1.2	0.8	N/A	N/A	N/A	N/A	N/A	*Anomalocera patersoni*	(Fisher et al., 1991b)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Maximum Efflux Rate for model (<1.0 d^{-1})	Mean Efflux Rate (d^{-1})	Mean Efflux Rate for model (<1.0 d^{-1})	Organism	Reference
S	49.4	64.3	56.85	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *Temora longicornis*	(Reinfelder and Fisher, 1991)
Se	95.5	98.7	97.1	N/A	N/A	N/A	N/A	N/A	*Acartia tonsa*	(Fisher and Reinfelder, 1991a)
Se	N/A	N/A	97.1	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *Temora longicornis*	(Reinfelder and Fisher, 1991)
Se	49.8	59.4	55	0.226	0.343	0.343	0.2845	0.2845	*T. longicornus*	(Wang and Fisher, 1998)
Se	75	90	82.5	0.34	0.69	0.69	0.515	0.515	*A. tonsa* (80%) and *T. longicornis* (20%)	(Wang and Fisher, 1996a)
Se	55.6	81.2	68.4	0.32	0.66	0.66	0.49	0.49	*Calanus sinicus*	(Xu and Wang, 2001a)
Se	60.7	62.4	61.55	0.64	0.87	0.87	0.755	0.755	*A. spinicauda*	(Xu and Wang, 2002)
Se	7.3	52.7	30	0.08	1.5	1	0.86	0.54	Calanoid sp.	(Xu et al., 2001b)
Zn	31.3	72	51.65	N/A	N/A	N/A	N/A	N/A	*Anomalocera patersoni*	(Fisher et al., 1991b)
Zn	N/A	N/A	55	N/A	N/A	N/A	N/A	N/A	*A. tonsa* and *A. hudsonica*	(Hook and Fisher, 2002)
Zn	32	37	34.5	N/A	N/A	N/A	N/A	N/A	*A. tonsa*	(Hutchins and Bruland, 1994)
Zn	26.8	47.3	37.05	N/A	N/A	N/A	N/A	N/A	*A. tonsa, A. hudsonica* or *Temora longicornis*	(Reinfelder and Fisher, 1991)
Zn	52.1	63.6	60	0.248	0.286	0.286	0.267	0.267	*T. longicornus*	(Wang and Fisher, 1998)
Zn	70	80	75	0.28	0.55	0.55	0.415	0.415	*A. tonsa* (80%) and *T. longicornis* (20%)	(Wang and Fisher, 1996a)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Maximum Efflux Rate for model (<1.0 d^{-1})	Mean Efflux Rate (d^{-1})	Mean Efflux Rate for model (<1.0 d^{-1})	Organism	Reference
Zn	27.7	52.1	39.9	0.33	0.376	0.376	0.353	0.353	*A. spinicauda*	(Wang et al., 2001)
Zn	43.7	69.6	56.65	0.205	0.304	0.304	0.2545	0.2545	*Calanus sinicus*	(Wang et al., 2001)
Zn	28.1	79	53.55	0.49	1.15	1	0.82	0.745	*Calanus sinicus*	(Xu and Wang, 2001a)
Zn	41.3	44.2	42.75	0.57	0.62	0.62	0.595	0.595	*A. spinicauda*	(Xu and Wang, 2002)
Zn	7.2	54.6	30.9	0.43	1.26	1	0.845	0.715	Calanoid sp.	(Xu et al., 2001b)

Table A2c. Mussel assimilation efficiencies and efflux rates reported in published studies.

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
Ag	1.8	19.3	10.55	0.038	0.062	0.05	*M. edulis*	(Baines et al., 2005)
Ag	3	15	9	0.0254	0.1639	0.09465	*M. galloprovincialis*	(Fisher et al., 1996b)
Ag	3.8	33.8	18.8	0.15	0.22	0.185	*M. edulis*	(Wang and Fisher, 1996a)
Ag	18.6	20.7	19.65	0.39	0.43	0.41	*M. edulis*	(Wang and Fisher, 1996b)
Ag	2	10	6	0.4536	0.7224	0.588	*M. edulis*	(Wang et al., 1995)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
Ag	5.4	17.6	11.5	0.2	0.43	0.315	*M. edulis*	(Wang et al., 1996c)
Ag	13	50	31.5	N/A	N/A	0.04	*M. edulis*	(Reinfelder et al., 1997)
Am	2.1	12.8	7.45	0.035	0.2151	0.12505	*M. edulis*	(Baines et al., 2005)
Am	5.4	6.7	6.1	0.011	0.020	0.015	*M. edulis*	(Fisher and Teyssie, 1986)
Am	4	6	5	0.0184	0.0472	0.0328	*M. galloprovincialis*	(Fisher et al., 1996b)
Am	2.9	3	2.95	N/A	N/A	N/A	*M. edulis*	(Reinfelder et al., 1997)
Am	0.7	6.2	3.45	0.2	0.25	0.225	*M. edulis*	(Wang and Fisher, 1996a)
Am	2	3.6	2.8	0.43	0.43	0.43	*M. edulis*	(Wang and Fisher, 1996b)
Am	2	10	6	0.1608	0.3888	0.2748	*M. edulis*	(Wang et al., 1995)
Am	0.6	1	0.8	0.35	0.52	0.435	*M. edulis*	(Wang et al., 1996c)
Cd	24.6	66.6	45.6	0.005	0.025	0.015	*M. edulis*	(Baines et al., 2005)
Cd	22	32	27	0.0008	0.0444	0.0226	*M. galloprovincialis*	(Fisher et al., 1996b)
Cd	37	96	66.5	0.02	0.05	0.035	*M. edulis*	(Reinfelder et al., 1997)
Cd	21	33	27	0.0167	0.0345	0.0256	*M. edulis*	(Wang and Fisher, 1997)
Cd	11.3	34.3	22.8	0.07	0.22	0.145	*M. edulis*	(Wang and Fisher, 1996a)
Cd	20	53.5	36.75	0.002	0.013	0.0075	*M. edulis*	(Wang and Fisher, 1996b)
Cd	20	50	35	0.024	0.0504	0.0372	*M. edulis*	(Wang et al., 1995)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
Cd	8	20.1	14.05	0.06	0.09	0.075	*M. edulis*	(Wang et al., 1996c)
Co (inorganic)	31.7	68.3	50	0.0346	0.1272	0.0809	*M. edulis*	(Baines et al., 2005)
Co (inorganic)	8	9	8.5	0.0107	0.0566	0.0337	*M. galloprovincialis*	(Fisher et al., 1996b)
Co (inorganic)	37	46	41.5	0.1	0.2	0.15	*M. edulis*	(Reinfelder et al., 1997)
Co (inorganic)	24	36	30	0.0271	0.0331	0.0301	*M. edulis*	(Wang and Fisher, 1997)
Co (inorganic)	19.5	43.2	31.35	0.23	0.27	0.25	*M. edulis*	(Wang and Fisher, 1996a)
Co (inorganic)	27.4	30.8	29.1	0.32	0.36	0.34	*M. edulis*	(Wang and Fisher, 1996b)
Co (inorganic)	15	25	20	0.3936	0.456	0.4248	*M. edulis*	(Wang et al., 1995)
Co (inorganic)	11.9	16.2	14.05	0.4	0.46	0.43	*M. edulis*	(Wang et al., 1996c)
Cr (III)	0.2	1.3	0.75	0.15	0.26	0.205	*M. edulis*	(Wang and Fisher, 1996a)
Cr (VI)	1.1	10.4	5.75	0.22	0.29	0.255	*M. edulis*	(Wang and Fisher, 1997)
Hg (inorganic)	41	44	42.5	0.146	0.242	0.194	*Perna viridis*	(Wang et al., 2004), (Wang, unpublished)
MeHg	84	87	85.5	0.021	0.038	0.0295	*Perna viridis*	(Wang et al., 2004), (Wang, unpublished)
N	27.8	84.4	60.9	N/A	N/A	0.0592	*M. edulis*	(Smaal and Vonck, 1997)
N	N/A	N/A	N/A	0.082	0.125	0.1035	*Musculista senhousia*	(Magni et al., 2000)
P	N/A	N/A	N/A	0.506	0.675	0.5905	*Musculista*	(Magni et al., 2000)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
							senhousia	
P	37.2	84.2	61.4	N/A	N/A	0.3163	*M. edulis*	(Smaal and Vonck, 1997)
Pb	56	64	60	0.0251	0.0281	0.0266	*M. galloprovincialis*	(Fisher et al., 1996b)
S	75.5	79.8	76.5	0.009	0.02	0.0145	*M. edulis*	(Wang and Fisher, 1996b)
Se	39.2	79.4	59.3	0.0176	0.044	0.0308	*M. edulis*	(Baines et al., 2005)
Se	75	86	80.5	0.02	0.05	0.035	*M. edulis*	(Reinfelder et al., 1997)
Se	72	82	77	0.018	0.0216	0.0198	*M. edulis*	(Wang and Fisher, 1997)
Se	13.7	71.9	42.8	0.04	0.07	0.055	*M. edulis*	(Wang and Fisher, 1996a)
Se	61.5	84.4	72.95	0.05	0.06	0.055	*M. edulis*	(Wang and Fisher, 1996b)
Se	70	80	75	0.0552	0.0648	0.06	*M. edulis*	(Wang et al., 1995)
Se	27.5	33.9	30.7	0.03	0.05	0.04	*M. edulis*	(Wang et al., 1996c)
Zn	12.3	55	33.65	0.0304	0.0601	0.0453	*M. edulis*	(Baines et al., 2005)
Zn	6.17	6.53	6.35	0.0053	0.0054	0.0053	*M. edulis*	(Fisher and Teyssie, 1986)
Zn	15	19	17	0.0133	0.0655	0.0394	*M. galloprovincialis*	(Fisher et al., 1996b)
Zn	43	62	52.5	0.05	0.07	0.06	*M. edulis*	(Reinfelder et al., 1997)
Zn	20	26	23	0.0123	0.0141	0.0132	*M. edulis*	(Wang and Fisher, 1997)
Zn	16	48.3	32.15	0.1	0.14	0.12	*M. edulis*	(Wang and Fisher, 1996a)

Element	Minimum Assimilation Efficiency (%)	Maximum Assimilation Efficiency (%)	Mean Assimilation Efficiency (%)	Minimum Efflux Rate (d^{-1})	Maximum Efflux Rate (d^{-1})	Mean Efflux Rate (d^{-1})	Organism	Reference
Zn	34.6	49.9	42.25	0.1	0.13	0.115	*M. edulis*	(Wang and Fisher, 1996b)
Zn	20	50	35	0.12	0.1872	0.1536	*M. edulis*	(Wang et al., 1995)
Zn	31.9	41.1	36.5	0.04	0.07	0.055	*M. edulis*	(Wang et al., 1996c)

Table 1. Summary of reported ingestion and growth rates from the literature.

	Ingestion Rate (g g^{-1} d^{-1})	**Growth Rate (g g^{-1} d^{-1})**
	Mean (min, max)	**Mean (min, max)**
Daphnia	1.9 (0.7, 3.1)	0.3 (0.01 , 0.6)
Marine Copepods	0.43 (0.05, 1.2)	0.3 (0.02, 1.0)
Mytilids	0.16 (0.02, 0.6)	0.05 (0.004, 0.12)

Mean rates were calculated from reported mean values across studies. Minimum and maximum values are from the entire range of values reported from all studies. See Appendix A, Table A1 for complete database and references.

Table 2. Summary of assimilation efficiencies (*A*) and efflux rate constants (K_e) from published studies.

	Daphnia			***Copepods***			***Mussels***		
Element	***A*, %** **Mean (min, max)**	***K_e*, d^{-1}** **Mean (min, max)**	**N**	***A*, %** **Mean (min, max)**	***K_e*, d^{-1}** **Mean (min, max)**	**N**	***A*, %** **Mean (min, max)**	***K_e*, d^{-1}** **Mean (min, max)**	**N**
Ag	31.8 (1, 62.6)	0.51 (0.26, 0.75)	1	13.7 (2.8, 21.9)	0.55 (0.34, 1)	5	15.3 (1.8, 50)	0.24 (0.025, 0.72)	7
Am	ND	ND	ND	3.3 (0.4, 9)	0.85 (0.77, 0.93)	4	4.3 (0.6, 12.8)	0.22 (0.011, 0.43)	8
Cd	36 (8.8, 76.7)	0.32* (0.07, 1.13)	3	53.7 (13.2, 96)	0.59* (0.31, 1.28)	10	34.3 (8, 96)	0.05 (0.0008, 0.22)	8
Co**	ND	ND	ND	25.8 (14.2, 48)	0.74 (0.38, 0.95)	2	28.1 (8, 68.3)	0.22 (0.01, 0.46)	8
Cr-III	26 (8.3, 43.7)	1.22* (0.12, 2.32)	1	ND	ND	ND	0.9 (0.2, 1.3)	0.21 (0.15, 0.26)	2
Cr-VI	ND	ND	ND	ND	ND	ND	5.8 (1.1, 10.4)	0.26 (0.22, 0.29)	1
Cu	ND	ND	ND	40.3 (29.9, 61.1)	0.07 (0.06, 0.08)	2	ND	ND	ND
Fe	ND	ND	ND	15.4 (5, 35)	0.06 (0.04, 0.08)	3	ND	ND	ND
Hg-II	21 (9.1, 47)	1.31* (0.55, 2.28)	2	18.3 (14, 39.7)	ND	4	42.5 (41,44)	0.19 (0.15,0.24)	1

	Daphnia			*Copepods*			*Mussels*		
Element	***A*, % Mean (min, max)**	***K_e*, d^{-1} Mean (min, max)**	**N**	***A*, % Mean (min, max)**	***K_e*, d^{-1} Mean (min, max)**	**N**	***A*, % Mean (min, max)**	***K_e*, d^{-1} Mean (min, max)**	**N**
MeHg	89 (64.2, 97.4)	0.095 (0, 0.22)	3	62	ND	1	85.5 (84, 87)	0.03 (0.02, 0.04)	1
Mn	ND	ND	ND	14.5 (3, 30)	ND	2	ND	ND	ND
N	ND	0.88* (0.07, 3.22)	5	56.4 (13.6, 99.1)	0.13 (0.05, 0.43)	5	60.9 (27.8, 84.4)	0.08 (0.06, 0.13)	1
P	73.5 (34, 98)	0.78* (0.08, 3.42)	7	64.8 (19.3, 99.2)	0.25 (0.06, 0.48)	4	61.4 (37.2, 84.2)	0.45 (0.32, 0.68)	1
Pb	ND	ND	ND	ND	ND	ND	60 (56, 64)	0.026 (0.025, 0.028)	1
Po	ND	ND	ND	37.4 (19.6, 55.1)	0.03 (0.01, 0.07)	1	ND	ND	
Pu	ND	ND	ND	0.8 (0.6, 1.2)	ND	1	ND	ND	
S	ND	ND	ND	56.9 (49.4, 64.3)	ND	1	76.5 (75.5, 79.8)	0.0145 (0.009, 0.02)	1
Se	36 (15.6, 64.6)	0.45 (0.16, 0.82)	3	70.2 (7.3, 98.7)	0.58* (0.08, 1.5)	7	62.6 (13.7, 86)	0.04 (0.02, 0.07)	7
Zn	15 (1.4, 66.5)	0.77* (0.3, 1.45)	3	48.8 (7.2, 80)	0.51* (0.21, 1.26)	11	30.9 (6.2, 62)	0.07 (0.005, 0.19)	9

Mean *A* and K_e were calculated from mean values across studies. When not reported, mean K_e and *A* values were calculated from the minimum and maximum values reported. Minimum and maximum values shown are from the entire range of values reported from all studies. Efflux rate constants were capped at 1.0 d^{-1} in all analyses. N = number of studies. ND = no data. See Appendix A, Table A2 for complete database and references. *Mean K_e (d^{-1}) used in model analyses for *Daphnia*: Cd, 0.30; Cr, 0.56; Hg-II, 0.82; N, 0.49; P, 0.50; Zn, 0.68; and Copepods: Cd, 0.56; Se, 0.52, Zn, 0.48. **Studies used inorganic cobalt.

