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Factors influencing metal accumulation in two estuarine fish

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Jessica Dutton

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Jessica Dutton

We, the dissertation committee for the above candidate for the
Doctor of Philosophy degree, hereby recommend
acceptance of this dissertation.

Nicholas S. Fisher – Dissertation Advisor
Distinguished Professor, School of Marine and Atmospheric Sciences

Anne E. McElroy – Chairperson of Defense
Professor, School of Marine and Atmospheric Sciences

David O. Conover
Professor, School of Marine and Atmospheric Sciences

Michael G. Frisk
Assistant Professor, School of Marine and Atmospheric Sciences

Christopher M. Wood
Professor, Department of Biology, McMaster University, Canada

This dissertation is accepted by the Graduate School

Charles Taber
Interim Dean of the Graduate School

Abstract of the Dissertation

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Small forage fish are a conduit for the transfer of metals from lower trophic levels to their predators and potentially human consumers of contaminated seafood. A metal bioaccumulation model has been developed for aquatic organisms, including fish, to determine: (1) the predicted body burden of metals at steady-state based upon the rate of metal uptake and loss from aqueous and dietary sources, and (2) the percentage of accumulated metal attributable to dietary exposure. However, this model assumes that rates of metal uptake and loss following aqueous and dietary exposure are constant and that fish only consume one type of prey. As a result, the kinetic parameters entered into the model may be over- or underestimated. Furthermore, the importance of ingestion rate and growth rate on the predicted body burden of metals has not been adequately researched in bioaccumulation studies. I performed laboratory experiments using a radiotracer technique to investigate the influence of water chemistry (salinity, dissolved organic matter concentration), prey type, and fish physiology (ingestion rate, growth rate) on the uptake, loss, and resulting tissue distribution of five metals (Am, Cd, Cr(III), Hg (as Hg(II) and methylmercury (MeHg)), and Zn) and two metalloids (As(V) and Se(VI)) in killifish (*Fundulus heteroclitus*) and Atlantic silversides (*Menidia menidia*).

The influence of salinity on metal uptake from the aqueous phase in killifish varied by metal; Cd showed an inverse relationship with salinity; As, Hg(II), and MeHg uptake increased with salinity; and Cr showed no relationship with salinity. Estuarine and marine fish drink to

osmoregulate, and Cd dissection data at the end of uptake showed that drinking may be an important uptake mechanism for Cd in these fish. Humic acids had a varying effect on metal uptake by killifish from the aqueous phase; Cd uptake showed no relationship with humic acid concentration, whereas As uptake increased with increasing humic acid concentration, and Cr, Hg(II), and MeHg uptake decreased with increasing humic acid concentration.

Following dietary exposure to radiolabeled amphipods and worms, assimilation efficiencies (AE) in killifish ranged from 0.2 to 4% for Cr and 92% for MeHg. Except for MeHg, AEs varied between prey type, and Hg(II) showed the greatest difference in AEs (14% amphipods, 24% worms). Calculated trophic transfer factors (TTF) indicated MeHg will biomagnify at this trophic step, whereas As, Cd, Cr, and Hg(II) will not. Killifish were intubated with radiolabeled sediment from three contaminated field sites; the AEs ranged from 0.01 to 0.03% for Cr and 10 to 14% for MeHg, and did not vary among field sites for each metal. Killifish do not actively consume sediment, but ingest it accidently while consuming benthic prey. The low AEs for all metals suggest that while sediment is a sink for metals in the estuarine environment, these metals are not readily bioavailable to killifish.

The metal bioaccumulation model was used to predict steady-state metal concentrations in killifish in three contaminated estuaries (Baltimore Harbor, MD; Elizabeth River, VA; and Mare Island, CA) with varying salinity and concentration of dissolved organic matter. Uptake and loss from the aqueous phase was monitored in water from each site and the calculated kinetic parameters were combined with the calculated metal assimilation and retention kinetic parameters from diet (amphipods). The predicted values matched independent field data from contaminated estuaries, indicating that the model can account for the major processes governing metal concentration in killifish. The model indicated that diet accounts for >99% of Cd and MeHg accumulation in killifish on a site-specific basis and that diet was also the major source of As, Cd, and Cr.

Finally, uptake and loss parameters were calculated for two populations of Atlantic silverside; Nova Scotia, at the northern end of the species range, with a higher rate of ingestion and growth, and South Carolina at the southern end of the species range, with slower physiological rates. Calculated body burdens of Am, Cd, and Zn were higher in South Carolina silversides and were attributed to higher AEs in South Carolina silversides and a higher growth rate in Nova Scotia silversides, resulting in somatic growth dilution of metals.

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Chapter I

Introduction

METAL ACCUMULATION IN AQUATIC ORGANISMS AND THE RESULTING EFFECTS

Metal accumulation in aquatic organisms has been a concern for decades due to the associated environmental risk and the possibility of humans consuming contaminated seafood. Studies began in the late 1950's/early 1960's following an outbreak of mercury poisoning in Minamata Bay, Japan, after a chemical company discharged mercury into the bay and the local population consumed seafood enriched in methylmercury (MeHg), resulting in serious health consequences including neurotoxic effects, death, and congenital abnormalities (Takeuchi et al. 1962; Harada 1995). In the following decades the number of studies increased due to the addition of radionuclides to the marine environment (e.g., ^{241}Am, ^{137}Cs, ^{238}Pu, ^{239}Pu,^{90}Sr) from nuclear weapons testing (Ribbe and Tomczak 1990; Robison and Noshkin 1999) and intentional and unintentional discharge from nuclear facilities (Charlesworth et al. 2006), resulting in elevated concentrations of radionuclides in the marine food web (Nonnis Marzano et al. 2000; Heldal et al. 2003; Fowler and Fisher 2004 and references therein; Buesseler et al. 2012; Madigan et al. 2012). During the 1970's and early 1980's, tributyltin (TBT) was used in antifouling paints applied to boat hulls and aquaculture cages. TBT leached into the water column, causing imposex in approximately 120 gastropod species (Garaventa et al. 2006). Legislation limiting the use of TBT was introduced during the late 1980's and a global ban was enforced in 2008. Since legislation was introduced, biomonitoring studies have shown that gastropod populations suffering from imposex are recovering (Evans et al. 1995; Rees et al. 2001; Castro et al. 2012). During the early 1980's, elevated Se levels were detected in ponds in the Kesterson National Wildlife Refuge (San Joaquin Valley, CA). Se is naturally found in elevated concentrations in western desert soils and entered the ponds in agricultural drainage water. Due to Se toxicity, reproductive failures and high mortality rates were observed in aquatic organisms and waterfowl (Ohlendorf 2002; Wu 2004).

Metal accumulation in estuarine and marine food chains in heavily industrialized and urbanized coastal areas is receiving increasing attention because field studies have found that sediments and water have elevated levels of heavy metals due to anthropogenic activities. This has resulted in elevated body burdens of metals in aquatic organisms, including fish (Kennish 1997 and references therein; Burger and Gochfeld 2005; França et al. 2005; Deshpande et al.

2009). Anthropogenic activities which can deliver heavy metals to estuarine and coastal waters include coal-fired power plants (e.g., As, Cu, Hg, Pb, Se; Donahue et al. 2006), mining and smelting (e.g., Cd, Cu, Hg, Pb; Dudka and Adriano 1997; Malm 1998), industrial activities (e.g., Cr, Fe, Pb, Zn; Desenfant et al. 2004), sewage treatment plants (e.g., Cd, Cr, Cu, Hg, Ni, Pb, Zn; da Silva Oliveira et al. 2007), agricultural run-off from land (e.g., Cd, Cr, Cu, Ni, Pb, Zn; Giuffré de López Camelo et al. 1997), motor vehicles (e.g., Cu, Pb, Zn; Lough et al. 2005) and boating practices (e.g., Cu and Zn; Singh and Turner 2009).

Understanding metal accumulation in fish is important for three reasons. First, for the health of the fish themselves and their predators. Studies have shown that fish exposed to elevated levels of As and MeHg have lower reproductive success (Hammerschmidt et al. 2002; Boyle et al. 2008), and an increased risk of developing physical deformities including spinal curvature and curved or stunted tails (Weis et al. 1981; Gonzalez et al. 2006). Second, for the health of human consumers of contaminated seafood. Within the last 60 years there have been several cases of chronic metal poisoning resulting in long term health effects and death. Apart from Minamata disease, MeHg poisoning was also responsible for the Iraqi poison grain disaster in the early 1970's when the Iraqi population consumed grain treated with a methylmercurial fungicide (Bakir et al. 1973). Cd was responsible for Itai-Itai disease which has been recognized since the 1950's in Japan, where people consumed rice contaminated with Cd from local mining practices, resulting in osteoporosis, osteomalacia, and kidney damage (Inaba et al. 2005). Prolonged exposure to hexavalent Cr (Cr(VI)) through drinking contaminated water can result in DNA damage and cancer. In California, 38% of the municipal sources of drinking water have a detectable level of Cr(VI) (Sedman et al. 2006). Human exposure to Pb, a known neurotoxin, through leaded gasoline, paint, occupational exposure (e.g. smelting, plumbing), and food and drink, can lead to damage to the central nervous system, kidneys and blood, and at extreme levels death (Tong et al. 2000). The risk of lead poisoning in developed countries has been reduced due to a ban on the use of lead in gasoline and paint and improved working conditions, whereas in developing countries Pb is still a public health concern, especially in children. Children exposed to Pb can suffer from neurobehavioral conditions and ADHD (Roy et al. 2009) and this is an increasing problem in developing countries in children of lead-exposed occupational workers (Khan et al. 2010). Mass metal poisoning has occurred in Bangladesh where people are exposed to As in drinking water from contaminated wells, resulting in keratosis

of the palms of hands and soles of feet, skin, bladder, and lung cancers, and death (Smith et al. 2000). Finally, understanding metal bioaccumulation in fish is important for regulatory agencies that set water quality standards based upon the ambient concentration of metal in the water and largely overlook the importance of the diet as a source of metals to fish (U.S. Environmental Protection Agency 1992, 2007).

METAL ACCUMULATION PATHWAYS IN FISH

Collecting fish from the field and determining metal concentrations in whole fish using mass spectrometry e.g. inductively coupled plasma mass spectrometry (ICP-MS) is useful to understand the body burden of different metals, for site comparisons, and whether the fish is suitable for human consumption. However, it provides no information on how the metal was accumulated (i.e. from the diet or the water), the rate of metal uptake and loss, and the mechanisms involved. Throughout this dissertation I will investigate several factors which influence the accumulation of metals (Am, Cd, Cr(III), Hg(II), MeHg, and Zn) and metalloids (As(V), Se(IV)) in two estuarine fish (the killifish *Fundulus heteroclitus* and the Atlantic silverside *Menidia menidia*) following dietary and aqueous exposure using a radiotracer technique, to further understand the influence of water chemistry, prey type, and fish physiology on metal bioaccumulation in fish, the resulting tissue distribution, and the relative importance of dietary versus aqueous exposure routes.