Figures

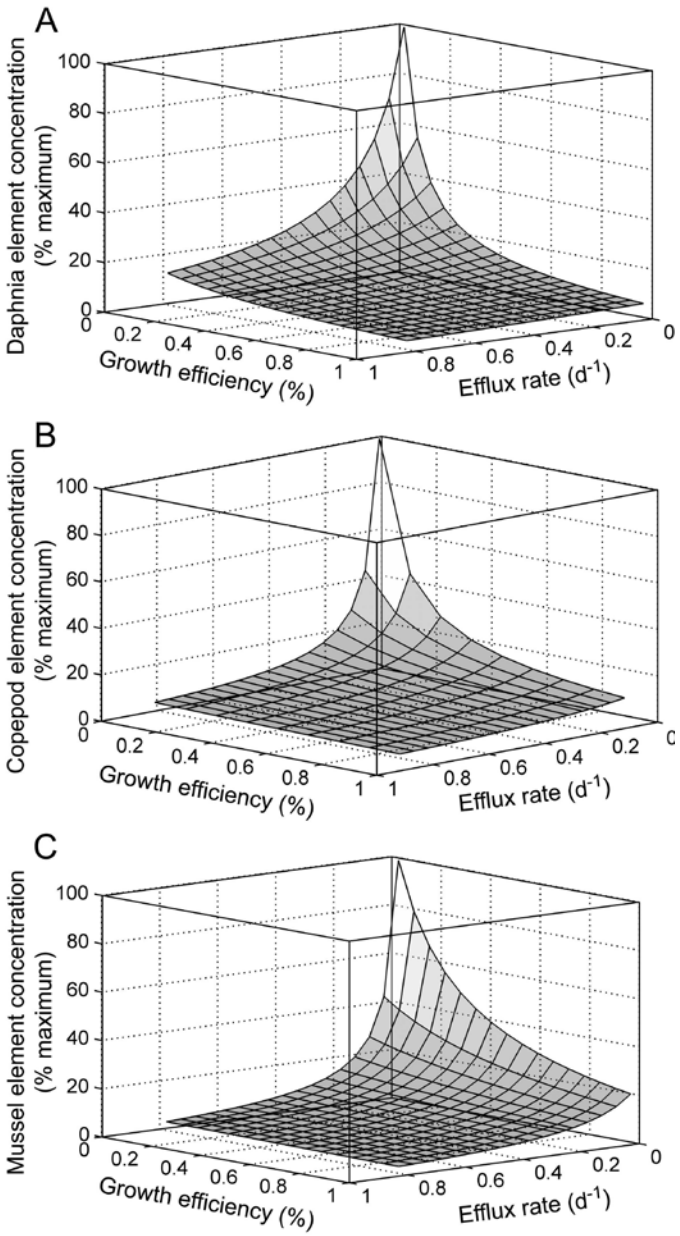

Figure 1. Element concentration response to growth efficiency and efflux. Steady state element concentrations in (A) *Daphnia*, (B) marine copepods and (C) mytilid mussels over a range of growth efficiencies and efflux rates.

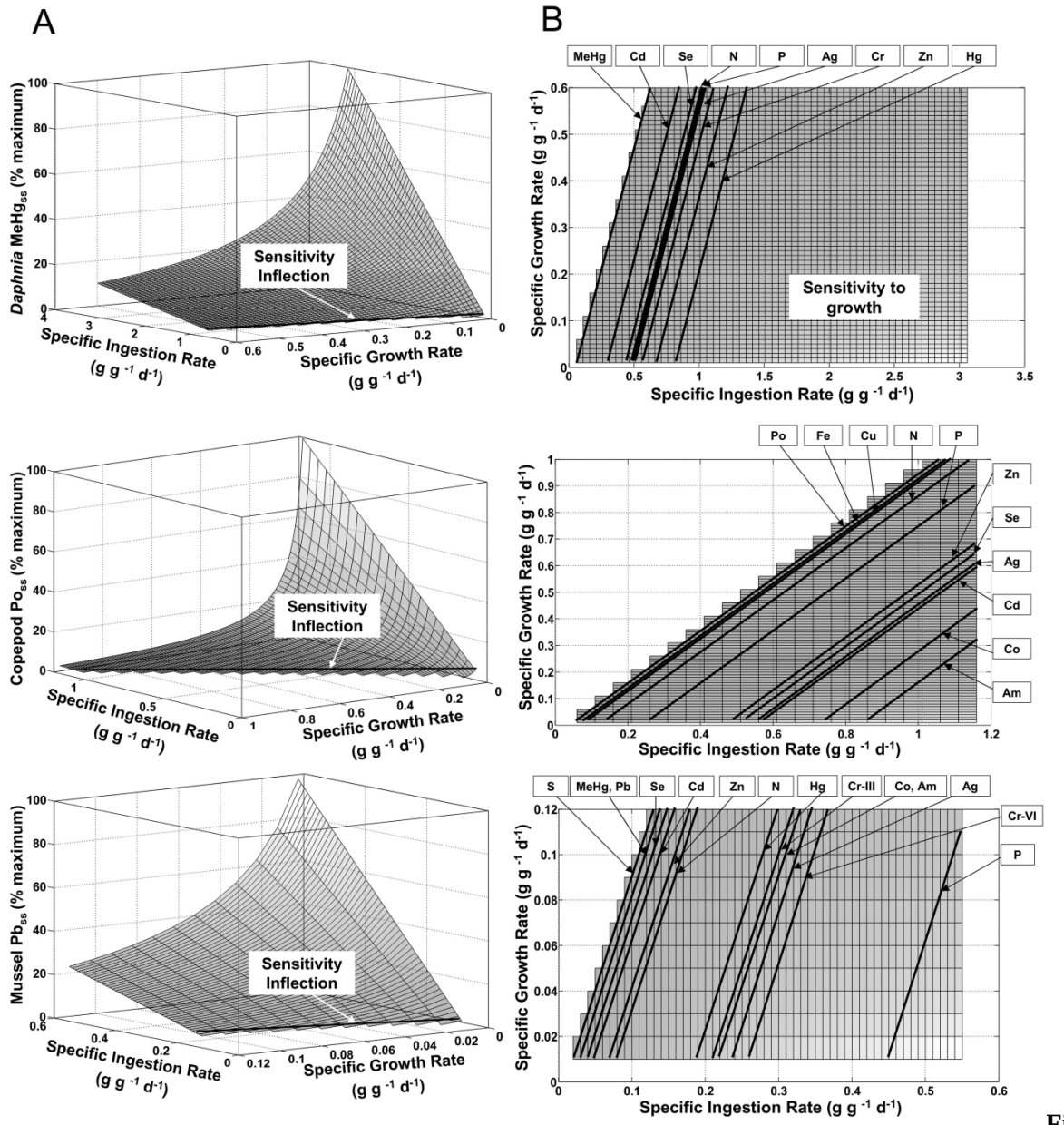

Figure 2. A (Left): Steady state element concentration in response to growth and ingestion rates. 'Sensitivity inflection' lines ($I = Ke + g$) indicate where element concentration is equally sensitive to growth and ingestion. Element concentration is more sensitive to ingestion above the line ($I < Ke + g$) and more sensitive to growth below the line ($I > Ke + g$). Nonessential metals with the lowest efflux rates (MeHg in *Daphnia*, Po in copepods, and Pb in mussels) are shown for comparison. For each animal, the same qualitative relationships were observed for all elements while the position of the inflection line varied. **B (Right): Sensitivity inflection lines for each element.** For each element, steady-state element concentration is relatively sensitive to growth to the right of the line and relatively sensitive to ingestion to the left of the line. The x-intercept of each inflection line is equivalent to element efflux rate (d^{-1}), increasing from left to right.

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Section II: Gregory A. Cutter

Metal Geochemistry in Sediments

Overview

The specific objectives for the geochemical part of this project using sediments from 3 sites are:

(1) Employ a unique combination of sediment leaching techniques, combined with careful measurement of ancillary parameters to establish the identity of the phases being solubilized (e.g., Ca in the carbonate leach, Fe and S in the AVS leach), to characterize as accurately as possible the phase speciation of As, Cd, Cr, and Pb in contaminated estuarine sediments.

(2) Utilize these phase speciation, total metal concentrations, and ancillary data as a function of sediment depth to describe the diagenesis of the four metals in contaminated sediments.

(3) Acquire sufficient phase speciation data, and data on metal concentrations in selected biota from the field sites (bivalves and polychaetes), to test the bioenergetic/kinetic model (see Fisher section).

(4) Assist the bioaccumulation effort in verifying that the phase speciation of the metals in the bioavailability experiments closely mimic those in the field.

(5) Help integrate the field data and lab studies to quantitatively describe the movement of sedimentary metals into benthic animals (processes and rates).

In 2008, the major task was to complete the phase speciation leaches and analyses of the leachate solutions for all of the sediments from the 3 field sites (2 stations per site): Elizabeth River (Virginia), Baltimore Harbor (Maryland), and Mare Island (California).

Sediment Phase Leaching Methods

The overall goal for the sediment phase leaches is to be able to quantify the concentrations of metals in the following phases (in this sequential order): exchangeable (easily adsorbed/desorbed), carbonates, iron sulfides, iron and manganese oxides, organic matter, and pyrite. Because the sediment phases can be altered by exposure to air, the first steps of this leaching scheme (all but the organic matter and pyrite leaches) are done in a nitrogen-purged glove box built especially for this project. The leaching sequence is shown in **Figure 1**, and we continue to monitor the tracer elements (e.g., Ca. Fe, Mn, S) to check recoveries and overall leaching efficiencies (via comparisons of total sedimentary metals with the sum of the leaches).

Elizabeth River (Virginia) Results

The Elizabeth River was selected for its organic-rich sediments in a mid-salinity estuarine environment with high contaminant metal concentrations due to military and industrial operations. Results for the Elizabeth River sediments are shown in **Figures 2-12**. Organic carbon concentrations ranged from 3-4%, nitrogen from 0.1-0.3%, and sulfur goes from a minimum of 0.5% near the surface to almost 4% at depth (**Figs. 2-3**), consistent with an accumulation of pyrite-S through anoxic diagenesis. The C:N ratios at both stations are well-above the classic marine Redfield value of 6.6, and indicate a large amount of terrigenous organic matter in these sediments.

The phase speciation (leach) data are shown in **Figures 4-12**. At both stations arsenic is primarily associated with the pyrite phases, with occasionally small fractions in the exchangeable phase (**Figs. 4** and **8**). In contrast, sedimentary cadmium is found in all phases except the exchangeable (**Figs. 5** and **9**), with the majority being in the AVS and organic phases at Station 1 (**Fig. 5**), while AVS and pyrite, then organic matter, having the most Cd at Station 2 (**Fig. 9**). Although it might seem contradictory that sulfides dominate the upper-most sediments, the oxic layer was only 2 cm deep, and the sediment itself was highly reworked (physically mixed by currents, dredging, etc), which could mix reduced sediments throughout the core. Indeed, the speciation patterns are rather constant with depth, suggesting considerable physical mixing. Chromium (**Figs. 6** and **10**) is very much like cadmium in that it is found in virtually every sediment phase, but in this case, the organic phase contains the most chromium. The relative amounts of chromium in the AVS, oxide, and pyrite phases are nearly constant with depth, suggesting little diagenesis in the time frame represented in these two cores. The phase speciation of lead at the two Elizabeth Rivers sites (**Figs. 7** and **11**) is dominated by AVS, with lesser amounts in the organic and oxide phases; lead is also found in the pyrite phase, but at very low concentrations.

Baltimore Harbor (Maryland) Results

Baltimore Harbor was selected as the second site due to its highly metal-contaminated sediments, the low salinity conditions in the upper Chesapeake Bay (potentially affecting the abundance of sulfur phases), and sediments rich in carbon. The CNS data in **Figures 12** and **13** show that these sediments provide a good contrast to those in the Elizabeth River. In particular, organic carbon ranges from a high of 5% to a low of ca. 2%, almost twice those in the Elizabeth River sediments (**Figs. 2** and **3**), while the C:N data (**Figs. 12** and **13**) show a similarly dominant terrestrial source (i.e., well above the marine Redfield ratio of 6.7). As expected, the total concentrations of sedimentary sulfur are lower than those in the more saline Elizabeth River, although they are still elevated relative to freshwaters. Whether this has an effect on metal phase speciation will be examined next.

The Baltimore Harbor metal phase speciation results are found in **Figures 14-21**. Although arsenic speciation at depth (**Fig. 14**) and in Station 2 (**Fig. 18**) is dominated by pyrite like the Elizabeth River (**Figs. 4** and **8**), the surprising observation is the predominance of As in organic phases at Station 1 (**Fig**. **14**) where the highest organic carbon values are also located (**Fig. 12**). At Station 1 cadmium is primarily bound in AVS and organic phases, with smaller but constant amounts in the pyrite and organic phases (**Fig. 15**); this contrasts slightly with Station 2 where the organic phase dominates, flowed by AVS, then pyrite (**Fig. 19**). In general, these phase speciation data for Baltimore are essentially identical to those in the Elizabeth River (**Figs. 5** and **9**). The first thing to notice about the chromium data (**Figs. 16** and **20**) is that the total concentrations (sum of all the phases) are 2-5 times those in the Elizabeth River and are likely derived from the steel plant near Station 1. At Station 1, chromium is evenly distributed between the organic and AVS phases (with smaller amounts in the oxide phase; **Fig. 16**), while at Station 2 (**Fig**. **20**) organic matter is the primary phase, followed by AVS then oxides. This observed behavior is almost identical to that in the Elizabeth River (**Figs. 6** and **10**). Lead in Baltimore Harbor is clearly found primarily in the AVS phase, while oxides and organic matter also contain some lead (**Figs. 17** and **21**). Like the other 3 metals, this behavior parallels that in the Elizabeth River (**Figs.7** and **11**).

Mare Island (California) Results

Mare Island in San Francisco Bay was sampled to acquire sediments from a higher salinity, low carbon environment with a long legacy of metal contamination (albeit much lower than Baltimore Harbor) from the Naval Shipyard. The results in **Figures 22** and **23** show the lowest carbon, nitrogen and sulfur concentrations of any of the sites. The C:N ratios are the lowest of the 3 sites, but whether this indicates more marine organic matter cannot be ascertained without carbon isotope data. Certainly the major composition of these sediments contrasts strongly with the east coast sites and may influence the phase speciation of the metals.

Although there are no data for arsenic at Station 2 due to recovery problems (samples currently being rerun), those from Station 1 (**Fig. 24**) are identical to those from the Elizabeth River and Baltimore Harbor – arsenic is primarily found in the pyrite phase. The phase speciation of cadmium at both stations is nearly the same, with organic matter having slightly more cadmium than AVS, and trace amounts in oxides and pyrite (**Figs. 25** and **28**). This contrasts sharply with the Elizabeth River and Baltimore results (**Figs. 5**, **9**, **15**, and **19**), where AVS and pyrite dominated (Baltimore Station 2 did have more Cd in the organic phase). Thus, the absolute amount of organic matter (Mare Island being the lowest) does not necessarily influence the phase speciation. The distributions of chromium in the sediment phases of Mare Island (**Figs. 26** and **29**) are the same as those in the Elizabeth River stations (**Figs. 6** and **10**) and Baltimore Harbor Station 2 (**Fig. 20**), with most chromium in the organic phase and the remaining amount being nearly equally divided between the AVS, oxide, and pyrite phases. Lead in Mare Island sediments (**Figs. 27** and **30**) is almost exclusively in the AVS phase, with small amounts in the oxide and organic phases as well. This pattern is identical to those in all the other sites.

Summary

The leaching scheme developed for this project is revealing speciation patterns for the four metals of interest that diverge widely and should provide a range of bioavailability potentials to the benthic invertebrates being studied in the Fisher lab. Further, the data suggest that the widely varying geochemical characteristics at the sites do affect the speciation of the metals, but not in a profound manner. The patterns are rather consistent for each of the metals, which may facilitate predictions of bioavailability for a given environment and metal. What remains to be done in the geochemical portion of the project is to complete determinations of metal concentrations in the resident biota at each of the sites so that these data can be used to

ground-truth the lab experiments. Determinations of metal concentrations in the overlying and pore waters must also be completed to evaluate this uptake pathway.

Sediment Phase Speciation Methods
(developed by this project)

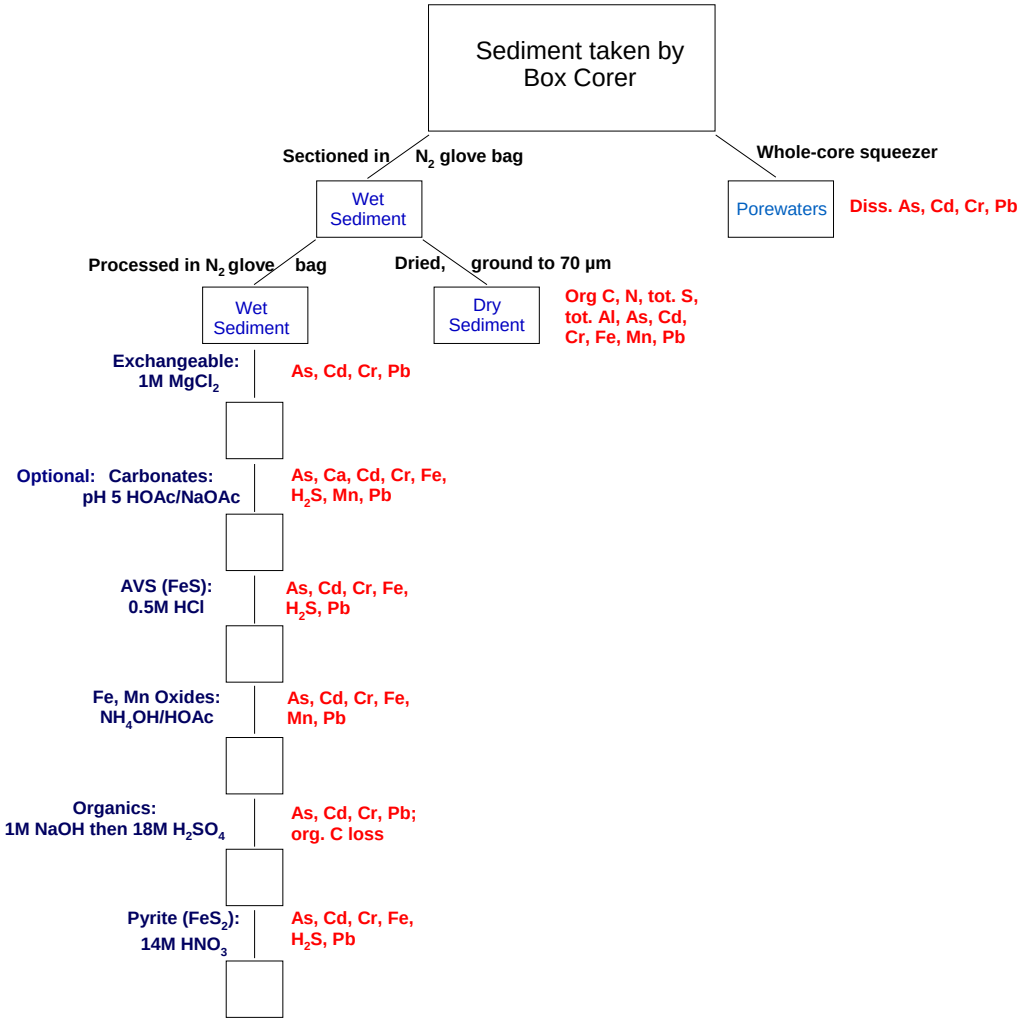

Figure 1. Sediment phase leaching sequence. Each step and its reagents are listed on the left in blue, while the parameters determined in the leach solutions are listed on the right (red). The carbonate phase leach is omitted if no carbonates are present.

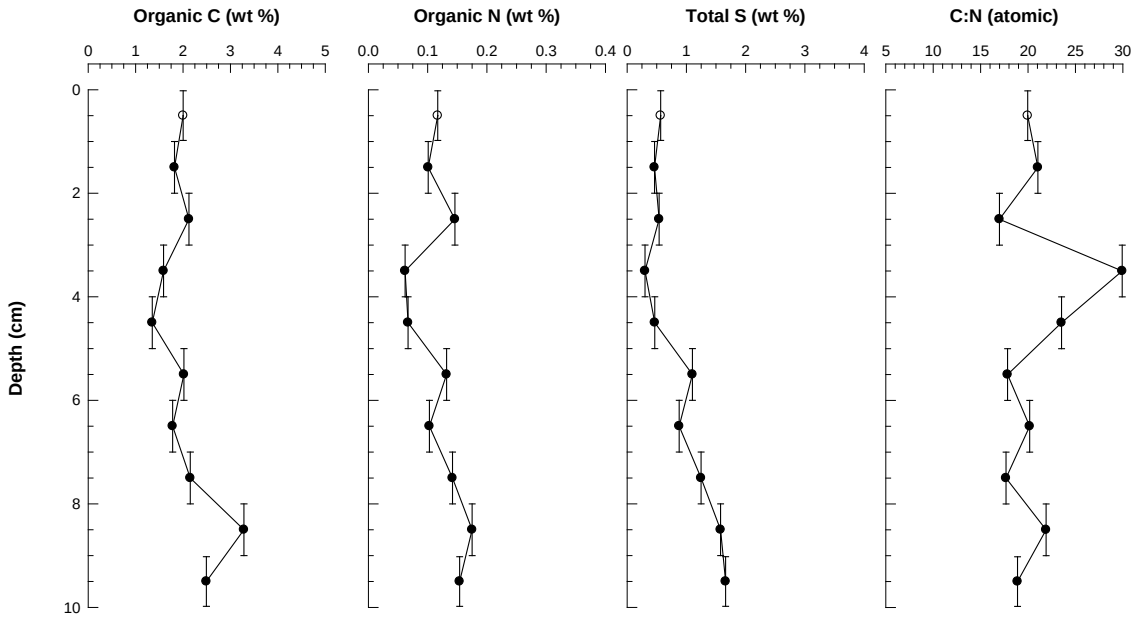

Figure 2. Depth profiles of CNS and C:N in sediments from Elizabeth River Station 1.

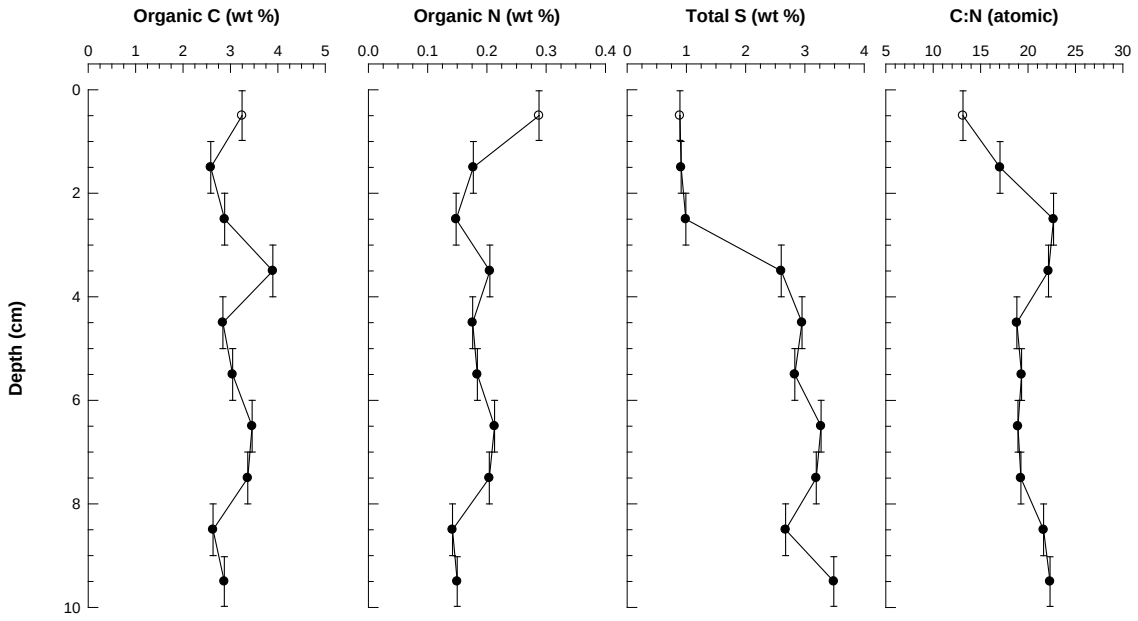

Figure 3. Depth profiles of CNS and C:N in sediments from Elizabeth River Station 2.

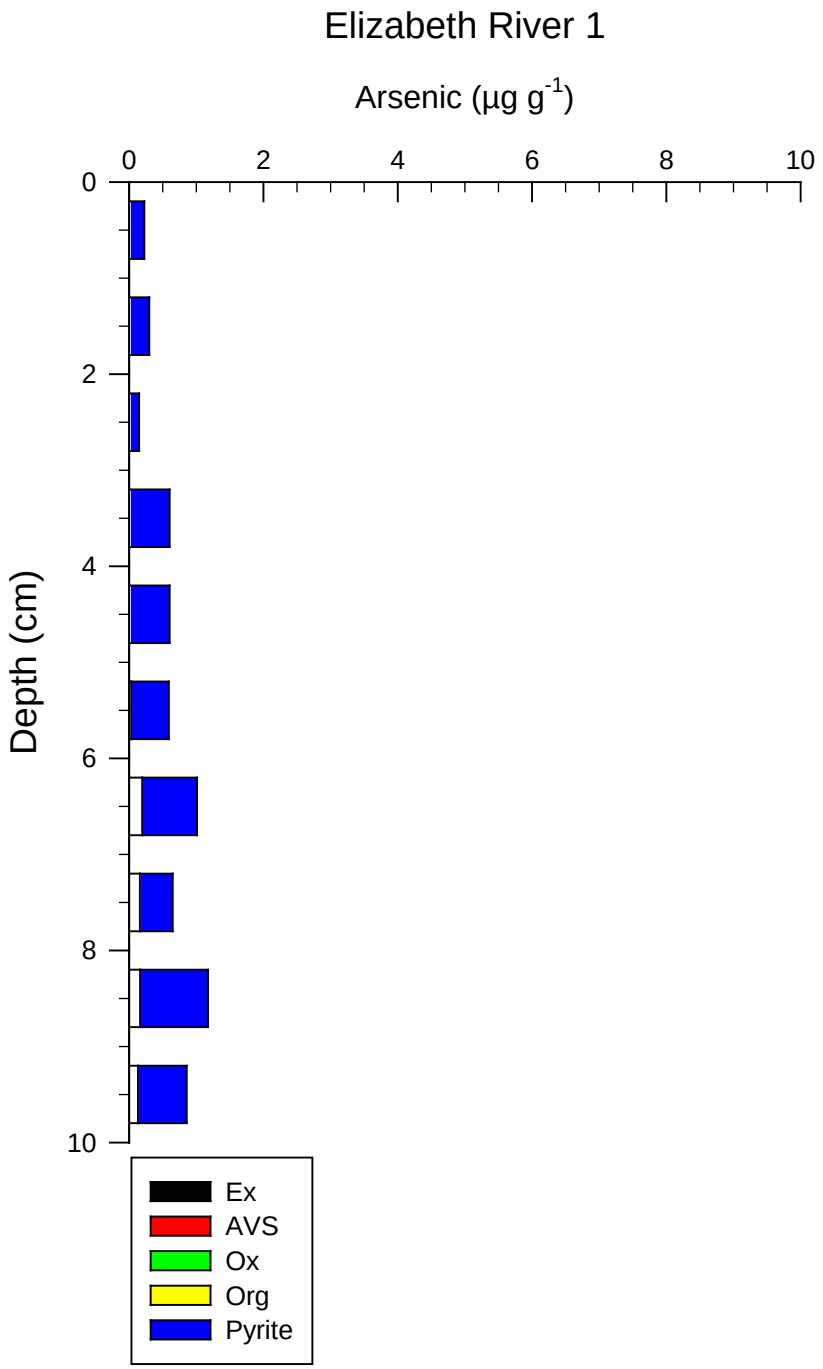

Figure 4. Depth profile of arsenic phase speciation in sediments of Elizabeth River Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

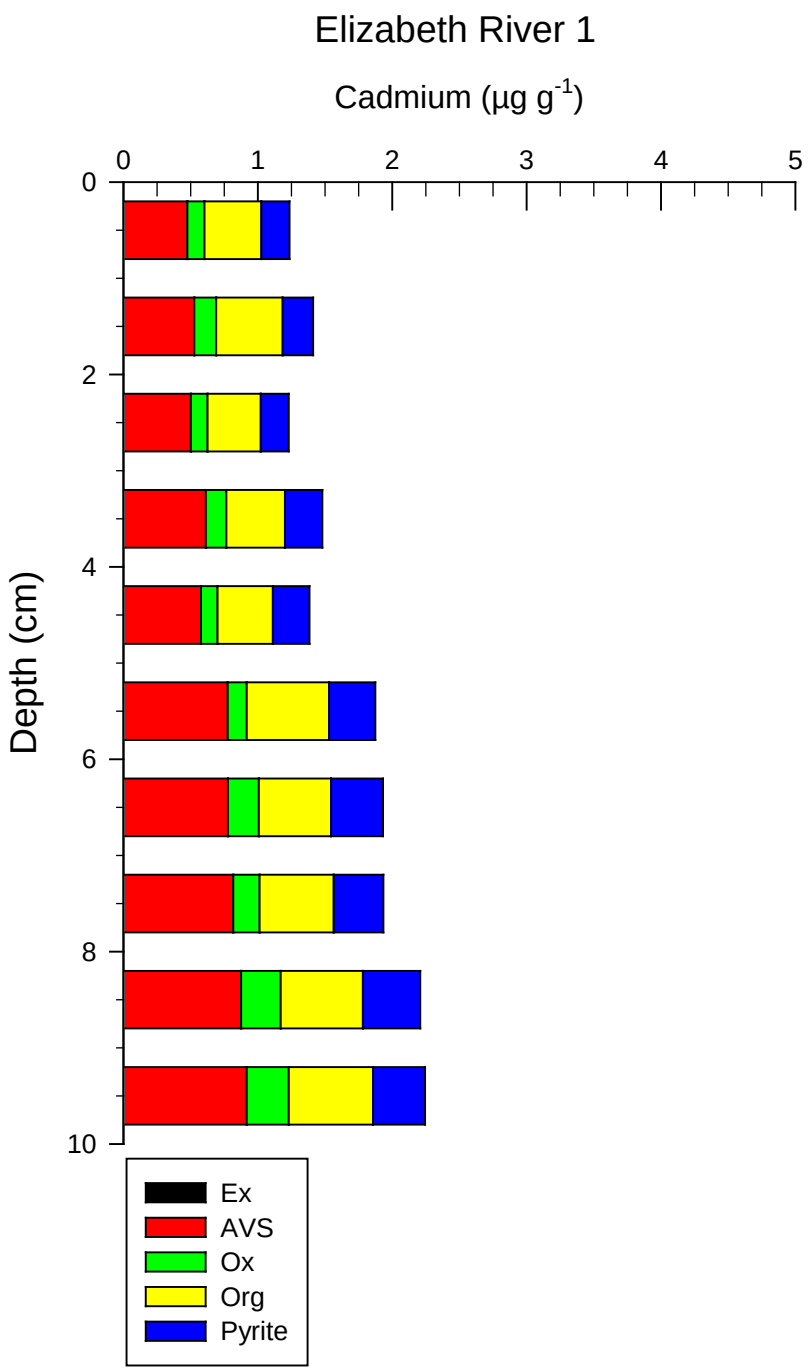

Figure 5. Depth profile of cadmium phase speciation in sediments of Elizabeth River Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

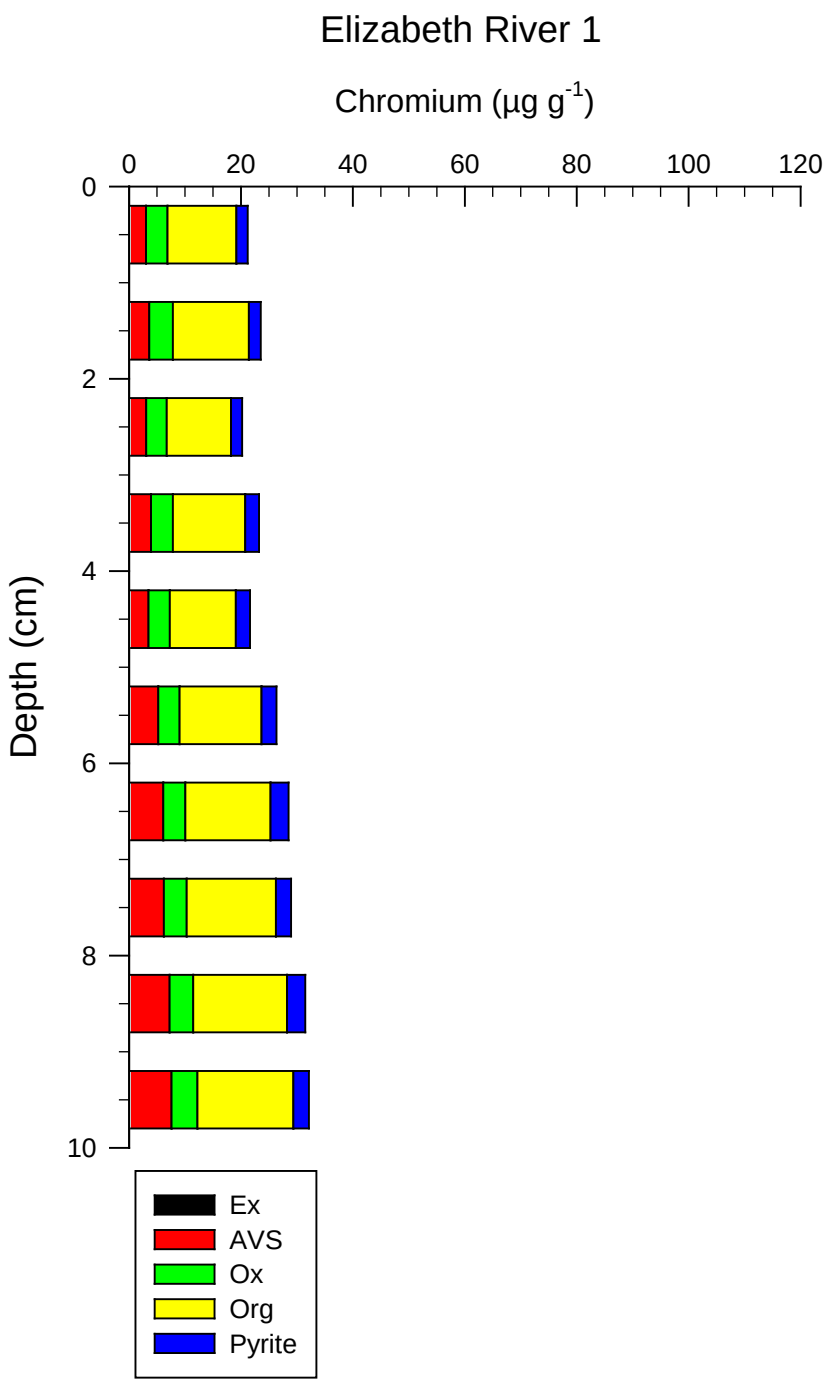

Figure 6. Depth profile of chromium phase speciation in sediments of Elizabeth River Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