Fish, like other aquatic organisms accumulate metals from their diet and surrounding water. However, studies to date have shown that the diet is the dominant uptake route for most metals. Pickhardt et al. (2006) calculated that >98% of MeHg and between 40-88% of Hg(II) accumulation in two freshwater fish (mosquitofish *Gambusia affinis* and redear sunfish *Lepomis microlophus*) is attributed to the diet, and Wang and Wong (2003) also concluded that the diet is the main exposure pathway for both Hg species in a marine fish (sweetlips *Plectorhinchus gibbosus*). Mathews and Fisher (2009) concluded that >90% of the Cd and Zn body burden in a benthic fish (turbot *Psetta maxima*) is attributed to the diet, whereas Xu and Wang (2002) stated that this percentage is dependent on the prey item; in their study when the mangrove snappers (*Lutjanus argentimaculatus*) were fed zooplankton prey dietary uptake dominated, but when the

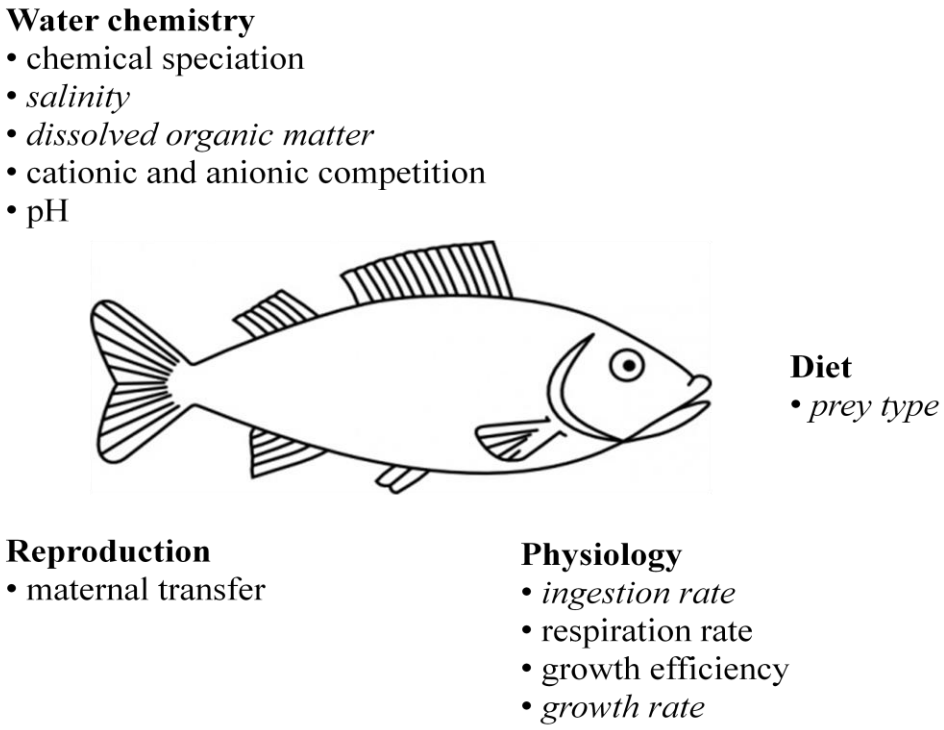
fish were fed planktivorous fish a greater proportion of metal accumulation was attributed to the aqueous uptake pathway. In comparison to Mathews and Fisher (2009), Alquezar et al. (2008) calculated that 85% of the Cd body burden in the estuarine toadfish (*Tetractenos glaber*) was attributed to aqueous exposure. Zhang et al. (2011) and Dang et al. (2009) calculated that the diet was the dominant uptake route for As(V) in the grunt (*Therapon jarbua*) and Cu in the black sea bream (*Acanthopagrus schlegeli*), respectively, and the relative importance of the aqueous phase as a source of As(V) and Cu was dependent on the concentration of metal in the prey and the ingestion rate of the fish. While the diet is the dominant uptake route for most metals, the aqueous phase should not be discounted. If the fish is an infrequent feeder, the assimilation efficiency of ingested food is low, the ingestion rate is low, or the concentration of metal in the prey is low, then aqueous exposure can become a relevant uptake route.

Most laboratory studies focus on calculating metal uptake and subsequent loss kinetics from either the aqueous phase (e.g. Wood et al. 2002; Jeffree et al. 2006; Zhang and Wang 2007a) or the diet (e.g. Reinfelder and Fisher 1994a; Ni et al. 2000; Baines et al. 2002; Leaner and Mason 2004; Kjoss et al. 2006; Mathews and Fisher 2008a,b; Mathews et al. 2008; Ng et al. 2008; Lapointe et al. 2009), and a limited number of laboratory studies combine the two exposure routes to calculate the total body burden of metal and the relative importance of dietary versus aqueous exposure (e.g. Pentreath 1977a,b; Pickhardt et al. 2006; Alquezar et al. 2008; Dang et al. 2009; Mathews and Fisher 2009; Zhang et al. 2011).

Studies have found that MeHg exposure interferes with fish reproduction and offspring hatch already contaminated with metals due to maternal transfer. The percentage of MeHg in eggs has been shown to account for 2% of the total MeHg body burden in female walleye (*Stizostedion vitreum*) and yellow perch (*Perca flavescens*) (Hammerschmidt et al. 1999; Johnston et al. 2001). Studies have found that the hatching success rate and embryonic heart rate of walleye declined with increasing waterborne MeHg concentration (Latif et al. 2001) and fathead minnows (*Pimephales promelas*) fed a diet labeled with MeHg delayed spawning by 5 d and had a lower spawning success rate (Drevnick and Sandheinrich 2003).

Understanding metal accumulation in fish is not straightforward as many factors play a role; these factors are summarized in Fig. 1 and the factors I have focused on in this dissertation are explained in more detail throughout the introduction.

Fig.1. Factors influencing metal accumulation in fish. The factors I have focused on throughout this dissertation are shown in italics.



KILLIFISH (*Fundulus heteroclitus*) AND THE ATLANTIC SILVERSIDE (*Menidia menidia*) AS MODEL ORGANISMS

Killifish (also known as mummichogs) and Atlantic silversides are small (15 cm maximum total length) forage fish found in bays, estuaries, and salt marshes along the eastern seaboard of North America, from Newfoundland to northern Florida (Johnson 1975; Abraham 1985). Killifish have a 3 year life span (Abraham 1985) whereas Atlantic silversides have a life span shorter than 2 years (Conover and Ross 1982). These two fish species were chosen as model organisms because they are readily available, not threatened or endangered, and are small enough (≤7 cm) to fit in the NaI(Tl) γ-detector that was used to analyze metal contents in these fish. Both species are not consumed by humans and apart from being used as baitfish they have

no commercial value. However, they are both a conduit for the transfer of metals up the estuarine food chain because they consume a varied diet and are predated upon by larger fish such as striped bass and bluefish (Hartman and Brandt 1995), dogfish (Rountree and Able 1996), blue crabs (Kneib 1986), and birds (Kneib 1986).

Killifish were used to investigate the influence of salinity, dissolved organic matter (DOM) concentration in the water, and prey type on metal accumulation. Killifish are euryhaline, tolerating a wide salinity range from freshwater to full strength seawater (Abraham 1985). Gut content analysis and laboratory observations have found that killlifish consume a varied diet consisting of crustaceans (e.g., amphipods and copepods), polychaetes, insects, crabs, algae, detritus, eggs, and small fish (Kneib and Stiven 1978; Allen et al. 1994; McMahon et al. 2005; Able et al. 2007). Furthermore, they have a limited swimming range (Abraham 1985) and can therefore be used to investigate metal accumulation on a localized site-specific basis, are tolerant of high contamination levels (Weis 2002), and can be used as a water quality indicator (Eisler 1986). Killifish are found in the most heavily polluted estuarine waters in the United States, including the Elizabeth River (VA) (Meyer et al. 2005).

The Atlantic silverside was used to investigate the importance of ingestion rate and growth rate on metal accumulation in fish. There is a counter-gradient variation in ingestion rate and growth rate throughout the species range, with northern Atlantic silverside populations having a much higher rate of ingestion and growth than southern populations (Conover and Present 1990; Present and Conover 1992). This evolved to overcome a shorter growing season at the northern end of the species range (4 months, compared to 9.5 months at the southern end of the range; Conover and Present 1990) and size-selective winter mortality as the northern populations migrate offshore during the winter where water temperatures are more stable (Conover and Murawski 1982). At the end of the growing season the total body length is the same throughout the specie's range, indicating that northern populations grow faster throughout a shorter growing season (Conover and Present 1990). However, there are trade-offs associated with higher rates of physiological processes; individuals at the northern end of the species range have reduced swimming performance and are at a greater risk of being predated on (Billerbeck et al. 2001; Lankford et al. 2001). A higher ingestion rate can result in a lower assimilation efficiency (AE) of ingested metals due a quicker gut passage time, and a higher growth rate can lead to growth dilution of metals. As a result, northern populations of juvenile Atlantic

silversides may have lower metal body burdens compared to individuals at the southern end of the range.

METAL(LOIDS) TO BE INVESTIGATED

Five metals (Am, Cd, Cr, Hg (as Hg(II) and MeHg) and Zn) and two metalloids (As and Se) were investigated throughout this dissertation due to their varying chemical characteristics and environmental interest. Most of the trace elements investigated do not have a biological function in fish, and are therefore considered non-essential, except for Se and Zn, which are required to build proteins (Bury et al. 2003). These trace elements can be further classified based on their binding affinity for either oxygen or sulfur, and therefore their association with protein (Nieboer and Richardson 1980). Am is classified as an oxygen-seeking Class A metal and typically displays little association with protein. Class A metals bind to the surface of cells and do not accumulate in the cytoplasm, as a result there is little to no trophic transfer of these elements (Fisher and Reinfelder 1995). Hg is classified as a sulfur-seeking Class B metal with a strong affinity for protein, and concentrates in the cytoplasm of cells so it is readily transferred up the food chain. Cd, Cr, and Zn are classified as borderline metals which have some affinity for protein and can therefore be somewhat assimilated and transferred up the food chain (Fisher and Reinfelder 1995). Finally, all of the investigated trace elements are found at elevated concentrations in industrialized coastal waters due to anthropogenic activities (Kennish 1997 and references therein) and are of concern due to their impacts on the health of the estuarine ecosystem and public health consequences.

Am is a trivalent cation and of interest because it is a particle-reactive synthetic actinide produced during the decay of ^{241}Pu in the nuclear power industry and previously from the detonation of atmospheric nuclear weapons. Cd is used for electroplating, in electronic items e.g., televisions, and to make batteries. Cd is also a byproduct from the mining of Zn. Cd is of interest in part due to its particle reactivity being salinity dependent, cationic competition with Ca^{2+} in fish (Franklin et al. 2005), carcinogenic effects in humans including liver, kidney, stomach, and prostate cancer (Waalkes 2000), and nutrient-like behavior in marine algae; studies

have shown that Cd can behave as a cofactor in carbonic anhydrase in at least one marine diatom when Zn is limited (Lee et al. 1995).

Zn can be discharged into the estuarine environment through mining practices and its use as a wood preservative and an antifouling paint. Zn is of interest due its biological necessity, and while it is toxic at high concentrations there is less environmental concern because toxicity levels are rarely reached. In comparison, there is concern about Se, which is also toxic at high concentrations, due to fossil fuel combustion and industrial waste (e.g. metal smelting) introducing elevated levels of Se into coastal waters, resulting in bioaccumulation of Se in the aquatic food chain (Eisler, 1985; Cutter 1989; Hamilton 2004). Se can naturally leach out of shale deposits, contaminating the groundwater, as observed in California (Tracy et al. 1990; Presser et al. 1994; Martens and Suarez 1997). Furthermore, Se is of interest due to its antagonistic relationship with Hg; studies have shown that fish accumulate less Hg as the aqueous concentration of Se increases (e.g. Chen et al. 2001). Selenate (Se(VI)) is the dominant form of Se in seawater, but selenite (Se(IV)) will be used in this dissertation. Se(VI) competes with sulfate for cellular uptake (Tuve and Williams 1961) and the sulfate levels in seawater are so high (28.2 mmol L^{-1}; Bruland 1983) that little to no Se(VI) will accumulate in fish from the aqueous phase.

As and Cr are of interest due to their redox chemistry and resulting toxicity. As enters the estuarine environment through the weathering of rocks, pesticide usage in agriculture, and its use as a wood preservative (Mandal and Suzuki 2002). In aquatic environments, arsenate (As(V)), a pentavalent oxyanion, is the dominant inorganic species and less toxic than arsenite (As(III)). In anoxic environments As(V) is reduced to As(III) (Neff 1997). On a cellular level As(V) and As(III) behave very differently; As(V) behaves as a P-analog and competes with PO_4 for uptake, and once inside the cell it interferes with phosphorylation reactions, while As(III) reacts with thiols in proteins (Hughes 2002).