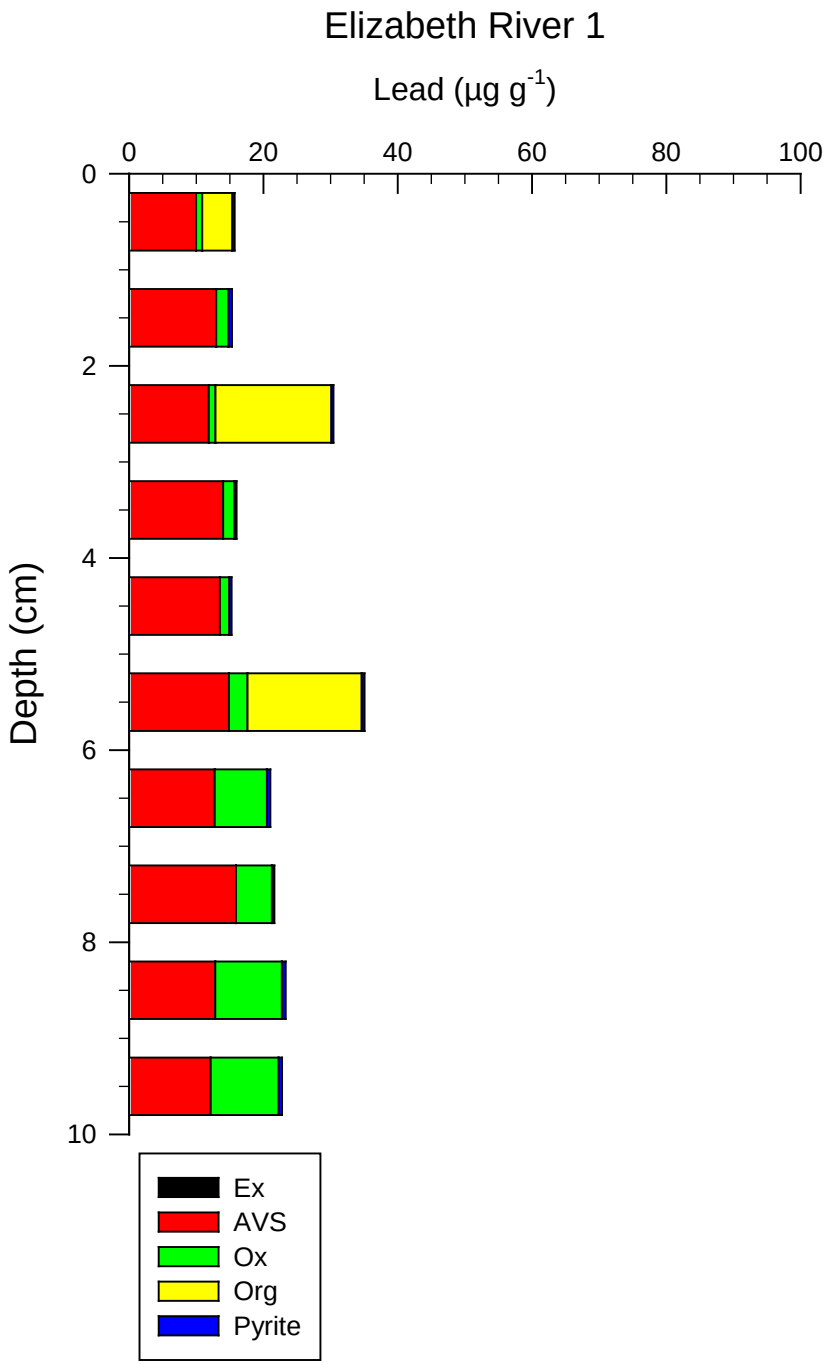

Figure 7. Depth profile of lead phase speciation in sediments of Elizabeth River Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

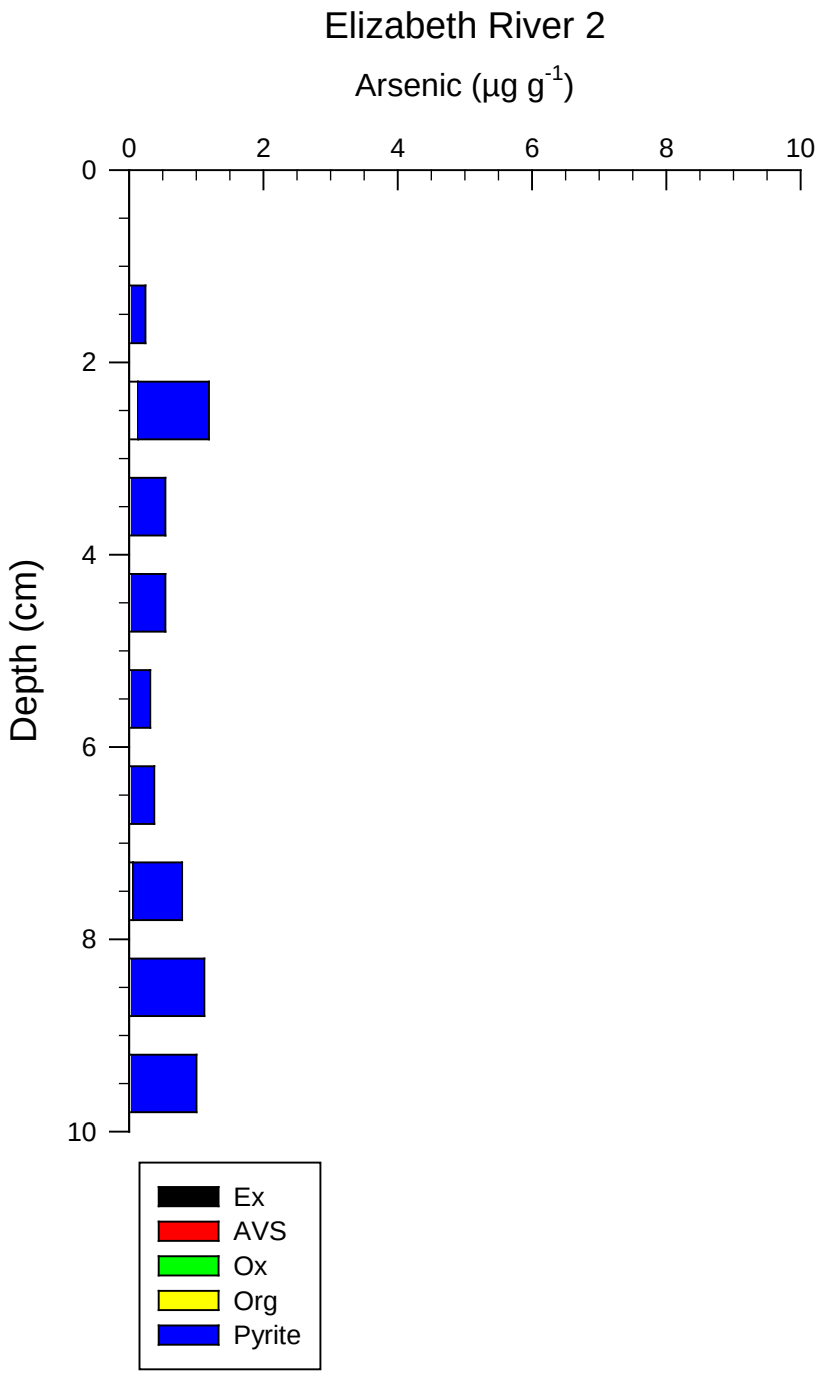

Figure 8. Depth profile of arsenic phase speciation in sediments of Elizabeth River Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

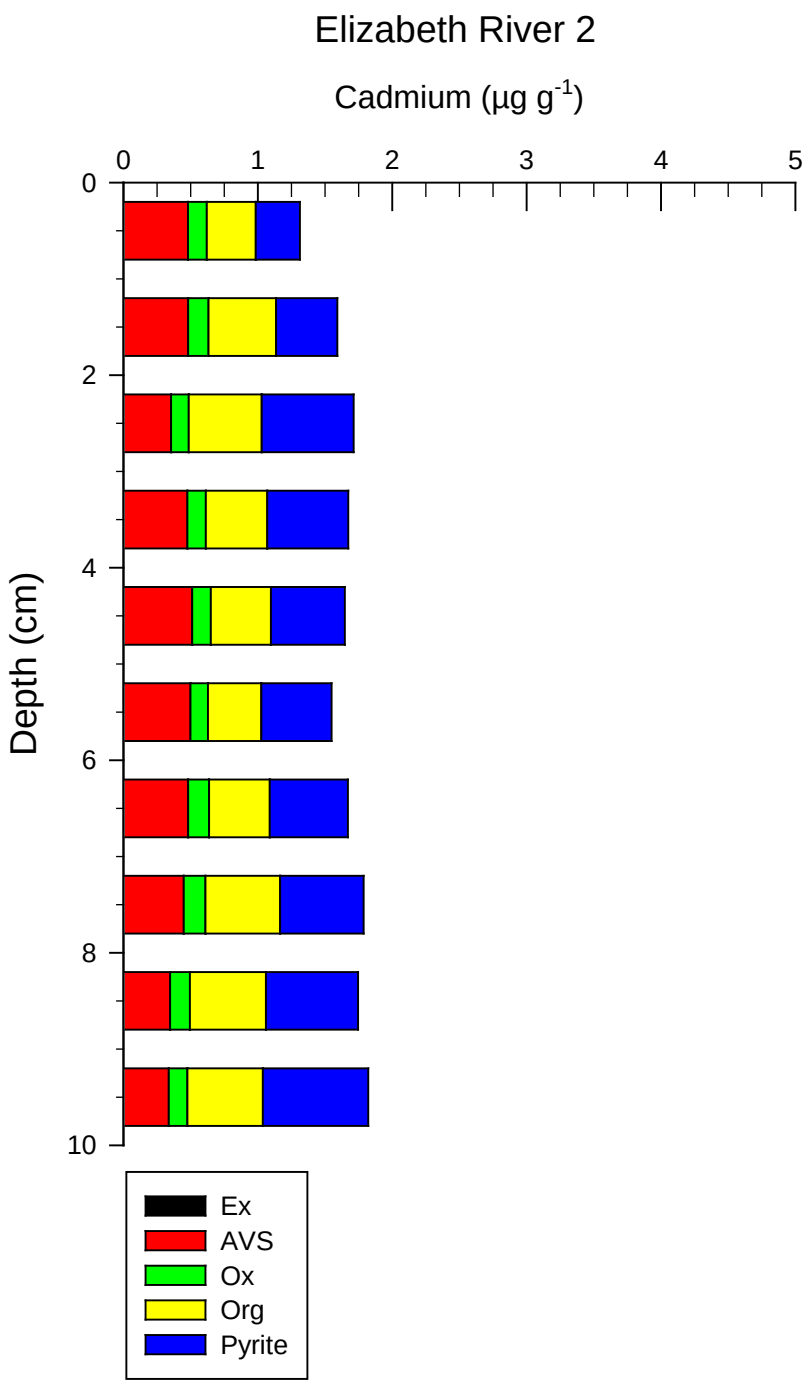

Figure 9. Depth profile of cadmium phase speciation in sediments of Elizabeth River Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

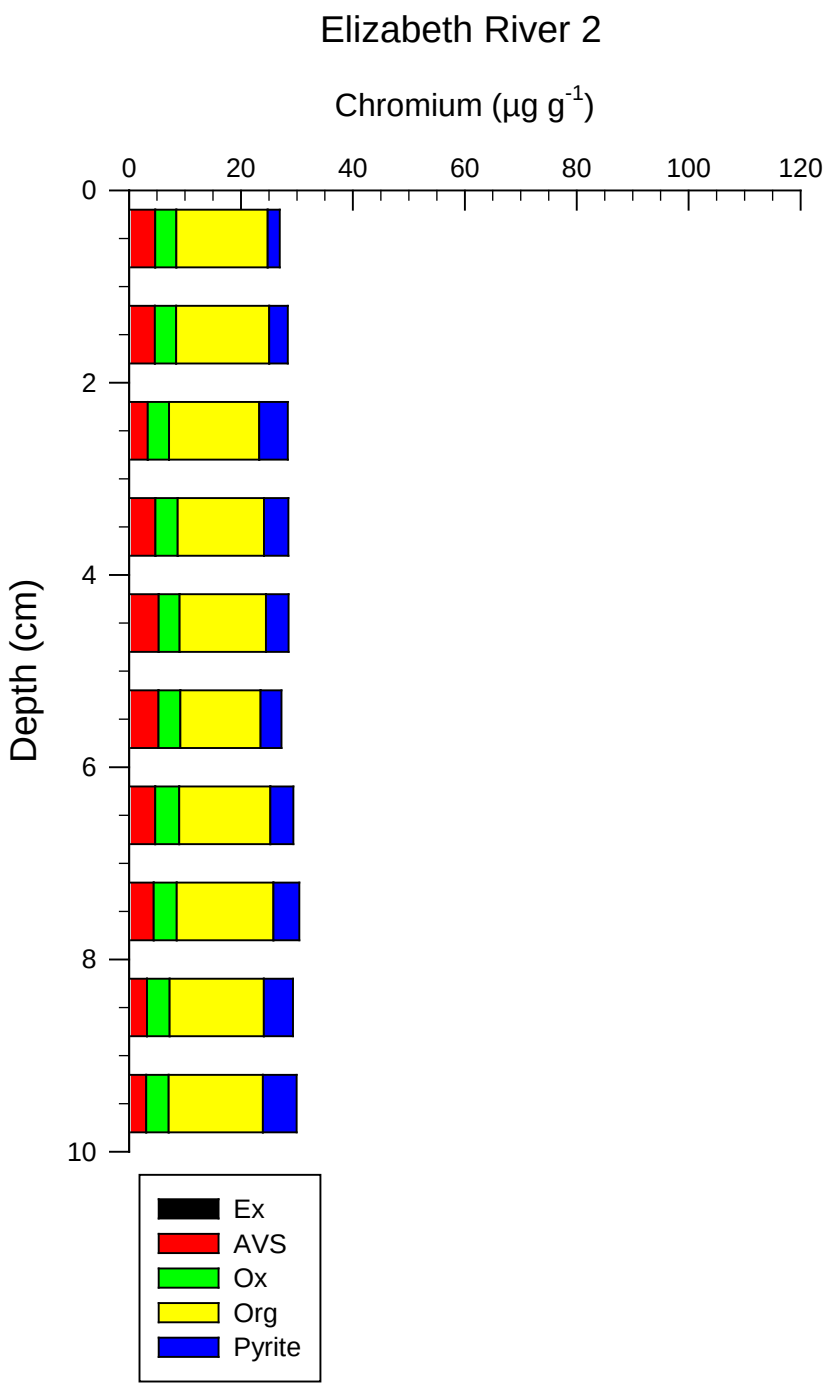

Figure 10. Depth profile of chromium phase speciation in sediments of Elizabeth River Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

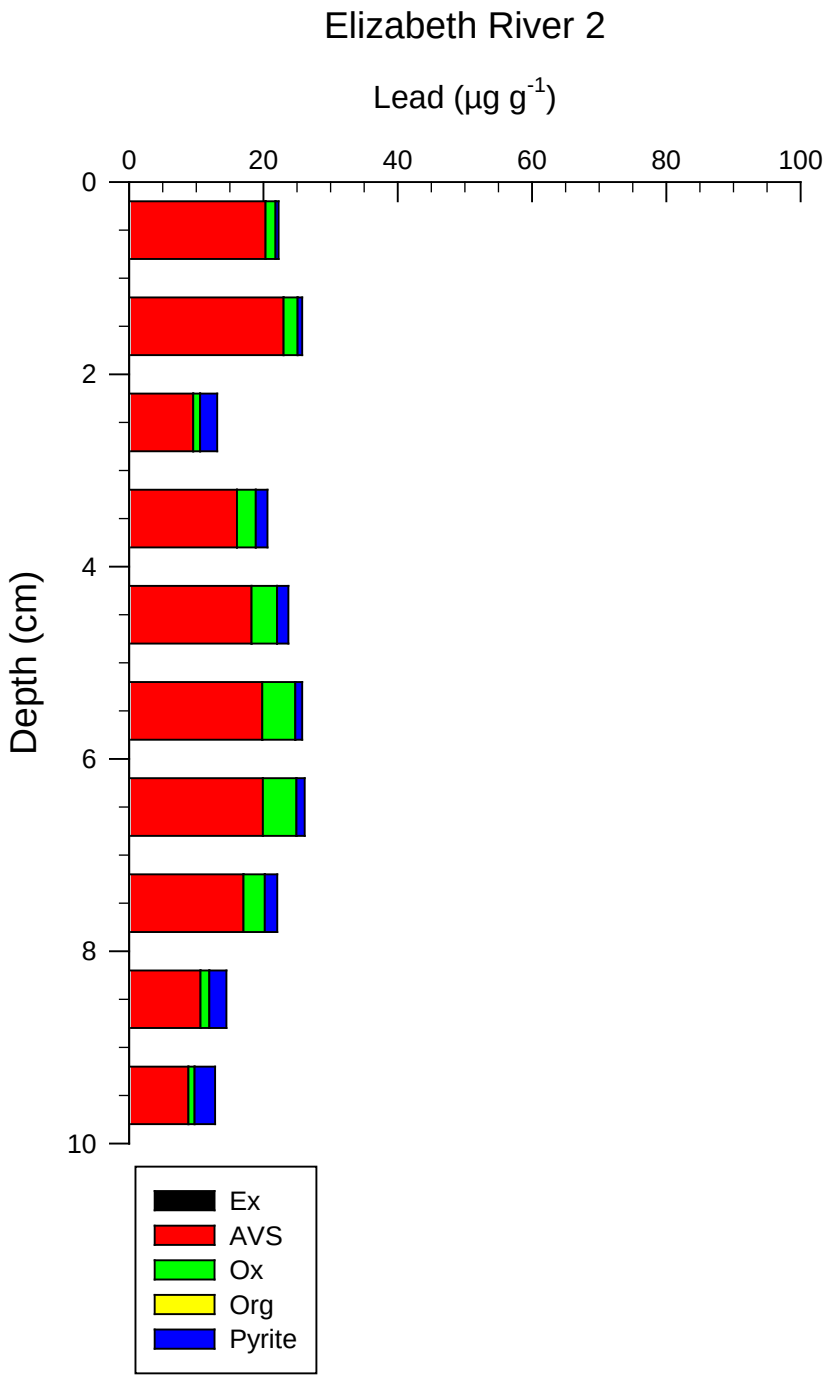

Figure 11. Depth profile of lead phase speciation in sediments of Elizabeth River Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

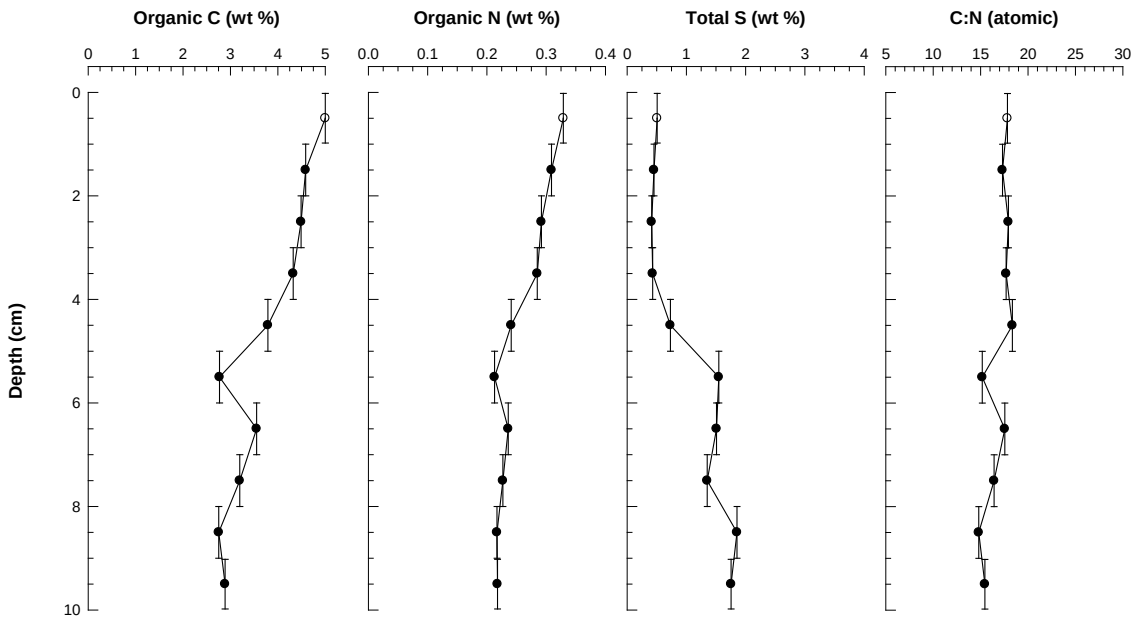

Figure 12. Depth profiles of CNS and C:N in sediments from Baltimore Harbor Station 1.

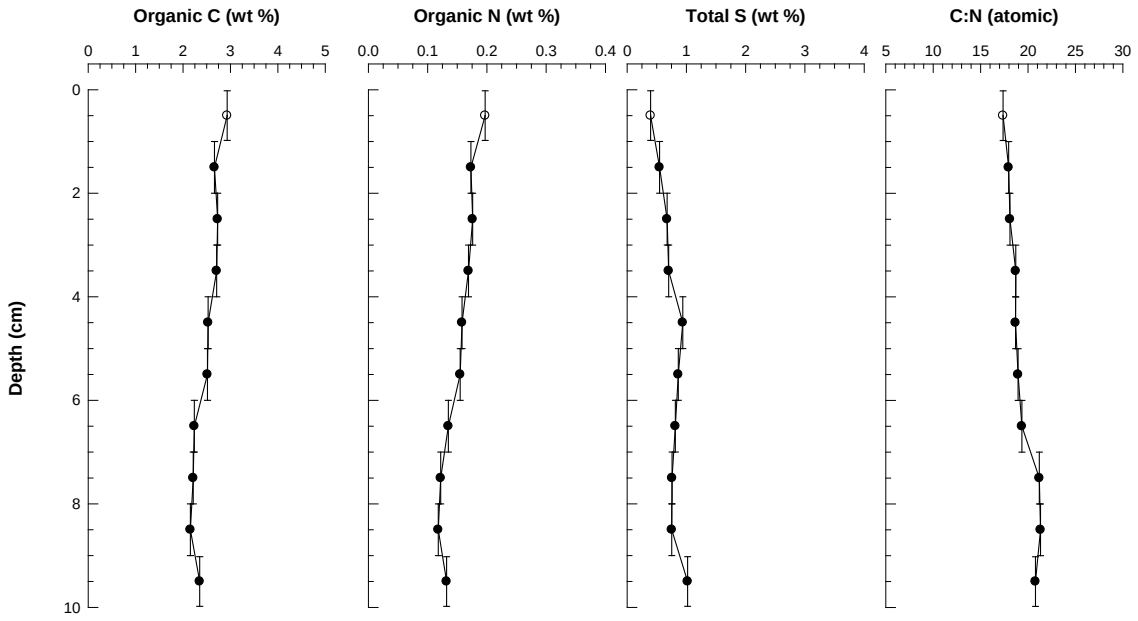

Figure 13. Depth profiles of CNS and C:N in sediments from Baltimore Harbor Station 2.

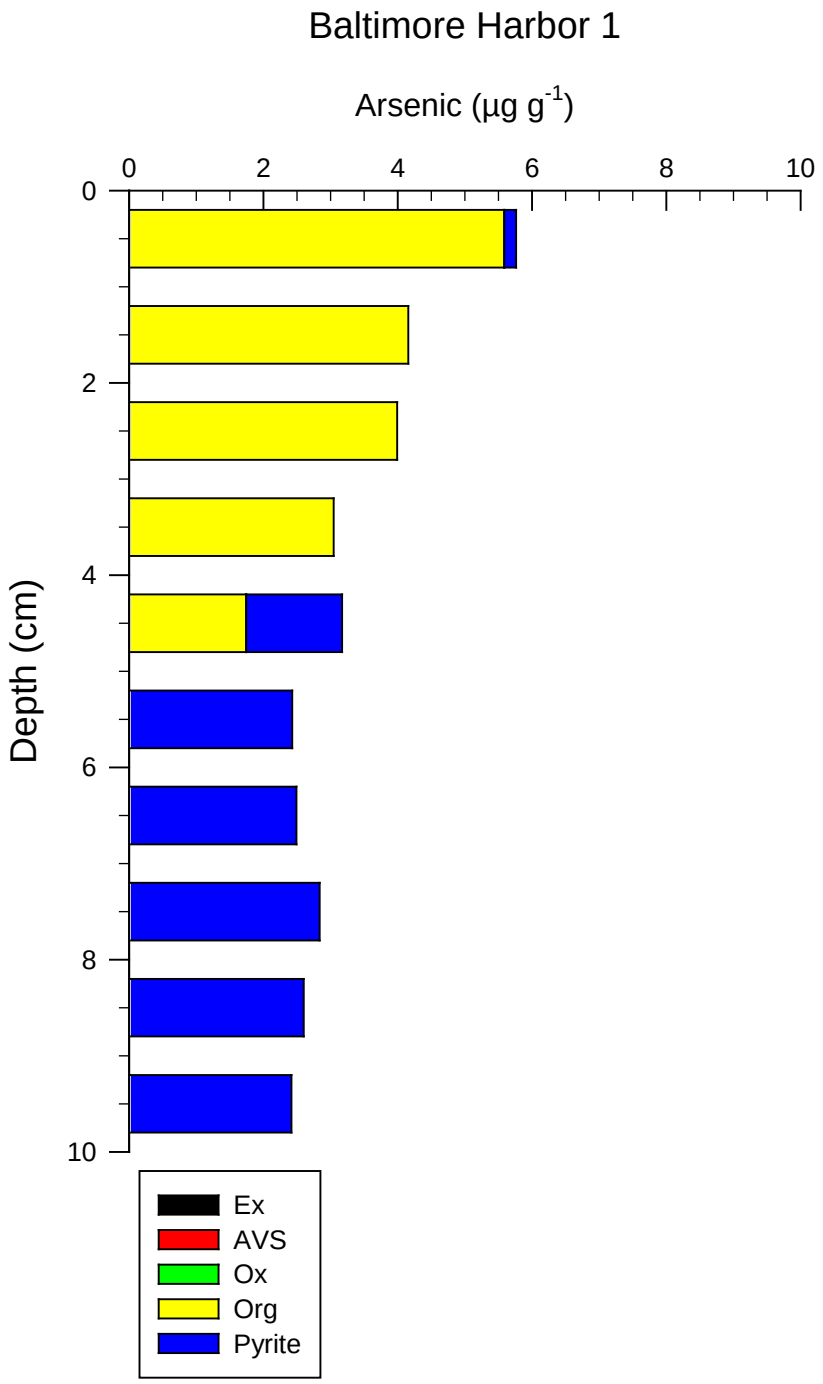

Figure 14. Depth profile of arsenic phase speciation in sediments of Baltimore Harbor Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

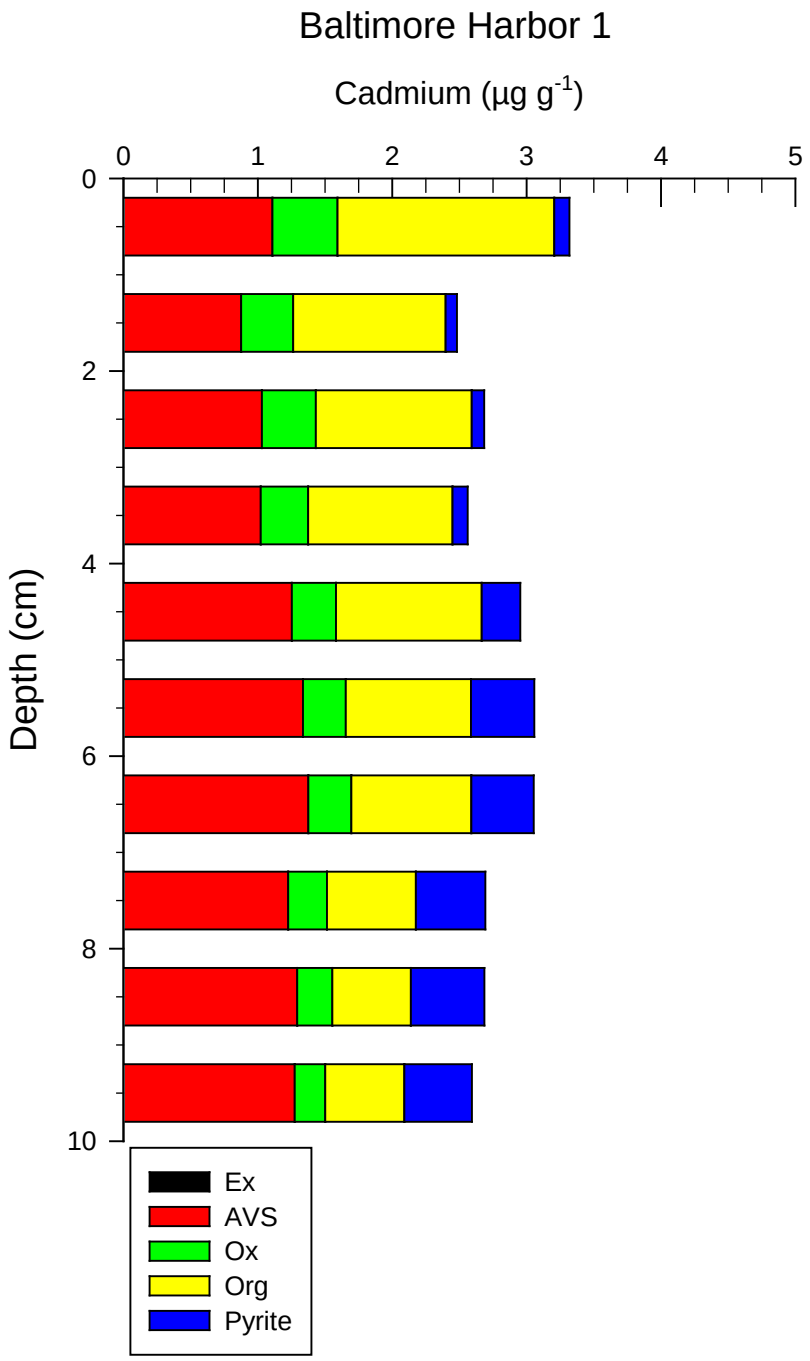

Figure 15. Depth profile of cadmium phase speciation in sediments of Baltimore Harbor Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

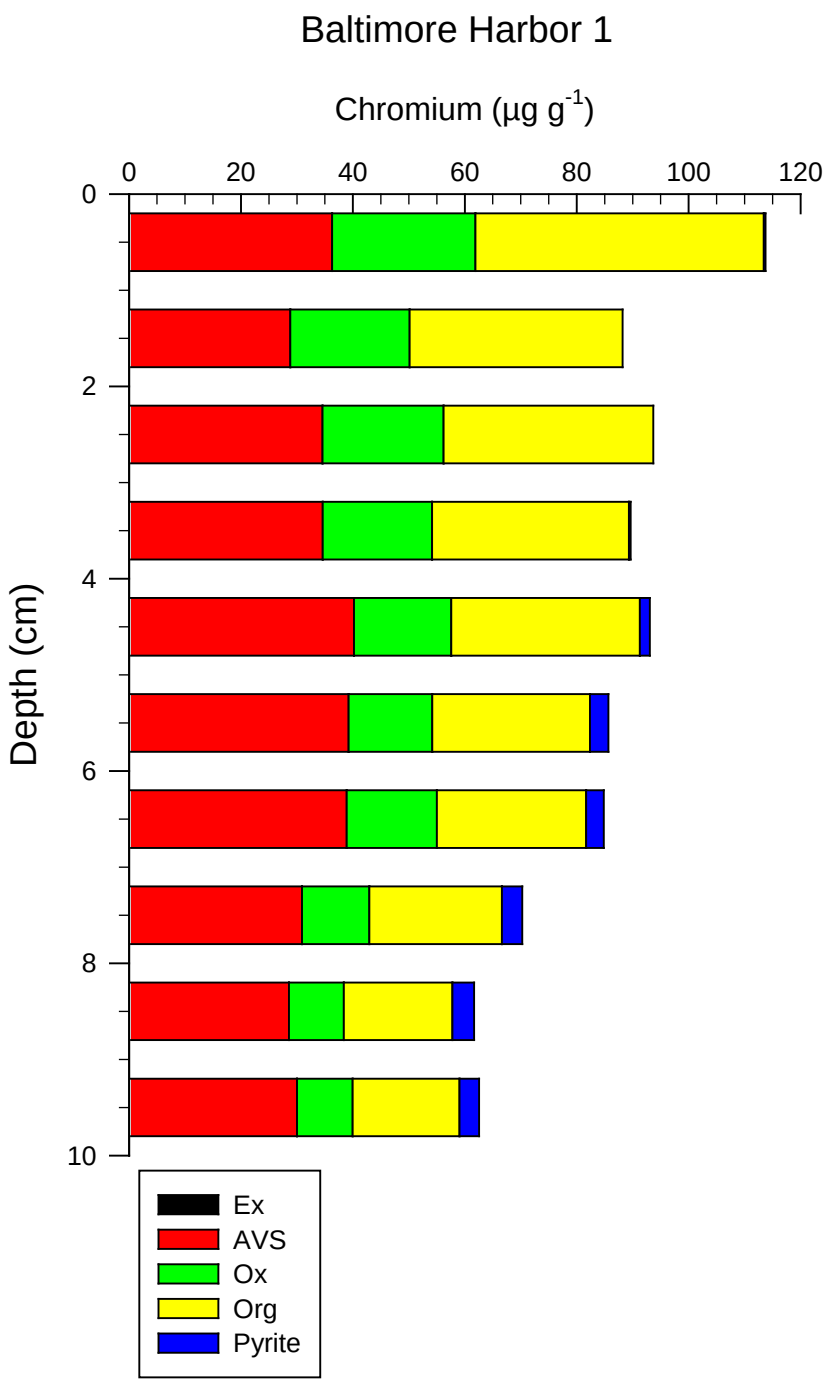

Figure 16. Depth profile of chromium phase speciation in sediments of Baltimore Harbor Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