Anthropogenic activities that can potentially discharge Cr into the estuarine environment include leather tanning, and its use as a pigment and dye, and wood preservative. Cr exists in seawater as either trivalent Cr (Cr(III)) or hexavalent Cr (Cr(VI)). In natural waters Cr(III) is oxidized to Cr(VI) and Cr(VI) is reduced to Cr(III); as a result these two species are not at steady-state concentrations (Pettine et al. 1991). In oxic environments, Cr is predominantly found as Cr(VI), whereas in anoxic environments Cr(III) dominates. Cr(VI) is considered to be

much more toxic than Cr(III). The chemical behavior of Cr, especially its particle reactivity and toxicity, is dependent on its oxidation state. Cr(III) is cationic and has greater particle reactivity, whereas Cr(VI) is anionic and has a higher solubility and greater oxidizing power. Cr(III) has no known biological function in fish, but is required for glucose, lipid, and protein metabolism in mammals (Florence and Batley 1980). However, Cr(VI) is very toxic because of its high permeability through negatively-charged cell membranes (Florence and Batley 1980; Wang et al. 1997). As and Cr are also of concern due to their human health impacts including cancers, cardiovascular disease, and neurological problems (Kapaj et al. 2006; Sedman et al. 2006). Seafood consumption is not thought to be a significant source of toxic inorganic As to humans (Neff 1997; Kirby and Maher 2002; de Rosemond et al. 2008). Organic species of As accumulate in fish fillet, particularly arsenobetaine, which is considered to be non-toxic and non-carcinogenic (Neff 1997; Kirby and Maher 2002).

Of all the metals examined, Hg in the form of MeHg poses the greatest risk to the health of the estuarine ecosystem and human consumers of contaminated seafood due to MeHg's neurotoxic effects. Most anthropogenic Hg enters the environment through coal combustion and waste incineration. Hg enters the aquatic environment as Hg(II), where it is converted to MeHg by bacteria in the sediment or water column (Topping and Davies 1981; Ikingura and Akagi 1999) and biomagnified up the food chain, or by gut bacteria in fish (Rudd et al. 1980). MeHg more readily crosses biological membranes than Hg(II); studies using fish and humans have found that MeHg binds to cysteine and crosses biological membranes as a MeHg-cysteine complex (Leaner and Mason 2002a; Simmons-Willis et al. 2002). MeHg can accumulate to such a high level in fish that it exceeds the health based maximum permitted level for human consumption. In adults, MeHg exposure is known to impact cognitive function as it is a known neurotoxin (Chang et al. 2008) and also increases the risk of myocardial infarction (Guallar et al. 2002). This has resulted in the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) issuing fish advisories regarding MeHg levels and seafood consumption aimed at pregnant women and nursing mothers because the fetus and young infants are more sensitive to MeHg exposure (U.S. Environmental Protection Agency 2004).

FACTORS INFLUENCING THE BIOAVAILABILITY OF METALS FROM THE AQUEOUS PHASE

In ocean water, the average concentration of As, Cd, Cr, Hg, Se, and Zn are as follows: 23 nmol L^{-1} As, 0.7 nmol L^{-1} Cd, 4 nmol L^{-1} Cr, 5 pmol L^{-1} Hg, 1.7 nmol L^{-1} Se, and 6 nmol L^{-1} Zn (Bruland 1983). The background concentration of Am (a synthetic radioactinide) in ocean water is 10^{-21} mol L^{-1} (Cochran et al. 1987). In estuarine water, the concentration of these metals can be higher due to natural weathering processes on land and anthropogenic activities. Hg has been speciated (an analytical testing procedure that separates and measures the different forms of Hg) in estuarine waters and MeHg has been found to account for 3% of the total Hg (Hammerschmidt and Fitzgerald 2006).

The gill accounts for about 50% of the external body surface in fish (Jezierska and Witeska 2004) and is presumed to be an important uptake site for aqueous metals. For example, Wood et al. (2002) calculated that ≤ 82% of the body burden of Ag in the freshwater eel (*Anguilla Anguilla*) and 23-58% of Ag in rainbow trout (*Oncorhynchus mykiss*) was associated with the gills at the end of aqueous uptake. Metals either passively sorb to the gill surface or are actively taken up across the gill membrane via uptake channels where they can potentially be redistributed around the body via the blood (Simkiss and Taylor 1985, 1989). Studies have shown that waterborne metals (e.g. Ag, Al, Cd, Cu) can interfere with gill function including gas and ion exchange (Jezierska and Witeska 2004). The first indication of increased metal exposure at the gill is the increased secretion of gill mucus, which is produced in excess and sloughed off to reduce the risk of the metal entering the bloodstream (Jezierska and Witeska 2004).

Fish can also accumulate metals across their body surface (dermal exposure), but the presence of scales and mucus has shown to provide a protective barrier. Epidermal mucus in fish is lipid-rich and has a range of functions, including respiration, increasing swimming performance, disease resistance and protection (Lewis 1971; Shephard 1994). One study exposed fish to waterborne Pb and Hg and both metals accumulated in their epidermal mucus due to binding to the glycoprotein component of the mucus. Once the fish were moved to metal-free water, the concentration of Pb and Hg in the epidermal mucus decreased within 24 h, due to the sloughing off of the metal-complexed mucus (Varanasi et al. 1975). The presence of fish scales increases swimming performance by reducing friction and drag, and protects the body from

injury. Studies have shown that fish scales also sequester metals and metalloids (e.g.As, Hg, Ni, Pb, Se), providing a protective role against the dermal uptake of metals (Coello and Khan 1996; Farrell et al. 2000).

The total dissolved metal concentration indicates how much metal an organism is exposed to in the aqueous medium, but it is the free-ion concentration which is of most interest because it is considered to be more chemically reactive, and is therefore more bioavailable to aquatic organisms, including fish. The Free-Ion Activity Model (FIAM) was developed to investigate whether metal toxicity was dependent on the aqueous speciation of the metal and has been well demonstrated using plankton (Sunda and Guillard 1976; Campbell 1995 and references therein).

Other models have since been developed to understand the effects of water chemistry on metal bioavailability and therefore toxicity to aquatic organisms. The Biotic Ligand Model (BLM) is used in freshwater systems to develop water quality criteria which are site-specific; it takes into account the pH of the water and metal complexation with DOM and inorganic ligands to predict metal speciation and therefore metal bioavailability to fish or other aquatic organisms (Paquin et al. 2002). Metals that are complexed to ions in seawater e.g. chloride, or bound to DOM or particulate matter are thought to be less bioavailable because they have a stronger affinity for the inorganic or organic ligand to which they are bound to than the biotic ligand (i.e. the gill). The BLM can predict the toxicity of dissolved metals based on metal accumulation at the receptor site (i.e., the gills). To date, the BLM has been successfully used to assess the toxicity of Ag, Cd, Cu, and Zn in rainbow trout *Oncorhynchus mykiss* (e.g. McGeer et al. 2000; Niyogi and Wood 2003 and references therein).

The rate of metal uptake and resulting toxicity to aquatic organisms can increase or decrease with increasing salinity. Studies to date using fish have focused on the uptake of Ag, Cd, Cu, and Zn across a salinity gradient from freshwater to full strength seawater; all studies determined that metal accumulation had an inverse relationship with salinity (Wood et al. 2004; Blanchard and Grosell 2005; Zhang and Wang 2007). However, several metals e.g. As, Cr, and Hg, which are known to be toxic at low concentrations have been largely overlooked.

Cd and Hg are known to chloro-complex in seawater. In freshwater, Cd is found mainly as the free-ion Cd^{2+} form or in carbonate complexes ($CdHCO_3^+$ and $CdCO_3^0$), whereas in seawater Cd is bound to chloride as $CdCl_2$ (Florence and Batley 1980; Bruland 1983). Studies

using crustaceans, oligochaetes, bivalves, crabs, and fish have determined that Cd accumulation and toxicity are greater at lower salinities (Hall and Anderson 1995 and references therein; Blackmore and Wang 2003; Jackson 2003) due to the greater particle reactivity and therefore bioavailability of Cd in the free-ion Cd^{2+} form. In freshwater Hg(II) and MeHg are found bound to hydroxide, whereas in seawater Hg(II) and MeHg are bound to chloride; in freshwater Hg(II) is predominantly found as $Hg(OH)_2$ and MeHg as CH_3HgOH, and in seawater Hg(II) is found as $HgCl_2$ and MeHg as CH_3HgCl (Fluorence and Batley 1980; Mason et al. 1996). Unlike for Cd, Hg accumulation shows no concise relationship with salinity; some studies conclude that the accumulation and therefore toxicity of Hg is greater at lower salinities for a range of organisms (Hall and Anderson 1995 and references therein; Farmer et al. 2010), whereas one study using the mangrove clam *Polymesoda erosa* determined that Hg accumulation was greater at higher salinities (Modassir 2000), and a study by Laporte et al. (1997) using the shore crab *Carcinus maenas* determined that Hg(II) and MeHg present as $HgCl_2$ and CH_3HgCl, respectively, are the most bioavailable species of Hg.

Unlike Cd and Hg, As and Cr do not chloro-complex in seawater (Bruland 1983) and instead are present in freshwater and seawater as oxyanions. In freshwater As is speciated between H_3AsO_4, $H_2AsO_4^-$, and $HAsO_4^{-2}$, whereas in seawater it is speciated as $HAsO_4^{2-}$ (Bruland 1983; Mandal and Suzuki 2002). No data are available investigating the uptake of As across a salinity gradient in fish, but a field study has shown that fish caught in higher salinity waters have a higher body burden of As (Larsen and Francesconi 2003). Cr is found bound to oxygen in freshwater and seawater; in seawater Cr is mainly speciated as CrO_4^{2-} and $NaCrO_4^-$ (Bruland 1983). Studies investing the aqueous accumulation of Cr(VI) in crustaceans, polychaetes, bivalves, and crabs as a function of salinity determined that Cr(VI) accumulation was greater at lower salinities (Hall and Anderson 1995 and references therein).

DOM is classified as organic material that passes through a 450 nm filter (Hedges 2002). It is recognized that this is an operational definition and includes the "colloidal" fraction that is between 1-450 nm and the truly dissolved fraction is < 1 nm (Hedges 2002). In the past, DOM was classified as organic material that could pass through a 0.7 µm filter; but this also included many bacteria and viruses which are clearly not "dissolved" (Ogawa and Tanoue 2003). DOM is considered to be one of the largest organic matter reservoirs on earth (Ogawa and Tanoue 2003). DOM is added to the coastal ocean from land via river discharge, which transports weathered

rock and soil, and decomposed plant material to the coastal ocean (Cauwet 2002), and from within the coastal ocean through the extracellular release of phytoplankton cell contents during cell growth, predation and cell lysis (Lee and Fisher 1994; Wetz and Wheeler 2007; Flynn et al. 2008). DOM complexes trace metals and metals complexed to organic ligands are typically less bioavailable to aquatic organisms. In theory, organisms should accumulate less metal from the dissolved phase in organic-rich waters.

The presence of DOM in aquatic systems has been shown to have a protective effect against metal toxicity in aquatic organisms. While this protective effect is well known in plankton, the presence of DOM as dissolved organic carbon (DOC) has been shown to have a nutritive effect in bivalves, where metal uptake (e.g., Ag, Cd, Co, Cr, and Zn) can increase in the presence of DOC due to DOC being absorbed by some bivalves, and metals bound to the DOC are taken up with it and redistributed around the body (Roditi et al. 2000a; Guo et al. 2001; Pan and Wang 2004a,b).

In fish, studies have focused on whether the presence of DOM has a protective effect against metals binding to gills, the mechanisms involved, and the physiological effects on the gills. Studies have not focused on the uptake of metals across a DOM gradient. Once metals bind to DOM, how the metal interacts with the gill is dependent of the relative equilibrium binding strengths between the DOM and the gill. If the binding strength is greater for the DOM, then a protective effect is observed, whereas if the gill has an equal or greater binding strength the protective effect is not observed. For example, Cu has a higher binding strength for DOM than for the gill and therefore the Cu-DOM complex has a protective affect against metal uptake at the gill of rainbow trout *Oncorhynchus mykiss* (Playle et al. 1993). In comparison, Cd has similar binding strengths for DOM and fish gills (Playle et al. 1993), and as a result studies have found that the presence of DOM does not have a protective role against aqueous Cd uptake in fish (Hollis et al. 1996; Richards et al. 1999).

Finally, metal uptake from the aqueous phase is also influenced by the presence of competing cations and anions, which at elevated concentration can reduce metal uptake to aquatic organisms. Cationic competition between Cd and Ca^{2+} in fish has been well established because both elements share the same uptake pathway in the gill and gut; in the presence of elevated Ca^{2+} the uptake of Cd is downregulated (Franklin et al. 2005). Anionic competition between As(V) and PO_4 has also been observed; in the presence of PO_4 the uptake of As(V) is

down-regulated, as observed in phytoplankton because As(V) and PO_4 share the same uptake pathway (Sanders and Windom 1980).

FACTORS INFLUENCING THE BIOAVAILABILITY OF METALS FROM THE DIET

Estuarine fish can accumulate metals through planktonic and benthic food chains. As metals are trophically transferred up the food chain to fish, the metals can bioaccumulate in organisms at each trophic level, and can also potentially biomagnify or biodiminish up the food chain. Most metals bioaccumulate; bioaccumulation is defined as the accumulation of metals over time in an organism because the rate of metal assimilation is greater than the rate of metal excretion (Bryan 1979). Cd and Hg are examples of metals which are known to bioaccumulate in organisms (Giguère et al. 2004; Chen et al. 2009; Fry and Chumchal 2012). Biomagnification is the increasing concentration of a metal in aquatic organisms with increasing trophic level, so the top predators e.g. fish, sharks, marine mammals have a much higher body burden than plankton at the bottom of the food chain. Cd in freshwater systems and MeHg in freshwater, estuarine, and marine systems are known to biomagnify (Watras et al. 1998; Baeyens et al. 2003; Croteau et al. 2005). In comparison, some metals biodimish, or decrease in tissue concentration with increasing trophic level, e.g. As in freshwater and estuarine food chains (Lindsay and Sanders 1990; Chen and Folt 2000; Culioli et al. 2009). Whether a metal bioaccumulates within an organism, and biomagnifies or biodiminishes with increasing trophic level in the food chain is dependent on a multitude of factors including the type of prey consumed, the tissue distribution of metal within the prey, the ability for the organism to solubilize the metal from the prey in its gut and assimilate it, and finally, the physiological turnover rate of the metal within the organism.

Within the planktonic food chain, the largest enrichment step occurs between the water and the phytoplankton (Fisher and Reinfelder 1995). Studies have shown that the concentration factor (CF; defined as the amount of metal per gram of organism divided by the amount of metal per gram of water) of metals in phytoplankton vary from 3×10^2 for Cd, to 9.5×10^4 for Hg, to 6.9×10^5 for Am (Fisher et al. 1984; Fisher 1986; Fisher and Reinfelder 1995), indicating that phytoplankton are much more enriched in metals than the surrounding medium. However, the CF in phytoplankton is directly related to the chemical speciation of metal in the water which

influences its particle reactivity, and therefore its bioavailability and potential trophic transfer up the food chain.

Studies have shown that while phytoplankton are greatly enriched in some metals, whether that metal will be trophically transferred to herbivores is dependent on what part of the phytoplankton cell the metal is associated with. Reinfelder and Fisher (1991) determined there was a direct 1:1 relationship between the assimilation efficiency (AE; defined as the percentage of ingested metal that crosses the gut lining) of metals in copepods and the percentage of metal associated with the cytoplasm in the diatom prey. This same relationship was also observed in juvenile and adult bivalves fed phytoplankton (Reinfelder and Fisher 1994b; Wang and Fisher 1996). The greater the percentage of metal associated in the cytoplasm of algal cells, the greater the likelihood the metal will be trophically transferred to higher trophic levels, including fish.

Whether fish assimilate metal from their prey is also determined by the fractionation of metal within different body compartments in the prey organism. Reinfelder and Fisher (1994a) investigated the AE of several metals to silversides fed zooplankton prey; this study concluded that the AE of each metal in fish is related to the percentage of metal associated with the "soft parts" of crustacean zooplankton. Another study comparing the assimilation of Hg(II) and MeHg to fish fed zooplankton prey saw a higher assimilation of MeHg due to a larger percentage of MeHg being associated with the zooplankton soft tissue (Lawson and Mason 1998).

The bioavailability of a metal to a predator is also dependent on whether the metal is stored in a detoxified form in the prey e.g. bound to metallothioneins or sequestered in lysosomes or calcium granules (George 1990). While the prey can accumulate high body burdens of metals, if the metals are stored in a detoxified form, then that metal is less bioavailable to its predator. A study determined that Cd sequestered in granules used for storing and detoxifying Cd in an oligochaete had a lower AE when ingested by the grass shrimp *Palaemonetes pugio* than when Cd was not sequestered (Wallace and Lopez 1997).

In benthic food chains, amphipods, polychaetes, and bivalves feed on sediment that can serve as a sink for metals. Field studies have found that sediment located close to industrialized areas can have elevated metal levels and are much more enriched than the overlying water column (Kennish 1997 and references therein). This provides a source of metals to benthic organisms, including polychates which assimilate 43-83% of MeHg, 7-30% of Hg(II), 1.5-59% of Cd, 1.2-12% of As, and 0.7-4.6% of Cr from ingested sediment (Wang et al. 1998, 1999;

Baumann and Fisher 2011), and can therefore act as a conduit for the transfer of these metals to fish.

MODELING METAL BIOACCUMULATION IN ESTUARINE FISH

In the early 1980's a biokinetic model was developed to calculate the body burden of metals at steady-state in aquatic organisms based upon the uptake and loss of metals following aqueous and dietary exposure (Thomann 1981). This model has since been modified (Wang et al. 1996; Reinfelder et al. 1998) and assessed using a radiotracer technique in zooplankton (Fisher et al. 2000), bivalves (Wang et al. 1996; Roditi et al. 2000b), and more recently fish (Pickhardt et al. 2006; Mathews and Fisher 2009). This model can be used to calculate the bioaccumulation potential of individual metals between different field sites and field conditions, to make intra- and inter-specific species comparisons, and the calculated values can be compared to independent field data to predict body burdens of metals in fish. Under steady-state conditions the equation is as follows:

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

where C_{ss} is the steady-state concentration of metal in an organism (μg g^{-1}), 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 (%; defined as the percentage of metal that crosses the gut lining), 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}).

Equation (1) can be rearranged to calculate the percentage of total body burden which is attributed to either aqueous (R_w) or dietary (R_f) metal exposure. The equations are as follows:

$$R_w = [(k_u \cdot C_w)/(g + k_{ew})]/C_{ss} \cdot 100 \quad (2)$$

$$R_f = [(AE \cdot IR \cdot C_f)/(g + k_{ef})]/C_{ss} \cdot 100 \quad (3)$$

Equation (1) can also be rearranged to calculate the trophic transfer factor (TTF) which describes the likelihood a metal will biomagnify at a particular trophic level, based upon the ratio of metal in the predator compared to metal in its prey. A TTF >1 indicates the metal is likely to biomagnify due to a high AE and low k_{ef}, whereas a TTF <1 indicates biomagnification is unlikely (Reinfelder et al. 1998). The equation is as follows:

$$TTF = (AE \cdot IR)/(g + k_{ef}) \quad (4)$$

The k_{ew} and k_{ef} values can be used to calculate the biological half-life ($tb_{½}$) of each metal in the whole fish following aqueous or dietary exposure. The $tb_{½}$ is defined as the time is takes for 50% of the metal to be excreted from the organism. A metal with a high k_{ew} or k_{ef} will have a short $tb_{½}$ and therefore a faster rate of physiological turnover. Assuming it takes 7 half-lives to excrete all the metal from the organism's body, the residence time can be calculated. The $tb_{½}$ is calculated as follows, where ln2 equals 0.693:

$$tb_{½} = \ln 2/k_{ef} \quad (5)$$

ISSUES WITH THE BIOKINETIC MODEL

The biokinetic model has proven to be an effective tool for understanding metal bioaccumulation in aquatic organisms, including fish; however there are several drawbacks which have been critically reviewed (Luoma and Rainbow 2005) and are summarized below.

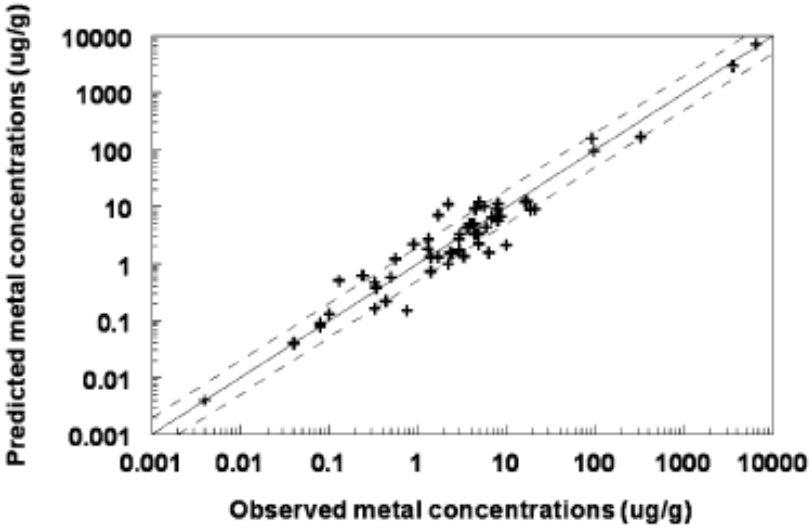
- When calculating the model parameters (k_u, AE, k_{ew}, k_{ef}) in controlled laboratory experiments it is assumed that all uptake rates and loss rates are constant, while in the natural environment they are not. For example, in the natural environment an estuarine fish would experience a change in salinity throughout the day due to the influence of the tides, and between seasons due to in the addition of meltwater decreasing the salinity of estuarine water in the late Spring and possible drought conditions increasing the salinity during the Summer; this in turn will influence the k_u value.
- k_u values are calculated using short term exposure periods (in the range of a few hours to a day; Wang and Wong 2003; Pickhardt et al. 2006) to remove the influence of metal storage and loss from the fish that would be associated with a long term exposure. However, during the first few hours of aqueous exposure, the metal is rapidly binding to the most reactive sites on the fish (presumably the gills), and once the number of free binding sites decrease then the k_u will decrease; therefore the k_u value used in the biokinetic model may be overestimated.
- In laboratory experiments, AE values are calculated using one prey organism, where in nature fish consume a varied diet, resulting in a wide range of AEs depending on the tissue distribution of metal within the prey. For example, a study investigating the importance of prey type on the AE of Zn in the grunt *Terapon jarbua*, determined that the AEs varied from 1.5% when fed barnacles to 52% when fed fish viscera (Zhang and Wang 2006). As a result, the AE value used in the biokinetic model could be over- or underestimated depending upon the chosen food source.
- The importance of ingestion rate has not been fully addressed in bioaccumulation studies. A study using the mangrove snapper *Lutjanus argentimaculatus* has shown that the IR strongly influences the AE of metals; as the IR in the mangrove snapper increased from 0.05 to 0.57 g g^{-1} d^{-1}, the AEs of Cd, Se, and Zn decreased (24 to 7%, 69 to 54%, and 43 to 17%, respectively; Xu and Wang 2002). This is thought to be attributed to a faster gut passage time, allowing less time for metal to be assimilated from the diet. Another study using the mussel *Mytilus edulis* also concluded that the AE of metals was higher when the gut passage time was longer (Wang and Fisher 1996).
- Laboratory studies have recognized the importance of k_{ew} and k_{ef} in metal bioaccumulation studies but have not provided mechanistic explanations for variability in

these parameters. k_{ew} and k_{ef} represent the physiological turnover of metal and are calculated on a whole-fish basis. However, the k_{ew} and k_{ef} basically integrate the physiological turnover rate of metals from difference tissue compartments following aqueous and dietary exposures to metals. Hogstrand et al. (2003) has calculated tissue-specific depuration rates in two freshwater fish following aqueous exposure to Ag, and Oliveira-Ribeiro et al. (1999) has calculated tissue-specific depuration rates in Arctic charr following dietary exposure to MeHg. More studies need to include a detailed dissection component to calculate tissue-specific metal influx or efflux rates, to further understand how metals are redistributed around the body and how metal uptake or loss from different tissues influences the whole-fish k_{ew} and k_{ef}.