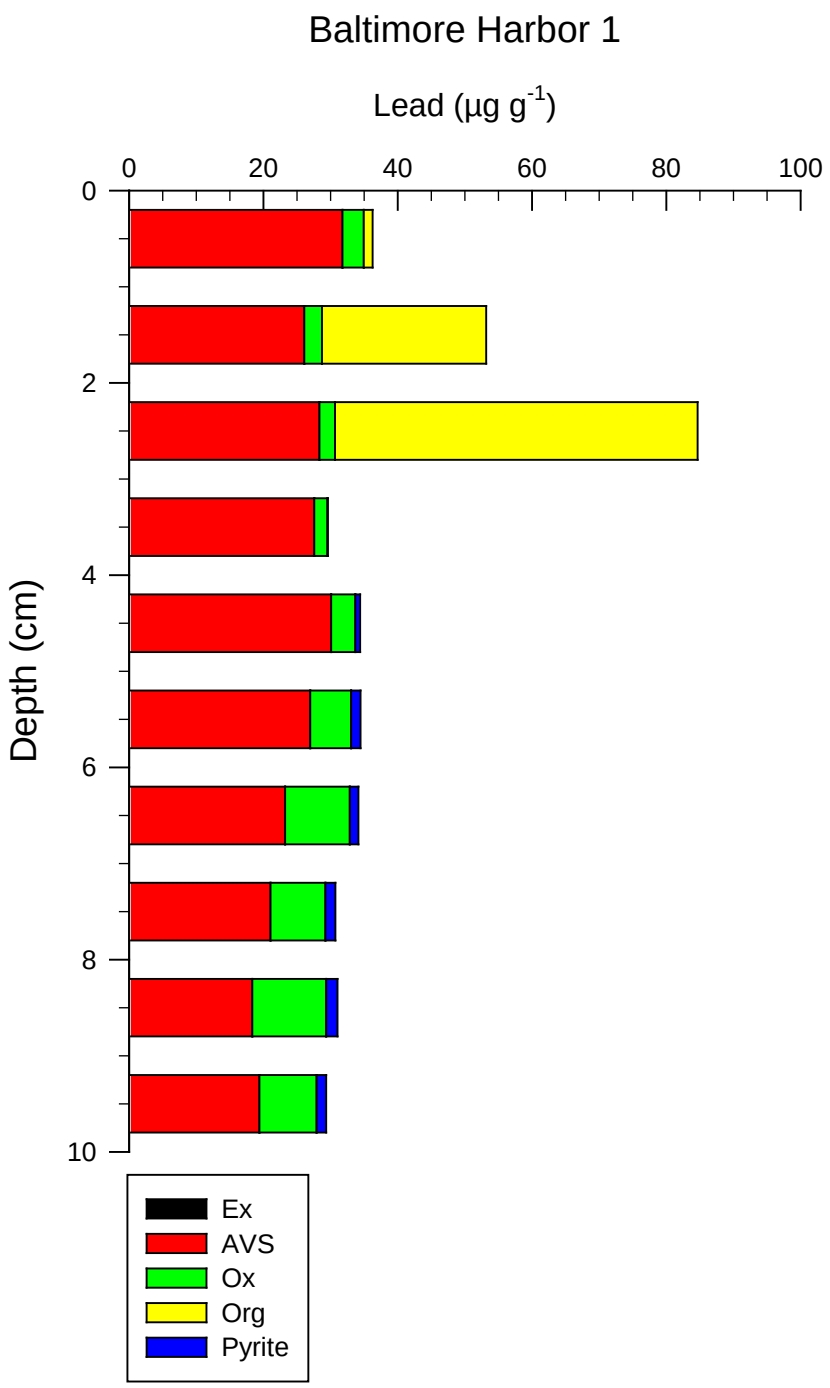

Figure 17. Depth profile of lead phase speciation in sediments of Baltimore Harbor Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

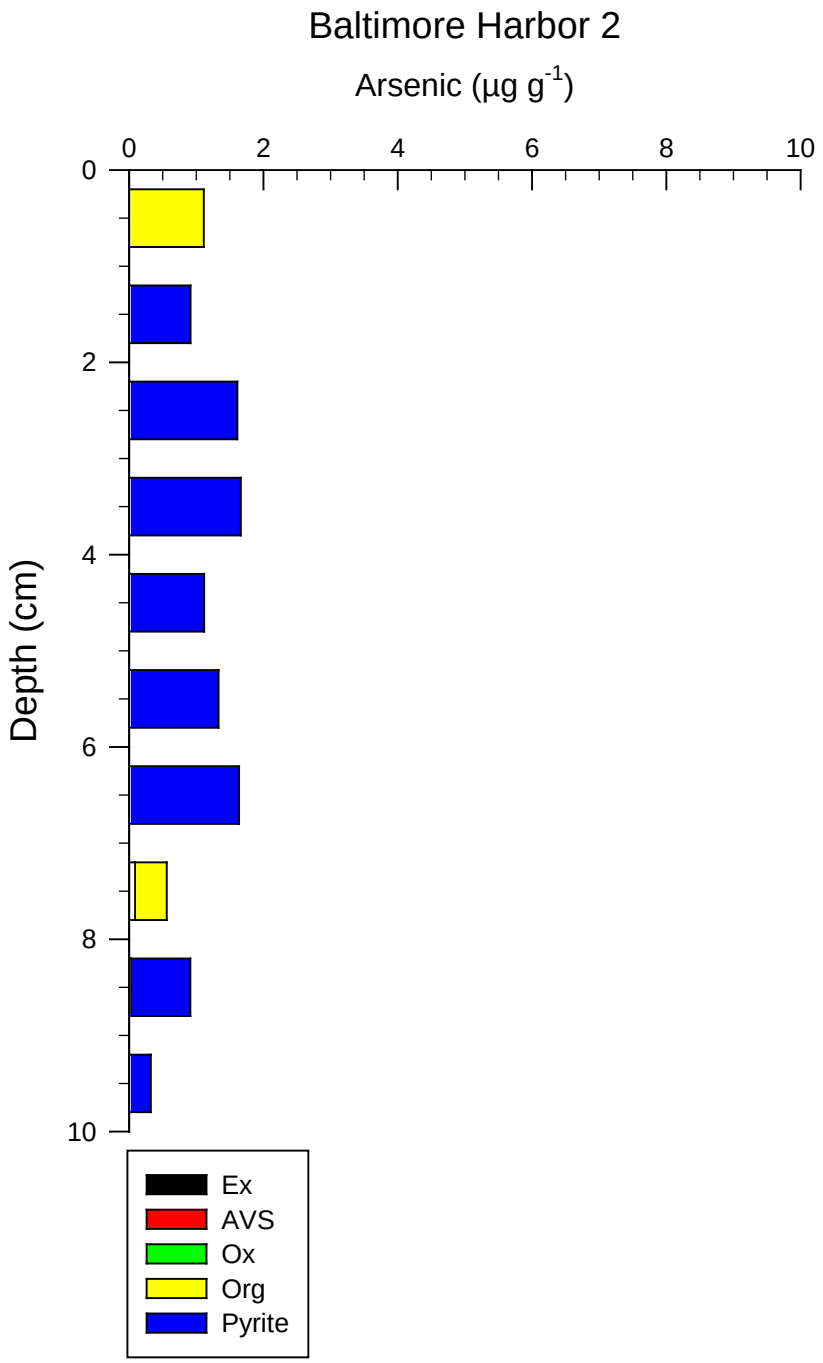

Figure 18. Depth profile of arsenic phase speciation in sediments of Baltimore Harbor Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

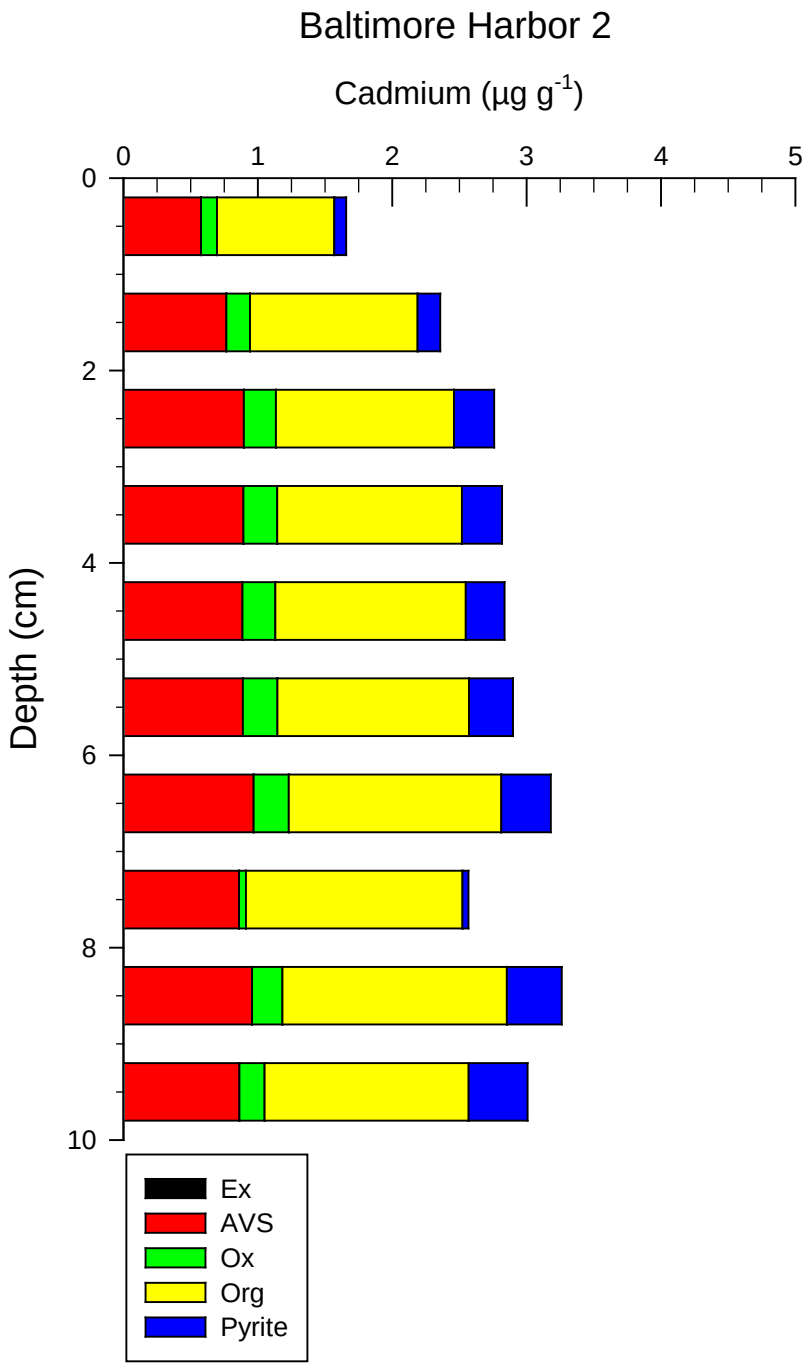

Figure 19. Depth profile of cadmium phase speciation in sediments of Baltimore Harbor Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

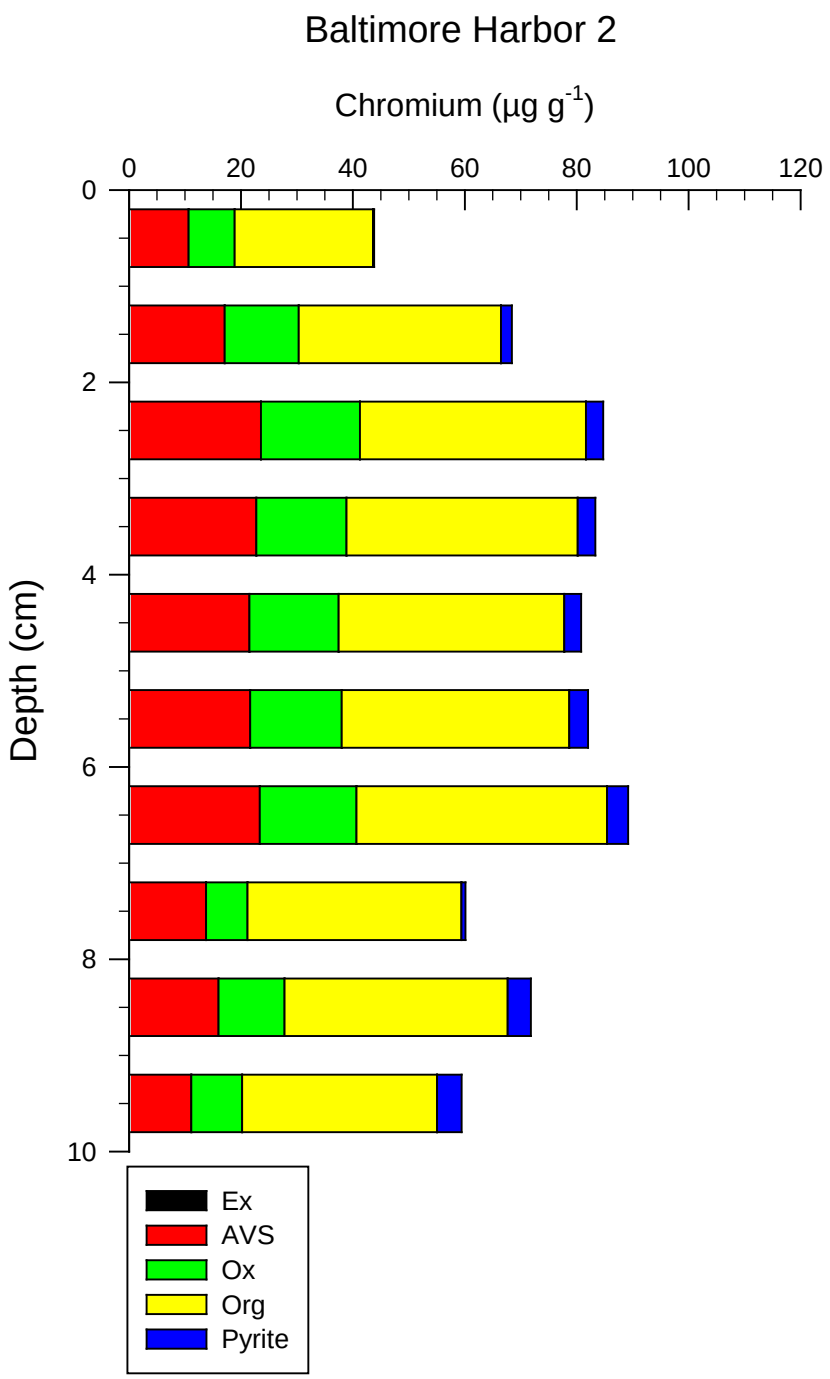

Figure 20. Depth profile of chromium phase speciation in sediments of Baltimore Harbor Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