- The k_u, k_{ew} and k_{ef} values may also be underestimated; it has been noted in other studies that the swimming activity and respiration rate of fish is often lower in laboratory experiments (Post et al. 1996; Pickhardt et al. 2006), possibly due to stress.
- Growth rate is a parameter which is often assumed to be negligible relative to k_e values in the biokinetic model, unless the investigated organism has a rapid rate of growth. It is important to consider the rate of growth because it can lead to the somatic growth dilution of metals in aquatic organisms. A laboratory study investigating the importance of food quality on MeHg accumulation in *Daphnia* found that *Daphnia* fed higher quality algae grew faster and had a lower MeHg body burden as a result (Karimi et al. 2007). A more comprehensive evaluation of this issue is presented in Karimi et al. (2010). A field experiment using the Atlantic salmon *Salmo salar* also concluded that faster-growing salmon had a lower Hg body-burden due to somatic growth dilution (Ward et al. 2010).

Nevertheless, despite these concerns, where model predicted metal concentrations have been generated for diverse marine animals and compared to independent field measurements it has generally been found that model predictions match field observations strikingly well, as shown in Fig. 2.

Fig. 2. Predicted versus observed metal concentrations in zooplankton, bivalve mollusks, barnacles, crabs, fish, and insects in eleven different ecosystems (from Luoma and Rainbow 2005)



ADVANTAGES OF USING A RADIOTRACER TECHNIQUE

All experiments were conducted using gamma-emitting radioisotopes (^{241}Am, ^{73}As, ^{109}Cd, ^{51}Cr, ^{203}Hg, ^{75}Se, ^{65}Zn). This method allowed non-destructive sampling of live animals, enabling metal uptake and loss to be monitored in the same individual fish throughout each experiment, therefore reducing biological variability. Radioisotopes, except for ^{241}Am and ^{203}Hg, can also be added at low, environmentally realistic concentrations. The background concentration of Am in seawater is too low to add a tracer concentration, and the specific activity of Hg is so low that a concentration higher than the background concentration of Hg(II) and MeHg needed to be added to detect a signal. Finally, because radioisotopes have different energy emissions (keV), fish can be labeled with two or more different radioisotopes simultaneously to reduce the number of experiments if the energy emissions do not overlap (e.g., As and Cr, Cd and Zn). ^{73}As, ^{109}Cd, ^{51}Cr, and ^{203}Hg were used for the killifish experiments, and ^{241}Am, ^{109}Cd, ^{203}Hg, ^{75}Se, and ^{65}Zn were used for the Atlantic silverside experiments.

OVERVIEW OF THE DISSERTATION

The objective of this dissertation was to investigate how water chemistry, prey type, and fish physiology influenced metal accumulation and tissue distribution in fish. This dissertation is presented in six research chapters, followed by an overall conclusions chapter.

- In Chapter II, I investigated the role of salinity on metal bioavailability to killifish from the aqueous phase over a 48 h (MeHg) or 72 h (As, Cd, Cr, Hg(II) time period. At the end of uptake the killifish were dissected to determine if salinity influenced tissue distribution.
- In Chapter III, I investigated whether dissolved organic matter (humic acid) influenced the bioavailability of metals to killifish from the aqueous phase over a 72 h exposure period. At the end of uptake the killifish were dissected to determine whether the presence of dissolved organic matter influenced the tissue distribution.
- In Chapter IV, I investigated whether the type of prey (amphipod and worm) fed to killifish influenced the AEs and k_{ef}s of ingested metals and calculated the likelihood of biomagnification at this trophic step. Dissections were carried out at the end of a 9 d depuration period after consuming both prey types to compare the tissue distribution, and fish were dissected regularly throughout the 9 d depuration period after feeding on amphipods to investigate how the metals were redistributed between different tissue compartments following dietary exposure.
- In Chapter V, I investigated whether metals bound to sediment and algae are bioavailable to killifish using an intubation procedure. Killifish do not actively consume sediment, but ingest a small amount through incidental uptake while consuming benthic prey.
- In Chapter VI, I focused on calculating rates of metal uptake and loss in killifish following aqueous exposure to metals in water from three contaminated field locations (Baltimore Harbor (MD), Elizabeth River (VA) and Mare Island (CA)). These three sites were chosen because they have varying salinities and dissolved organic matter concentrations, are heavily contaminated, and are part of a larger study. These values were entered into the biokinetic model with the dietary parameters calculated in Chapter IV to calculate the steady-state concentration of metals in killifish at each field site and

the percentage of the body burden attributed to the diet. Killifish were dissected at the end of 9 d depuration following aqueous exposure at all three field sites to compare tissue distributions. Five killifish in Baltimore Harbor water were dissected at the end of metal uptake and on day 1, 3, 6, and 9 of depuration to monitor the redistribution of aqueous metals around the body and calculate efflux rates from different tissue compartments.

- In Chapter VII, I investigated the uptake and loss of metals from the aqueous phase and the diet in two populations of the Atlantic silverside (Nova Scotia and South Carolina) as a function of ingestion rate and growth rate. The calculated kinetic parameters were entered into the biokinetic model to calculate the body burden of metals at steady-state in both populations and the percentage attributed to the diet. The fish were dissected at the end of 6 d depuration following aqueous and dietary exposure to metals to investigate whether tissue distribution varied between exposure route and the two populations.

Chapter II

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. The present study 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 (salinities ≤ 6 ppt) and Cr were regulated by the fish, whereas Cd, Hg(II), and MeHg were not. Cadmium, Hg(II), and MeHg are chloro-complexed, 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 (0.79-2.29 L g^{-1} d^{-1}), followed by Hg(II), Cd, Cr, and lowest for As (0.0004-0.0008 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, whereas 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.

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INTRODUCTION

Field studies have shown that fish caught in heavily urbanized and industrialized estuaries have elevated metal levels (Kennish 1997; Edwards et al. 2001). As with other aquatic organisms, fish are exposed to waterborne metals through the aqueous phase and their diet (Xu and Wang 2002; Wang and Wong 2003; Pickhardt et al. 2006; Mathews and Fisher 2009). 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 (Fisher and Reinfelder 1995). 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 (Xu and Wang 2002; Pickhardt et al. 2006; Mathews and Fisher 2009). However, the uptake of dissolved metal still has 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 as a result of chloro-complexation of the metal ion; Cd uptake has an inverse relationship with salinity (Blackmore and Wang 2003; Jackson et al. 2003), 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 (Laporte et al. 1997; Modassir 2000; Pan and Wang 2004). 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 (Baldisserotto et al. 2005; Franklin et al. 2005). Competition between anionic arsenate (As(V)) and PO_4 influences As(V) uptake, as noted for phytoplankton (Sanders and Windom 1980). As a result, metal uptake as a function of salinity varies by metal.

Aqueous exposure studies have quantified metal uptake into fish (Xu and Wang 2002; Wang and Wong 2003; Jeffree et al. 2006; Pickhardt et al. 2006; Mathews et al. 2008), but information on how metal uptake in fish varies with salinity is scarce. Studies have reported the influence of salinity on the acquisition of Cu in *Fundulus heteroclitus* (Blanchard and Grosell

2006), Ag in the gulf toadfish (*Opsanus beta*) (Wood et al. 2004), Cd in the tidewater silverside (*Menidia beryllina*) (Jackson et al. 2003), and Cd and Zn in the black sea bream (*Acanthopagrus schlegeli*) (Zhang and Wang 2007a). 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 (Thomann 1981; Wang et al. 1996).

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 (Abraham 1985) and limited swimming range. Furthermore, they are euryhaline (Abraham 1985), 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 (Kneib 1986; Hartman and Brandt 1995; Rountree and Able 1996). 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 because of their binding preferences for N or S; As, Cd, and Cr are borderline metals/metalloid, whereas Hg is a sulfur-seeking Class B metal (Nieboer and Richardson 1980). Cadmium, Hg(II), and MeHg are known to be chloro-complexed, whereas As and Cr are not (Bruland 1983). 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 (Kennish 1997; Edwards et al. 2001) 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. Fish were acclimated to experimental conditions for at least four weeks prior to the beginning of experiments, and fed a diet of TetraCichlid™ cichlid flakes (Tetra Holding) 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) 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). 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, 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. All experiments were carried out in glass beakers that 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 were 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. 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) were used 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) and held in 0.1 M HCl, ^{51}Cr ($t_{½}$ = 27.7 d) was purchased from PerkinElmer and held in 0.5 M HCl, and ^{203}Hg(II) ($t_{½}$ = 46.6 d) was purchased from Eckert & Ziegler Isotope Products 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 (Imura et al. 1971; Rouleau and Block 1997; Bancon-Montigny et al. 2004), and held in deionized water (MilliQ). 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 ethyl 3-aminobenzoate methanesulfonate (MS-222); 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 dry weight of the fish in grams 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 concentration factors can be converted to wet weight CFs by dividing by 4 (dry weight is ~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 deep-well NaI(Tl) γ-detector. This allows the same fish to be counted throughout uptake, reducing biological variability. Counting time did not exceed 5 min to reduce stress on the fish, and we generally obtained propagated counting errors of ≤ 5%. For ^{73}As and ^{51}Cr , however, propagated errors reached up to 25% because of 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 as a function of salinity for *F. heteroclitus* after 10 h (Cr), 14 h (MeHg) and 72 h (As, Cd, and Hg(II)) of 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 concentration factors 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. A strong positive linear relationship between salinity and CF was seen for As ($p < 0.01$), and no relationship between salinity and CF for Cr and MeHg ($p > 0.05$). No statistically significant relationship between Cd CFs and salinity was seen ($p > 0.05$); 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) of 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 sixfold 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. Although 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, 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 associated mainly with the body (54-64%), and least with the gills (3-8%). Chromium was associated with either the head (28-40%) or the viscera (24-46%) and least with the body (8-16%). Inorganic Hg was associated predominantly with the body (38-41%), followed by similar distributions between the head and the gills (27-28% and 23-30% respectively), and lowest in the viscera (4-8%) 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; whereas 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 (7-12%; $p > 0.05$). 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, no relationship was observed between the percentage of MeHg associated with the body and salinity ($p > 0.05$). The greatest percentage of MeHg was associated with the body (45-49%).

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, and 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 be predominantly at the gills (Jezierska and Witeska 2004; Franklin et al. 2005). 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 resulting from 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 (Neff 1997); 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 have been reported (Larsen and Francesconi 2003). Nevertheless, it is recognized that As is largely acquired through diet (Neff 1997), 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 for which salinity had no influence on uptake; like As, it does not chloro-complex in seawater but instead binds to oxygen forming oxyanions (Bruland 1983). It was added as trivalent Cr (Cr(III)), which is highly particle reactive, yet uptake was still low (resulting in CFs < 1), suggesting that Cr(III) does not readily cross biological membranes in living organisms. In one experiment, 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; Wang et al. 1997). In comparison, although hexavalent Cr (Cr(VI)) is not as particle-reactive as Cr(III), it more readily crosses biological membranes and accumulates in marine organisms (Wang et al. 1997). 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 might have been reached for both As and Cr before 10 h of exposure, but because of 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*) (Mathews et al. 2008) and after several days for the dogfish (*Scyliorhinus canicula*) (Jeffree et al. 2006), and did not reach equilibrium after 2 weeks for the turbot (*Psetta maxima*) (Jeffree et al. 2006). For the dogfish and turbot, CFs were < 1 throughout the two 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}) (Mathews et al. 2008) was somewhat higher than the values noted in this study.

Cadmium, Hg(II), and MeHg are all chloro-complexed in seawater (Bruland 1983). 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 (0-2 h), uptake of MeHg was rapid at 25 ppt, after which uptake slowed; this decreased uptake could be due to some of the remaining MeHg being bound to DOC 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 dissolved organic matter was not determined in our study.

The present study showed that preferential uptake of Hg(II) and MeHg occurs in the mercuric chloride form, compared to the hydroxide form found in freshwater. The octanol-water partition coefficients (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 (Faust 1992; Mason et al. 1996). 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 that 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 (2007) 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 (Andres et al. 2002; Pickhardt et al. 2006). Although we are unable to compare our Hg(II) and MeHg k_u values with 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 (Wang and Wong 2003; Pickhardt et al. 2006). However, uptake rate and toxicity data for aquatic invertebrates showed that Hg uptake increased with decreasing salinity (Hall and Anderson 1995; Pan and Wang 2004), the reverse of what has been observed in fish.

Cadmium uptake decreased as salinity increased because of 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 $CdCl_3^-$ (Zhang and Wang 2007a), reducing its bioavailability to organisms. Cadmium chloride has a very low k_{ow} (0.21) (Mason et al. 1996) compared to $HgCl_2$ and CH_3HgCl, so it is unable to penetrate biological membranes easily because of its low lipophilicity. Other Cd bioaccumulation and toxicity studies using fish and invertebrates support our findings of higher Cd accumulation at lower salinities (Hall and Anderson 1995; Blackmore and Wang 2003; Jackson et al. 2003: Zhang and Wang 2007a).

Dissolved organic carbon 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 as a result of organic carbon release from fish. This increased DOC might 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, the tissue distribution of Cd changed in *Fundulus heteroclitus*. In freshwater, Cd was predominantly associated with the gills, whereas in seawater it was associated with the viscera, comparable to the findings of an earlier study (Zhang and Wang 2007a). 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 (Zhang and Wang 2007a), which would support this conclusion. However, Ca uptake also increases at lower salinities (Zhang and Wang 2007a), and studies have shown that Cd and Ca share the same uptake pathway, so there is competitive uptake (Franklin et al. 2005); 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 cannot be explained by drinking; as the salinity increased from 0 to 25 ppt, the Hg(II) concentration in the gills increased by 3.5-fold and the by 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).

Tissue distribution data with which to compare our results are limited, 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 (Jeffree et al. 2006). At the end of uptake, As, Hg(II), and MeHg were associated predominantly 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 (NY, USA).

	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 methylmercury (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 methylmercury (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 percentage of metal in each tissue compartment are represented by asterisks (* $p < 0.05$, ** $p < 0.01$). MeHg = methylmercury.

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 are mean ± 1 standard error; n = 5 per salinity. Units are kBq g^{-1} for Cd, Hg(II), and methylmercury (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

Fig. 1. Accumulation of aqueous metals (Bq g^{-1} dry wt) 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.

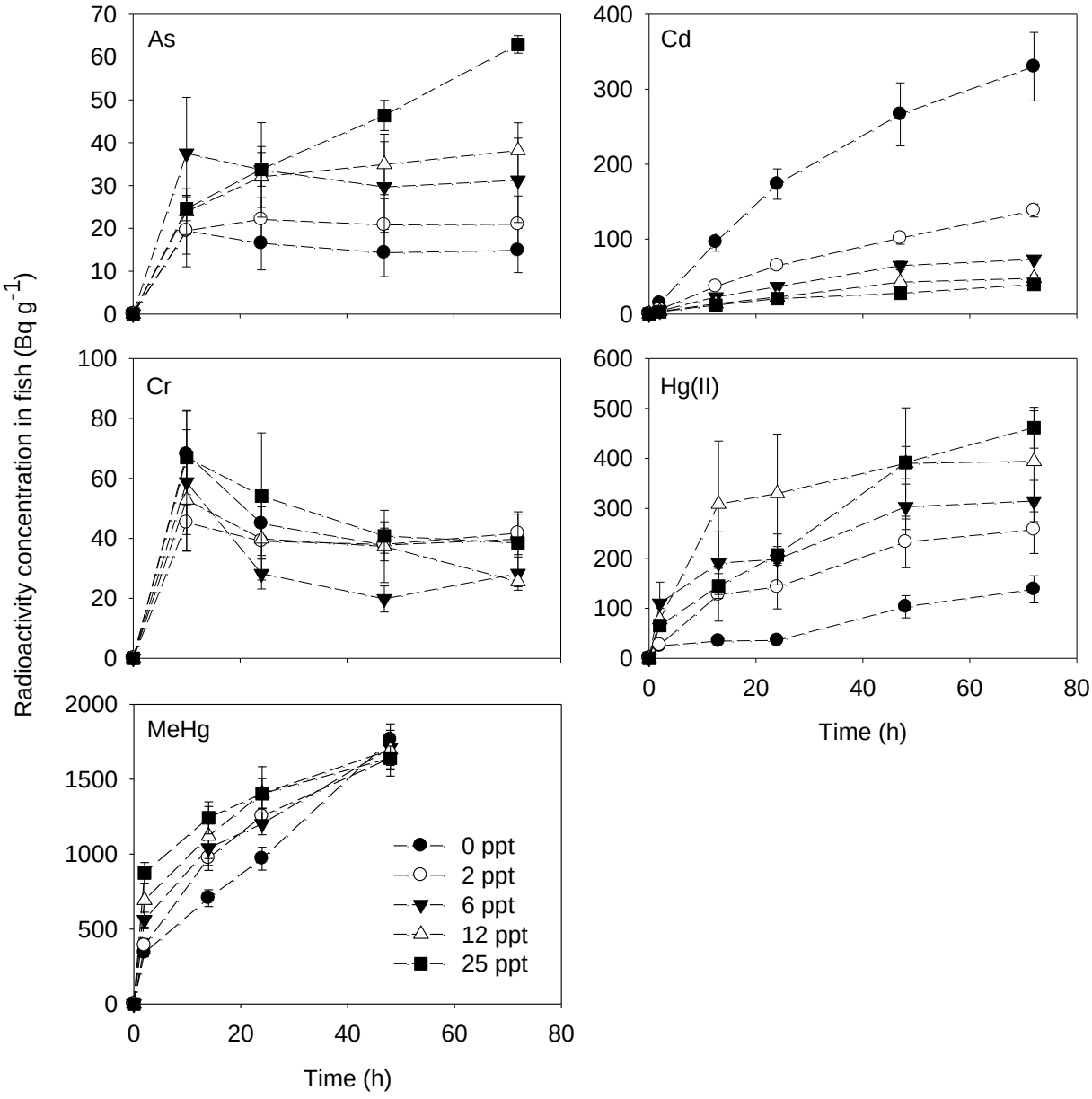
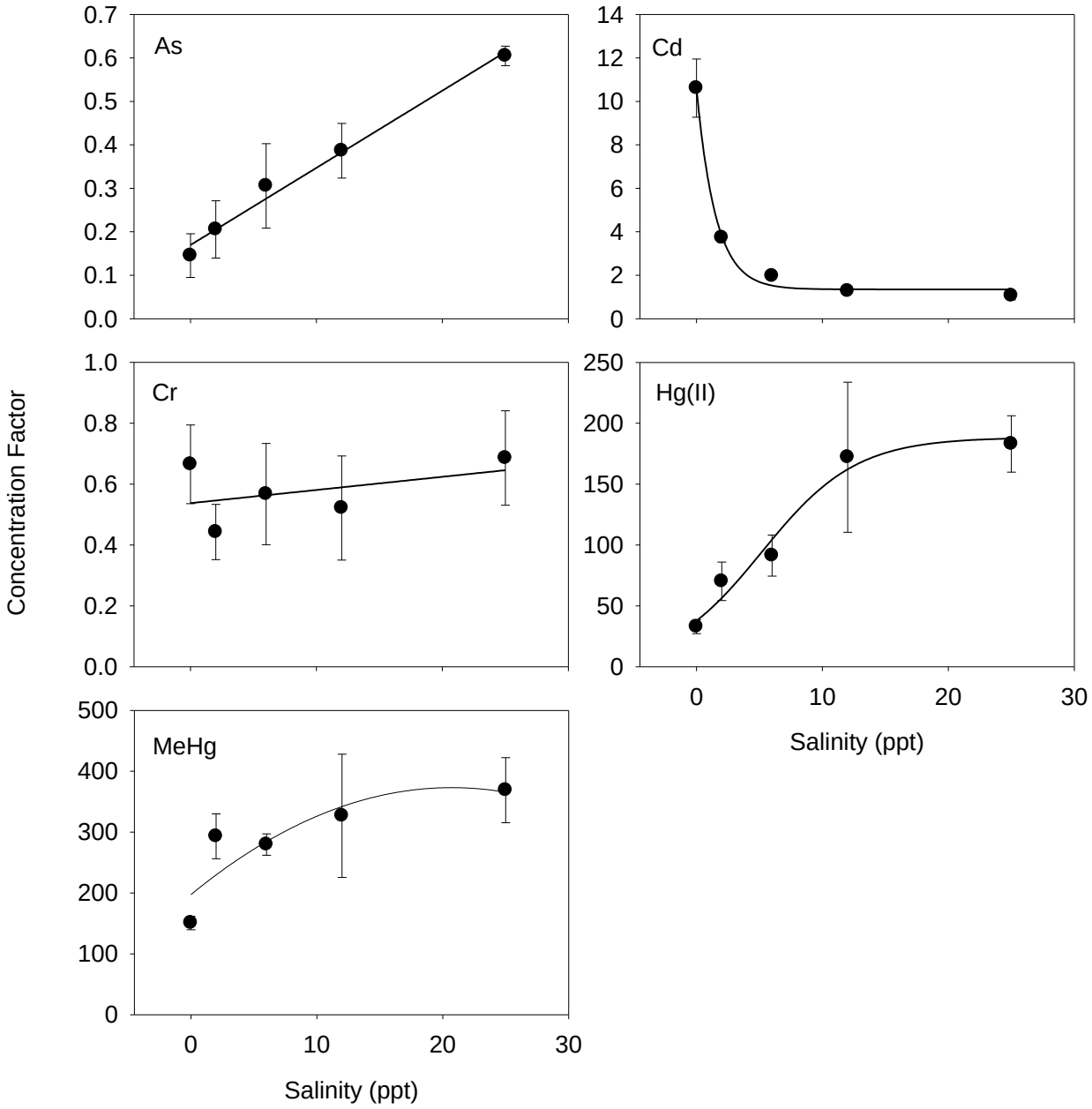


Fig. 2. Metal concentration factors (CFs) as a function of salinity for killifish (*Fundulus heteroclitus*) after 10 h (Cr), 14 h (methylmercury, (MeHg)), or 72 h (As, Cd, Hg(II)) exposure. Values represent means ± 1 standard error; $n = 5$ per salinity. Regression equations and r^2 values describing the relationships 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 $y = -0.410x^2 + 16.966x + 197.450$, $r^2 = 0.76$.



Chapter III

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

ABSTRACT

The role of humic acids was investigated 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 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 concentration factors <1 at the end of a 72 h exposure. The uptake of As and Cr reached steady-state within the 72 h exposure, whereas uptake of Cd, Hg(II) and MeHg did 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. The present 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.