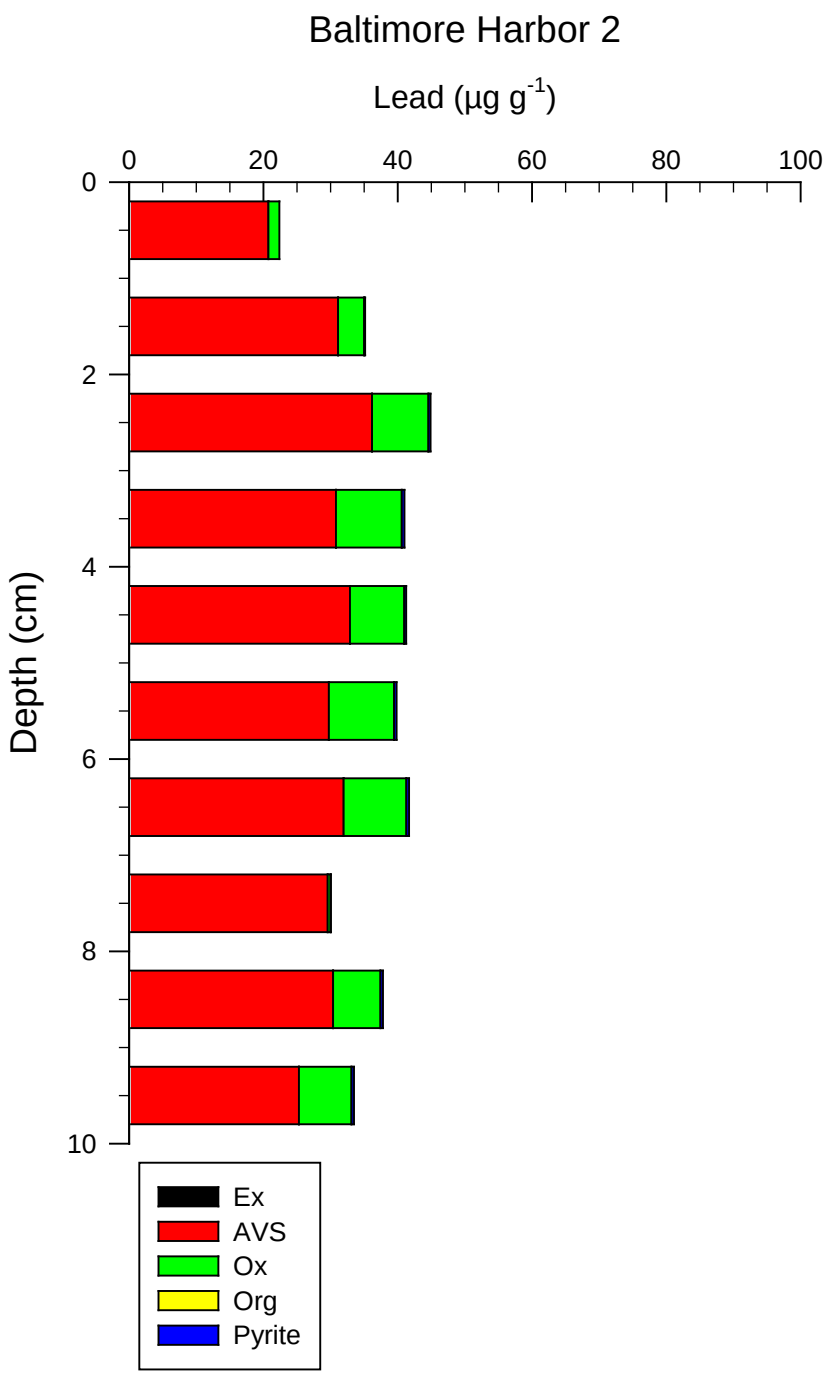

Figure 21. Depth profile of lead phase speciation in sediments of Baltimore Harbor Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

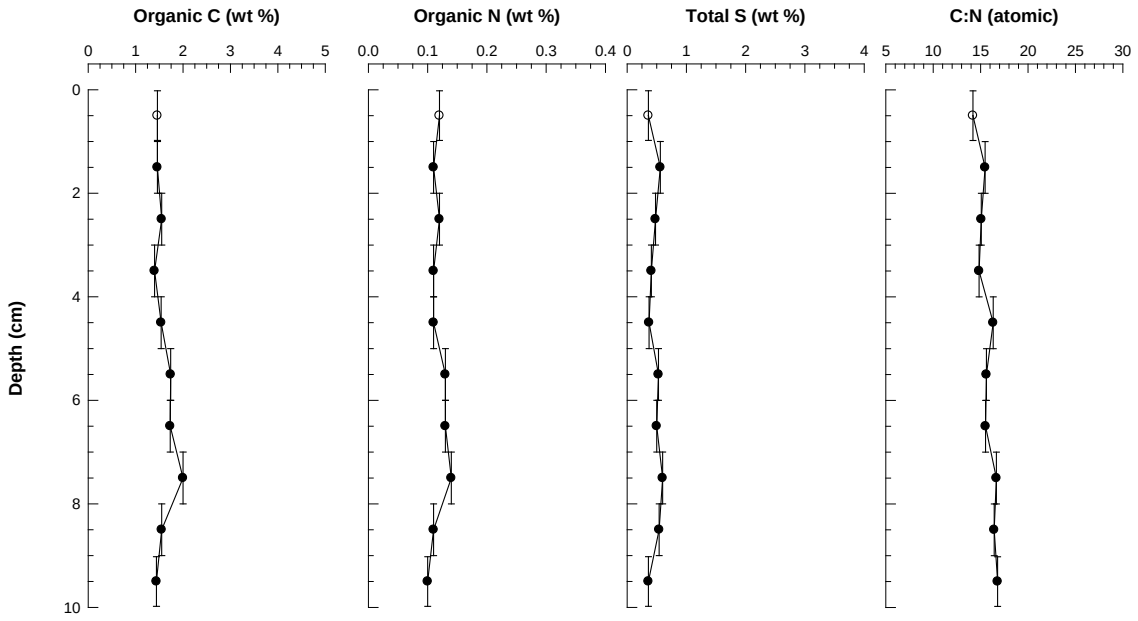

Figure 12. Depth profiles of CNS and C:N in sediments from Mare Island Station 1.

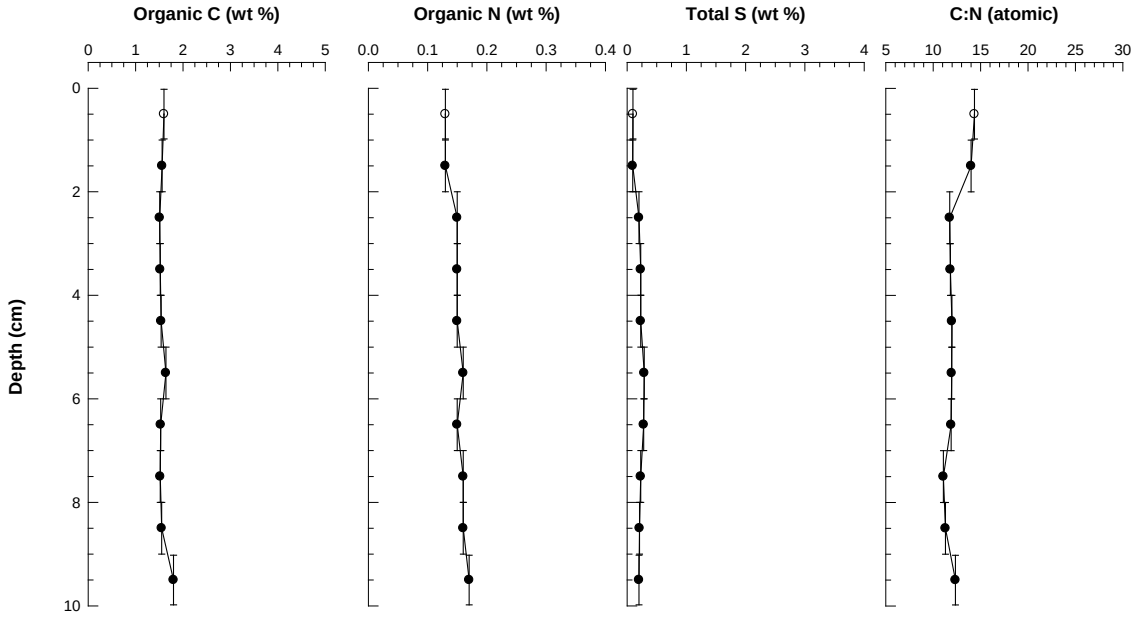

Figure 23. Depth profiles of CNS and C:N in sediments from Mare Island Station 2.

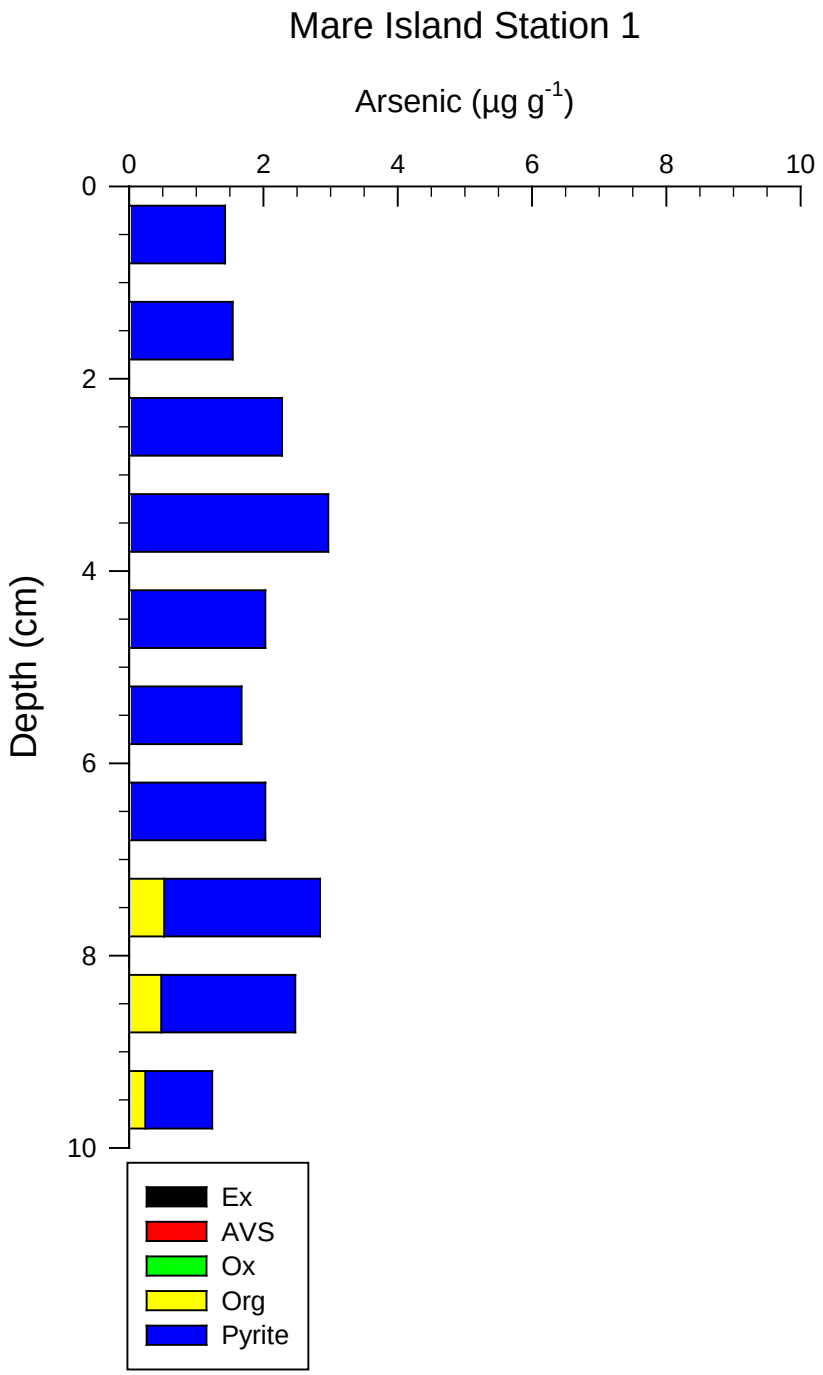

Figure 24. Depth profile of arsenic phase speciation in sediments of Mare Island Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

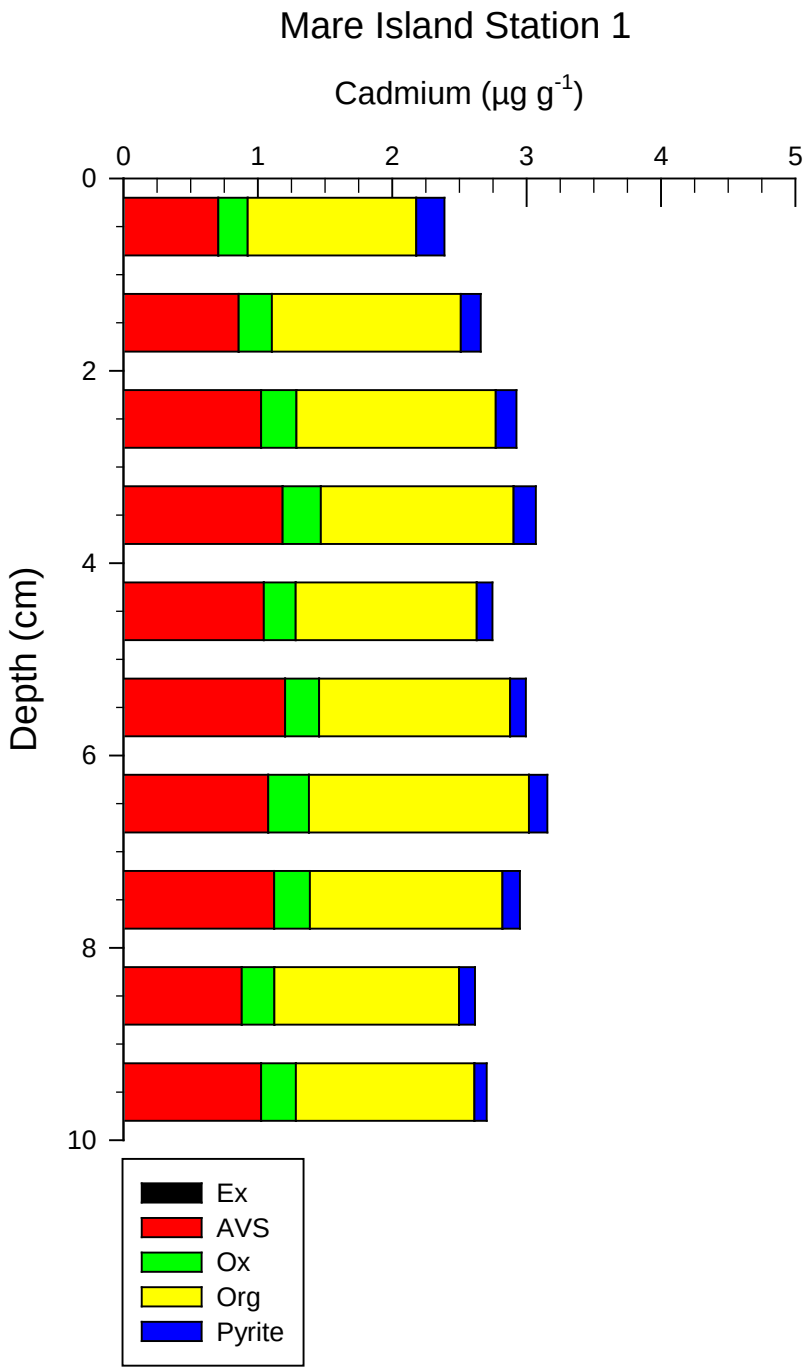

Figure 25. Depth profile of cadmium phase speciation in sediments of Mare Island Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