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INTRODUCTION

It has been well demonstrated that dissolved organic matter (DOM) tends to diminish metal uptake and toxicity in aquatic organisms (Campbell 1995; Richards et al. 1999). Studies originally focused on lower trophic levels, including phytoplankton (Sunda and Lewis 1978), which typically have the highest bioconcentration (Fisher and Reinfelder 1995). 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 has greater chemical reactivity and has been shown to be more bioavailable (Campbell 1995). The general applicability of this pattern to other organisms is questionable. For example, studies using bivalve mollusks have shown that metal bioavailability can either increase or decrease with increasing DOM concentration (Roditi et al. 2000a; Guo et al. 2001; Pang and Wang 2004a; Nadella et al. 2009).

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 and metal complexation with DOM and inorganic ligands) and its impact on metal speciation, and therefore bioavailability to fish (Di Toro et al. 2001; Paquin et al. 2002). 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 (McGeer et al. 2000; Niyogi et al. 2003, 2004).

The gills account for approximately 50% of the external body surface in fish (Jezierska and Witeska 2004). As a result, the gills are an important uptake site for dissolved metal, due to either metal passively binding to the gill surface, or entering the gills through uptake channels (Simkiss and Taylor 1989). Furthermore, studies have investigated the binding affinity of metals to gills compared to the binding affinity of metals to DOM and calculated stability constants (Playle 1998). To date, most studies investigating the role of DOM on metal accumulation in fish have determined whether the presence of DOM reduces metal accumulation, the uptake mechanisms involved, and the resulting physiological changes in the gills (Choi et al. 1998; Richards et al. 1999; Jezierska and Witeska 2004). However, further studies are needed to understand how the presence of DOM affects the rate of metal uptake across a wide range of DOM concentrations for potentially toxic metals, including As, Cd, Cr, and Hg.

The objective of the present 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 methylmercury (MeHg))) 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 (Abraham 1985), and have varying sensitivity to aquatic contaminants. Furthermore, killifish are found in fresh water, brackish water, and saltwater (Abraham 1985), which experiences a large variation in DOM concentration. Humic acid was the chosen DOM because it is an effective chelating agent in natural waters (Mantoura et al. 1978).

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 (Kennish 1997). 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.

MATERIALS AND METHODS

Fish acclimation

Field-collected (Taylor River, Hampton, NH, USA; salinity = 25-30 ppt) killifish, *F. heteroclitus*, (mean wet wt 2.06 g ± 0.31 standard deviation) purchased from Aquatic Research Organisms were used in the present 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) 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 period, 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) amended to 1 ppt using Instant Ocean (Aquarium Systems), and either 0, 2.5, 5, 10, or 20 mg L^{-1} Suwannee River humic acid. The humic acid was purchased from the International Humic Substances Society as Suwannee River humic acid standard batch 2.

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

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) because humic acid dissolves more readily at a higher pH (Aiken et al. 1992). Humic acid was added to the required concentration and dissolved by sonicating for 15 min. The pH was then adjusted back to 7.8-8 using trace metal grade nitric acid (2N HNO_3; GFS Chemicals). Salinity was adjusted to 1 ppt using Instant Ocean, and samples were collected for DOC and chloride ion analysis. The experimental water (250 ml) was poured into individual 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.

High specific activity gamma-emitting radioisotopes were used in the present study (274 kBq/µg ^{73}As, 470 kBq/µg ^{109}Cd, 4292 kBq/µg ^{51}Cr, 122 kBq/µg ^{203}Hg(II) and 133 kBq/µg MeHg). In all, ^{73}As (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), ^{51}Cr (as Cr(III), $t_{½}$ = 27.7 d, in 0.5 M HCl) from PerkinElmer, and ^{203}Hg (as Hg(II), $t_{½}$ = 46.6 d, in 1 M HCl) from Eckert and Ziegler Isotope Products. Inorganic mercury was methylated in our lab to $CH_3{}^{203}$Hg(II), methylmercury, following a well described method (Imura et al. 1971; Rouleau and Block 1997; Bancon-Montigny et al. 2004) and held in

deionized water. Equimolar concentrations of sodium hydroxide in microliter quantities were added to the experimental water to neutralize the acid associated with the radioisotopes, so the pH remained unchanged. Both ^{73}As 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 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), dissected into head, gills, viscera, and body (backbone, fillet, and 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 wt) 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 wt) by the radioactivity in 1 ml water. Concentration factors were calculated at 72 h for As, Cd, Cr, and Hg(II), and at 47.5 h for MeHg due to a potential drawdown of MeHg at 72 h in the experimental media. 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 significances of concentration factors, 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 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 min 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 min using an inter-calibrated LKB Pharmacia-Wallac 1282 CompuGamma CS gamma counter. 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. By the end of the 72 h exposure, As uptake increased 2.6-fold as the humic acid concentration increased from 0 to 20 mg L^{-1}. 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 3.7–fold, and Cr accumulation at the end of uptake was similar at 5, 10, and 20 mg L^{-1}. Arsenic and Cr uptake stabilized, and decreased for Cr at some humic acid concentrations, by the end of uptake, indicating steady-state 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 to 0.07% of As, 1.9 to 2.4% of Cd, 0.06 to 0.3% of Cr, 8 to 21% of Hg(II), and 50 to 86% of MeHg added at the start of the experiment was associated with the fish. Through mass balance calculations, we could account for >95% of all of the metal used in each experiment. For As,Cd, Cr, and Hg(II), the

metal was in the water primarily, whereas for MeHg it was mostly in the fish. Any metal not accounted for in the water or fish was presumed to be adsorbed to the glass beaker.

Concentrations factors 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 concentration factors were >1 at 0 mg L^{-1} throughout uptake, but <1 at 2.5-20 mg L^{-1}. Cadmium concentration factors did not exceed 1 until 12 h exposure at 0, 2.5, 10, and 20 mg L^{-1} (data not shown). Figure 2 shows concentration factors of each metal as a function of humic acid concentration after 47.5 h (MeHg) or 72 h (As, Cd, Cr, Hg(II)) of 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. A statistically significant relationship was found between humic acid concentration and the concentration factor 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 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 were lowest for As. 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 0.58 ml g^{-1} d^{-1} as humic acid concentration increased from 0 to 10 mg L^{-1}. 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 Hg(II) k_u value decreased as humic acid concentration increased. Methymercury k_us decreased as humic acid concentration increased; at 0 and 2.5 mg L^{-1} the highest k_u was observed between 0 and 2.5 h of exposure, whereas for 5, 10, and 20 mg L^{-1} the highest k_u was observed between 23.5 and 47.5 h of exposure. Like Cr, the largest decrease in MeHg k_us was noted

between 0 and 2.5 mg L^{-1}. No statistically significant relationship was found between k_u and humic acid concentration for As and Cd ($p > 0.05$), whereas there was a statistically significant relationship for Cr, Hg(II), and MeHg ($p < 0.05$).

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 as humic acid concentration increased from 0 to 20 mg L^{-1}, whereas it increased in the body. Cadmium was mainly associated with the gills, and least with the viscera and body at each humic acid concentration. As humic acid concentration increased from 0 to 20 mg L^{-1}, the percentage of Cr associated with the head and gills decreased, whereas it increased in the viscera and body. Inorganic Hg was mainly associated with the body and least with the viscera at each humic acid concentration. Methylmercury was mainly associated with the body, followed by the head, and then nearly evenly distributed between the gills and viscera at each humic acid concentration. 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.

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 concentration factors were <1 at all humic acid concentrations. The low CFs and k_u values observed in the present 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 could possibly be attributed to a low binding affinity of As for the gill, saturation of all available As binding sites

on the gill, downregulation of the As transporter protein, or internal regulation of As within the fish, in which 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 (Dutton and Fisher 2011a). 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 arsenite in the presence of humic acid (Palmer et al. 2006). After the experimental medium 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 the present study are similar to (CFs) or slightly lower (k_us) than those calculated when As uptake was measured as a function of salinity and a background DOC concentration of 2.9 mg L^{-1} (Dutton and Fisher 2011a).

Chromium uptake decreased inversely with humic acid concentration. 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 $\geq$ 5 mg L^{-1} there was little difference in CFs (0.28 to 0.33) and k_u values (0.58 to 0.92 ml g^{-1} d^{-1}), suggesting strong binding of Cr by humic acid at concentrations $\geq$ 5 mg L^{-1}. The decrease in CFs and k_us in the presence of humic acid also suggests that Cr(III) either has a greater binding affinity for humic acid than fish gills or as the humic acid concentration increases there are more free binding sites on the humic acid than the 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 (Wang et al. 1997). In the presence of humic acid, steady-state was reached within the first 24 h of exposure and within 48 h when no humic acid was added, indicating a low binding affinity of Cr for the gill or internal regulation within the fish. In other studies Cr uptake reached steady-state 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 steady state in the turbot (*Psetta maxima*) throughout a 14 d exposure period (Jeffree et al. 2006; Mathews et al. 2008; Dutton and Fisher

2011a). 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 the present 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 (Dutton and Fisher 2011a).

Cadmium was the only metal for which humic acid concentration had no influence on metal uptake. Prior studies have shown that the presence of DOM did not have a protective role against Cd binding at the gills in rainbow trout (*Oncorhynchus mykiss*) (Hollis et al. 1996; Richards et al. 1999). 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) (Playle et al. 1993). 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) (Playle et al. 1993). However, another study using the common carp (*Cyprinus carpio*) demonstrated that the presence of humic acid provided a protective role against Cd uptake from water (Van Ginneken et al. 2001). 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 (Dutton and Fisher 2011a). The CFs and k_u values 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 and k_u values decrease due to the chlorocomplexation of Cd reducing metal accumulation (Dutton and Fisher 2011a). The data for Cd at 5 mg L^{-1} were not shown in the figures and tables of the present study; 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 although 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.

The uptake of Hg(II) and MeHg decreased inversely with humic acid concentration. 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 (Pickhardt et al. 2006) and a freshwater diatom (Luengen et al. 2012). It should be noted that the shape of the Hg(II) and MeHg uptake curves shown in Figure 1 and the calculated k_u values may possibly be a result of the drawdown of metal in the experimental media reducing the concentration of Hg the fish are exposed to over time. 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 (Dutton and Fisher 2011a).

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 species 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 $K_{Hg\text{-}NOM}$ and log $K_{Hg\text{-}gill}$ = 18.0) (Klinck et al. 2005). 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 the present study (Averett et al. 1994). 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 (Pickhardt et al. 2006; Dutton and Fisher 2010, 2011a).

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 humic acid, thereby reducing the bioavailable free metal ion. This resulted in a significant decline in the total body burden of Cr. No significant trend in Cr concentrations were found 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, Cd 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 (Dutton and Fisher 2011a).

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. No consistent trends were found 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 (Dutton and Fisher 2011a).

Importance of humic acid concentration when modeling metal bioaccumulation

Bioaccumulation studies using fish often focus on the trophic transfer of metals because the diet is considered to be the dominant exposure route (Pickhardt et al. 2006; Mathews and Fisher 2009; Dutton and Fisher 2010). The present study as well as another (Dutton and Fisher 2011a) concludes that changes in water chemistry can influence the accumulation of aqueous metals in killifish and should be considered when modeling metal bioaccumulation in these fish. If fish migrate between fresh water, brackish water, and saltwater with varying DOM concentrations, then the rate of metal uptake from the aqueous phase can change. This can influence the overall body burden of metals in fish and the importance of aqueous exposure as a source of metals to fish. Furthermore, studies investigating the aqueous accumulation of metals in fish often use short-term exposure periods to remove the complications of metal storage and

loss associated with longer exposure periods. The resulting k_u value is assumed to be constant, but as the present study shows, the k_u value changes over time and can therefore be over- or underestimated, and should be considered when modeling metal bioaccumulation in fish.