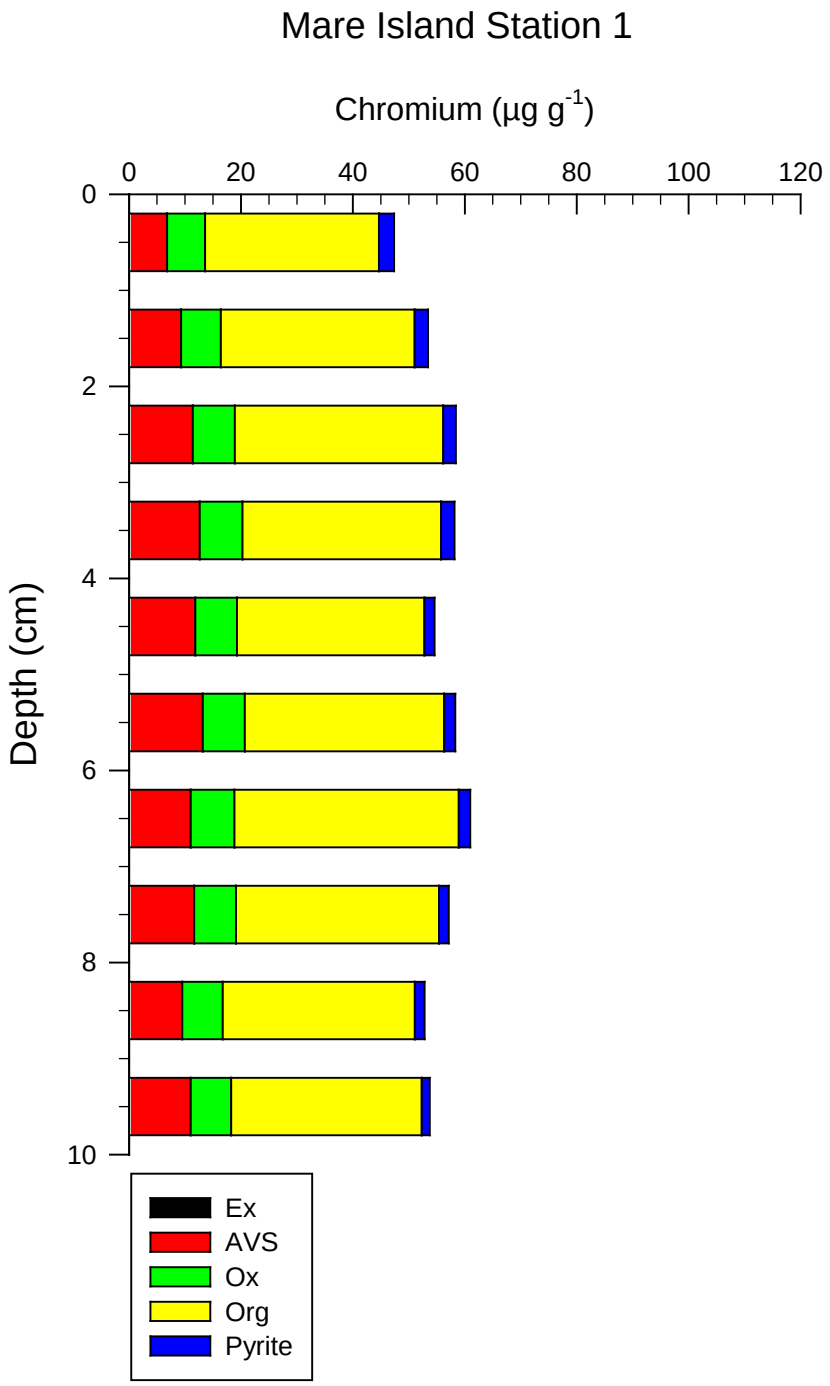

Figure 26. Depth profile of chromium phase speciation in sediments of Mare Island Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

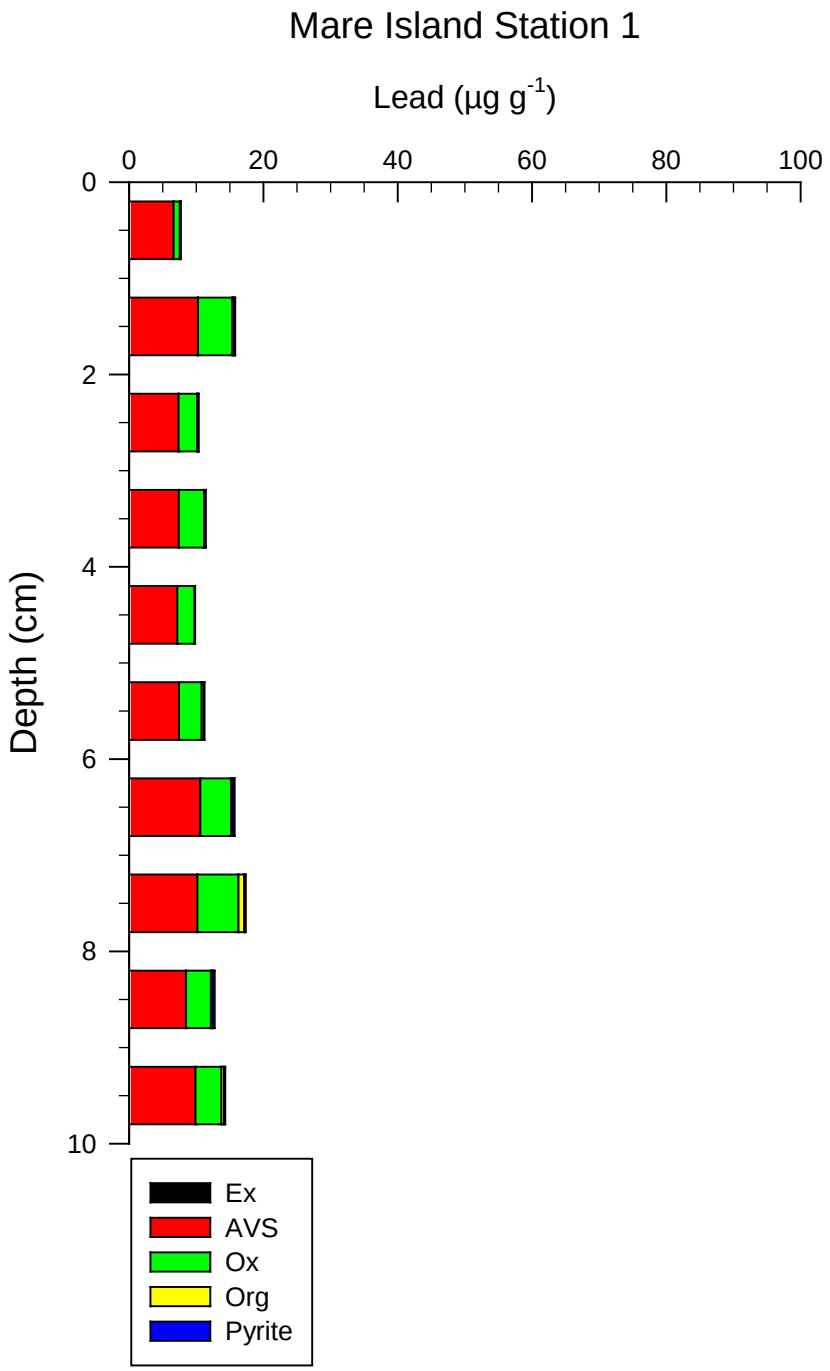

Figure 27. Depth profile of lead phase speciation in sediments of Mare Island Station 1. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

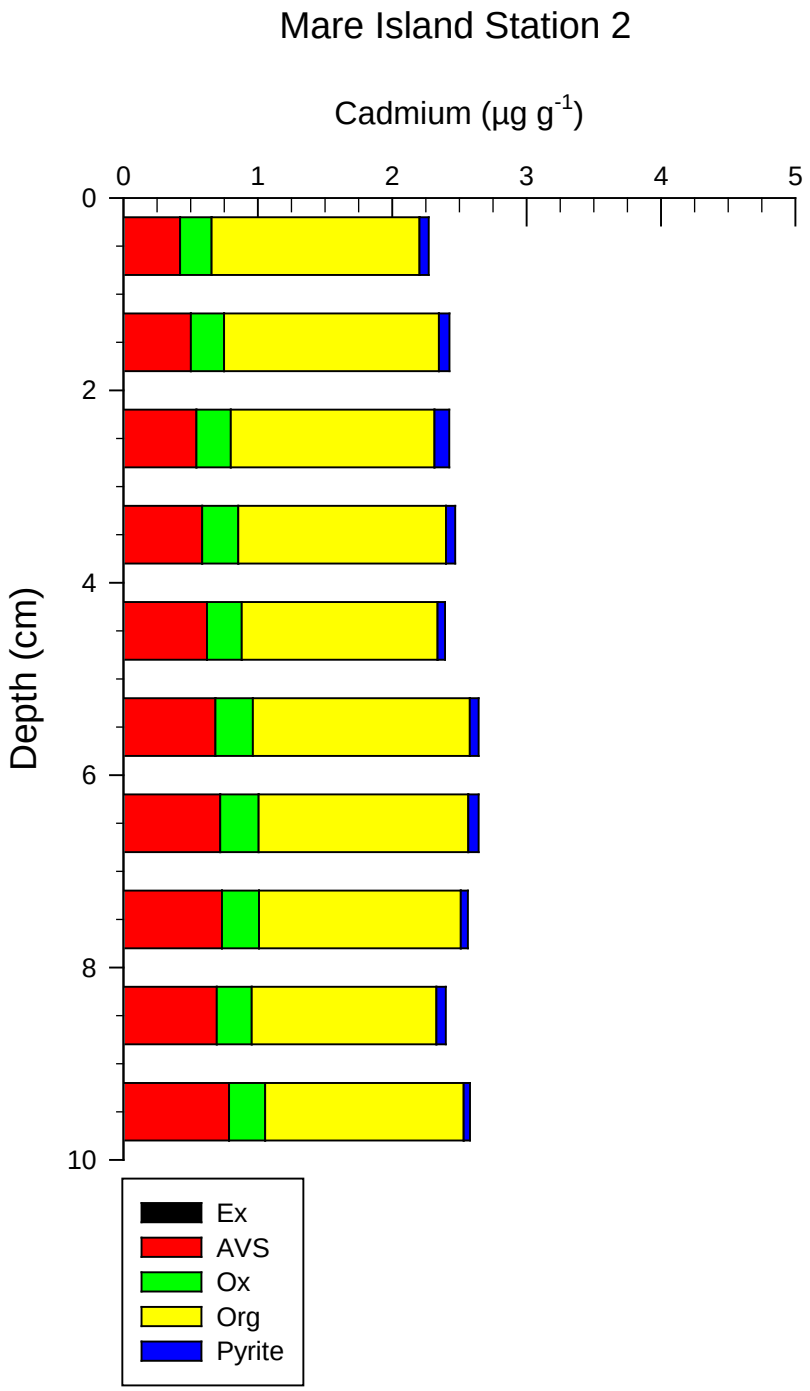

Figure 28. Depth profile of cadmium phase speciation in sediments of Mare Island Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

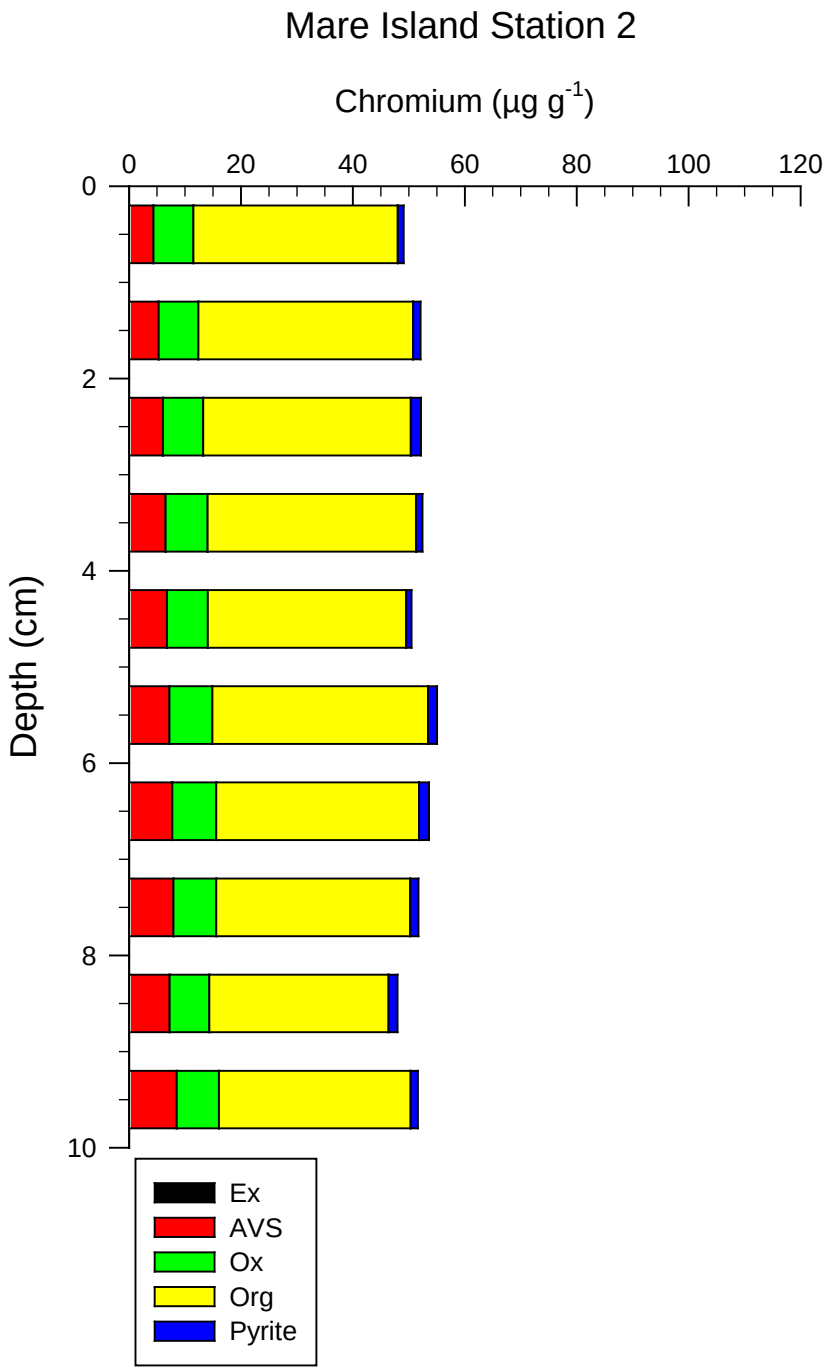

Figure 29. Depth profile of chromium phase speciation in sediments of Mare Island Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.

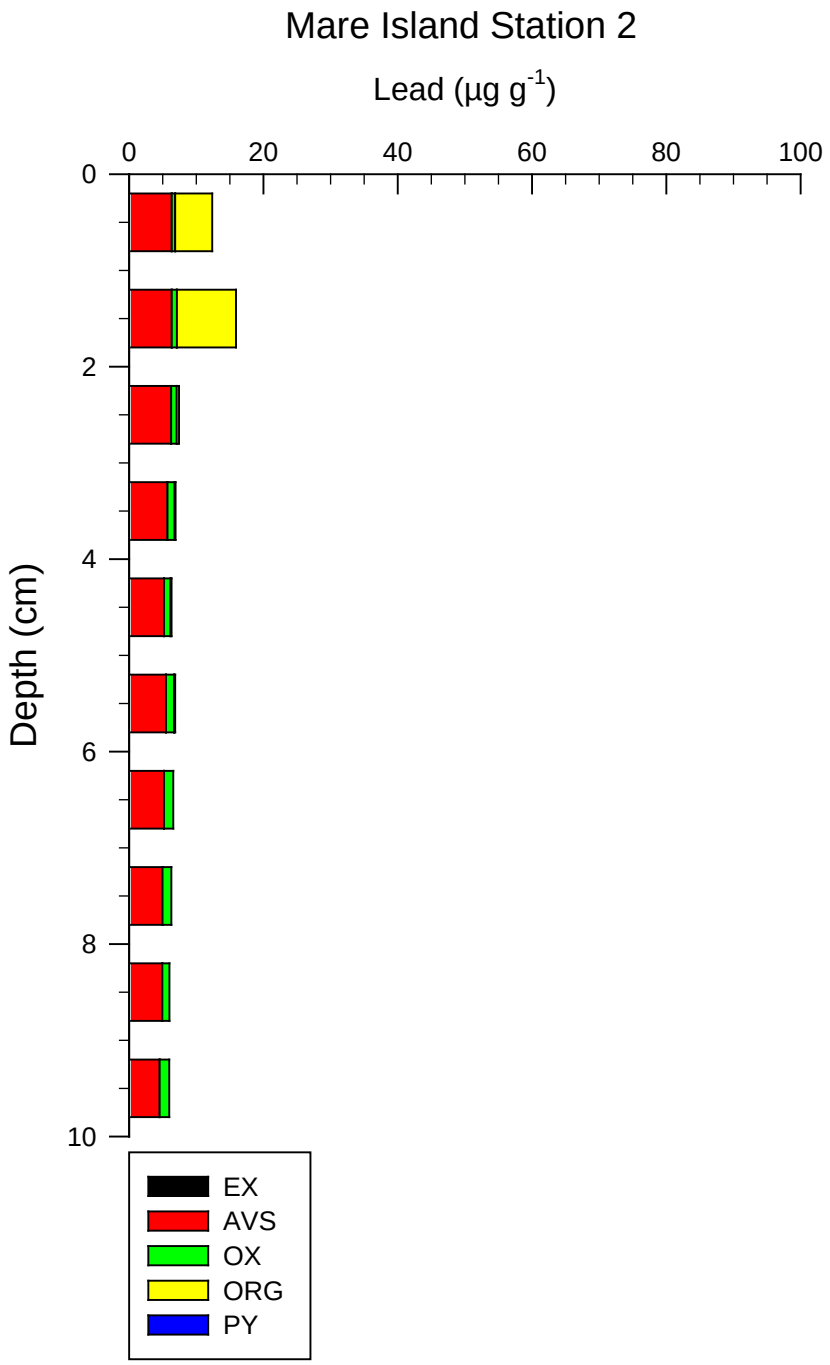

Figure 30. Depth profile of lead phase speciation in sediments of Mare Island Station 2. Ex is the exchangeable phase, AVS is acid-volatile sulfide, Ox is iron/manganese oxides, Org is organic matter, and Pyrite is the pyrite phase.