Future studies are required to understand whether the uptake patterns observed in the present study are a result of the chemical speciation of metal in the water, due to fish physiology, or whether both factors play a role. The results from the present study are not applicable across a wide range of salinities. Studies are needed to investigate metal uptake as a function of DOM concentration at higher salinities because due to the change in the chemical reactivity of metals in the presence of chloride ions, the influence of DOM on metal uptake may vary with salinity.

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 % DOC, 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 concentration (mg L^{-1})	Chlorinity (mM)	DOC (mg L^{-1})	% DOC	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 of 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 of 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

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

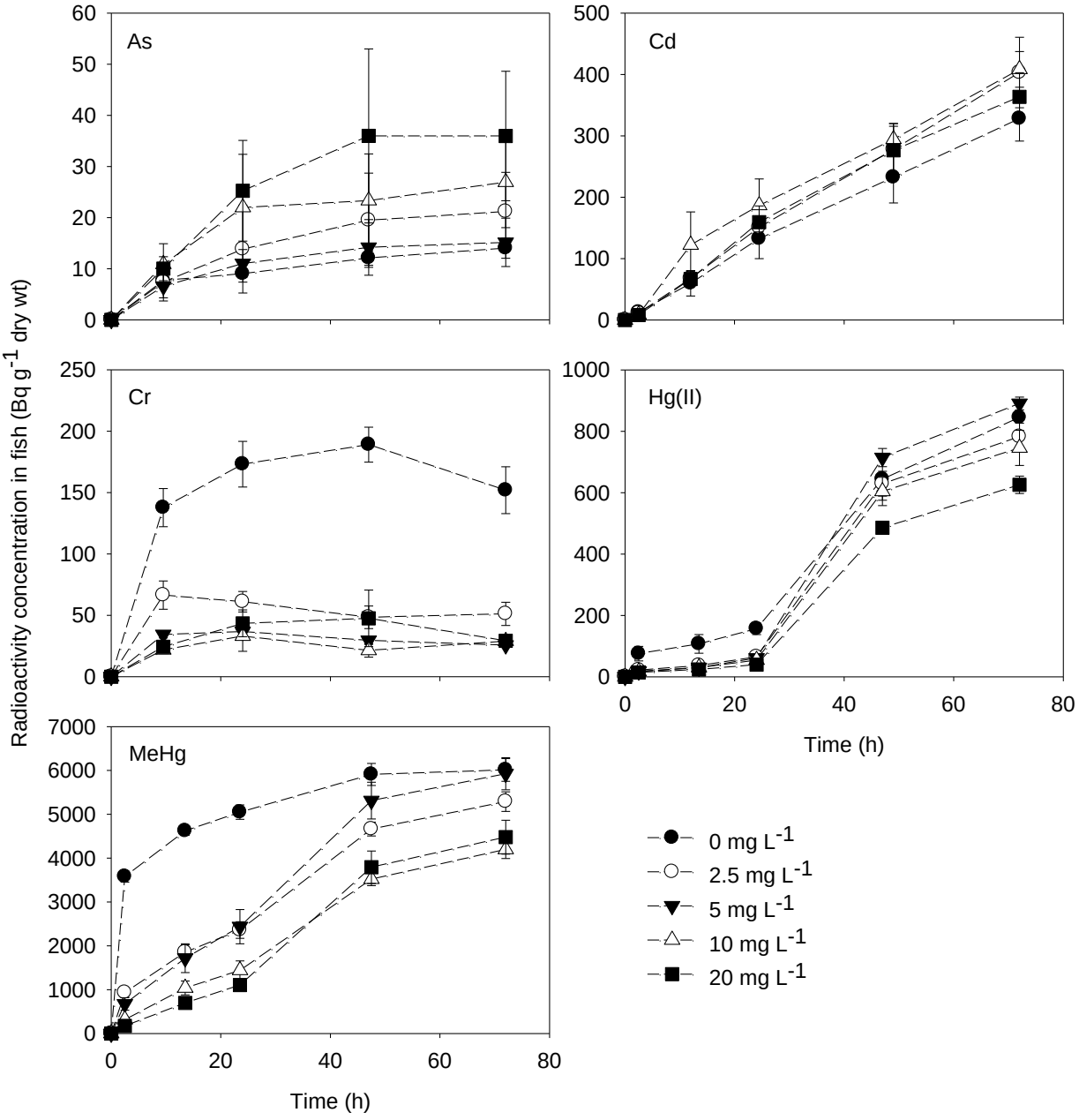
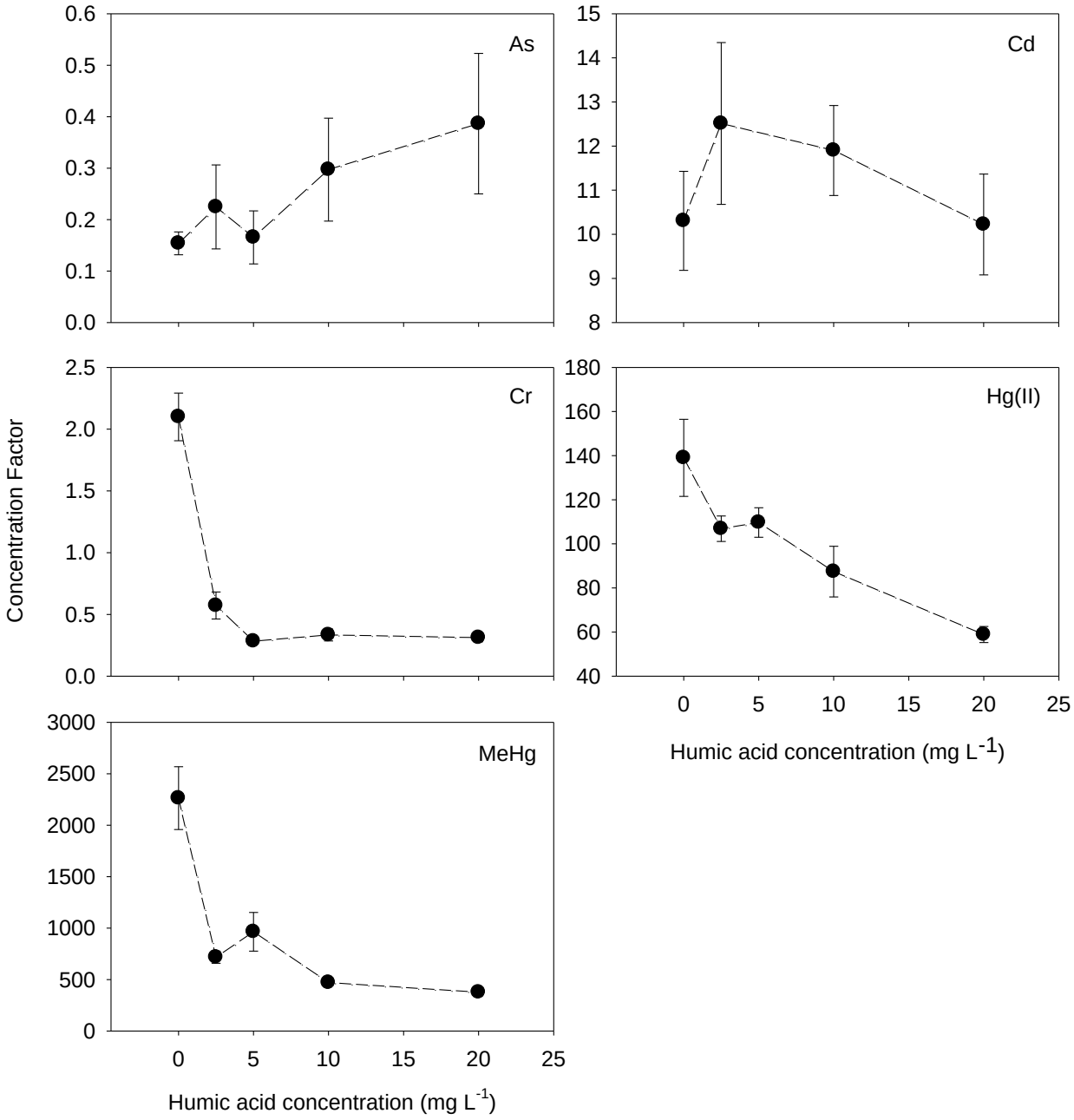


Fig. 2. Metal dry wt concentration factors (CFs) in killifish (*Fundulus heteroclitus*) after 47.5 h (methylmercury [MeHg]) or 72 h (As, Cd, Cr, Hg(II)) aqueous uptake as a function of humic acid concentration. Values represent means ± 1 standard error; *n* = 5 per humic acid concentration.



Chapter IV

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.

Dutton J, Fisher NS (2011) Bioaccumulation of As, Cd, Cr, Hg(II), and MeHg in killifish (*Fundulus heteroclitus*) from amphipod and worm prey. Science of the Total Environment 409: 3438-3447

INTRODUCTION

It is well known that metals can bioaccumulate in aquatic food chains and in some cases biomagnify as well (Wang 2002; Xu and Wang 2002; Wang and Wong 2003; Mathews and Fisher 2008a,b; Mathews et al. 2008). 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 (Harada 1995; Chang et al. 2008), 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 (Xu and Wang 2002; Wang and Wong 2003; Pickhardt et al. 2006). Recent studies have shown that the diet is the dominant exposure route for many metals (Xu and Wang 2002; Pickhardt et al. 2006; Mathews and Fisher 2009). 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 have a direct influence on metal assimilation efficiencies (defined as the percentage of ingested metal crossing the gut lining) in fish (Xu and Wang 2002; Wang and Wong 2003; 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 its wide distribution; is 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 1994a). 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).

MATERIALS AND METHODS

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.

Prey organisms and fish

California blackworms (*L. 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 (*L. 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 (*F. 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 h prior to feeding on radiolabeled prey to allow for gut clearance.

Metal uptake from amphipods

The euryhaline green algae *D. 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 (4×10^5 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 min 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 water 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 h, 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 h 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.

Metal uptake from worms

To radiolabel blackworms, radioisotopes were added to 300 ml of Stony Brook tap water and allowed to equilibrate for 1 h, after which 60 ml was poured into two containers, and 30 worms added to each. Worms were radiolabeled for 48 h, with a water change after the first 24 h. At the end of labeling, the worms were removed, rinsed and held in non-radiolabeled tap water for 5 min, 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.

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 (Imura et al. 1971; Rouleau and Block 1997; Bancon-Montigny et al. 2004). ^{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 min 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.

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:

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

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} (Pauly 1989; Garnier-Laplace et al. 2000). 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.

RESULTS

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 h 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.

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 h 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).

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 h 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.

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.

DISCUSSION

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 (Wang and Wong 2003; Pickhardt et al. 2006), 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 cannot digest the chitinous amphipod exoskeleton, and therefore assimilate a limited amount of metal associated with the exoskeleton (Reinfelder and Fisher 1994a). 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 min 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%) (Wang and Wong 2003; Pickhardt et al. 2006). Copepods and *D.pulex* may accumulate more Hg(II) than the *L. 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 1994a). 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%) (Ni et al. 2000; Baines et al. 2002; Mathews and Fisher 2008b). 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 are 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 (1994a) 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 (Lindsay and Sanders 1990; Chen and Folt 2000; Culioli et al. 2009).

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 2007b). 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}; Wang and Wong 2003; Pickhardt et al. 2006; Mathews and Fisher 2008a). 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; Xu and Wang 2002; Mathews and Fisher 2008b), but higher than noted in another killifish study after feeding on *D. pulex* (0.03 d^{-1}; Mathews and Fisher 2008a).

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 that 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.

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; Wang and Wong 2003; Mathews and Fisher, 2008a), 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; Xu and Wang 2002; Mathews and Fisher 2008a,b; Mathews et al., 2008); however, Cd has been shown to biomagnify (TTF > 1) in freshwater food webs (Croteau et al. 2005).

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% (Wang and Wong 2003; Mathews and Fisher 2008a). 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.

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

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.

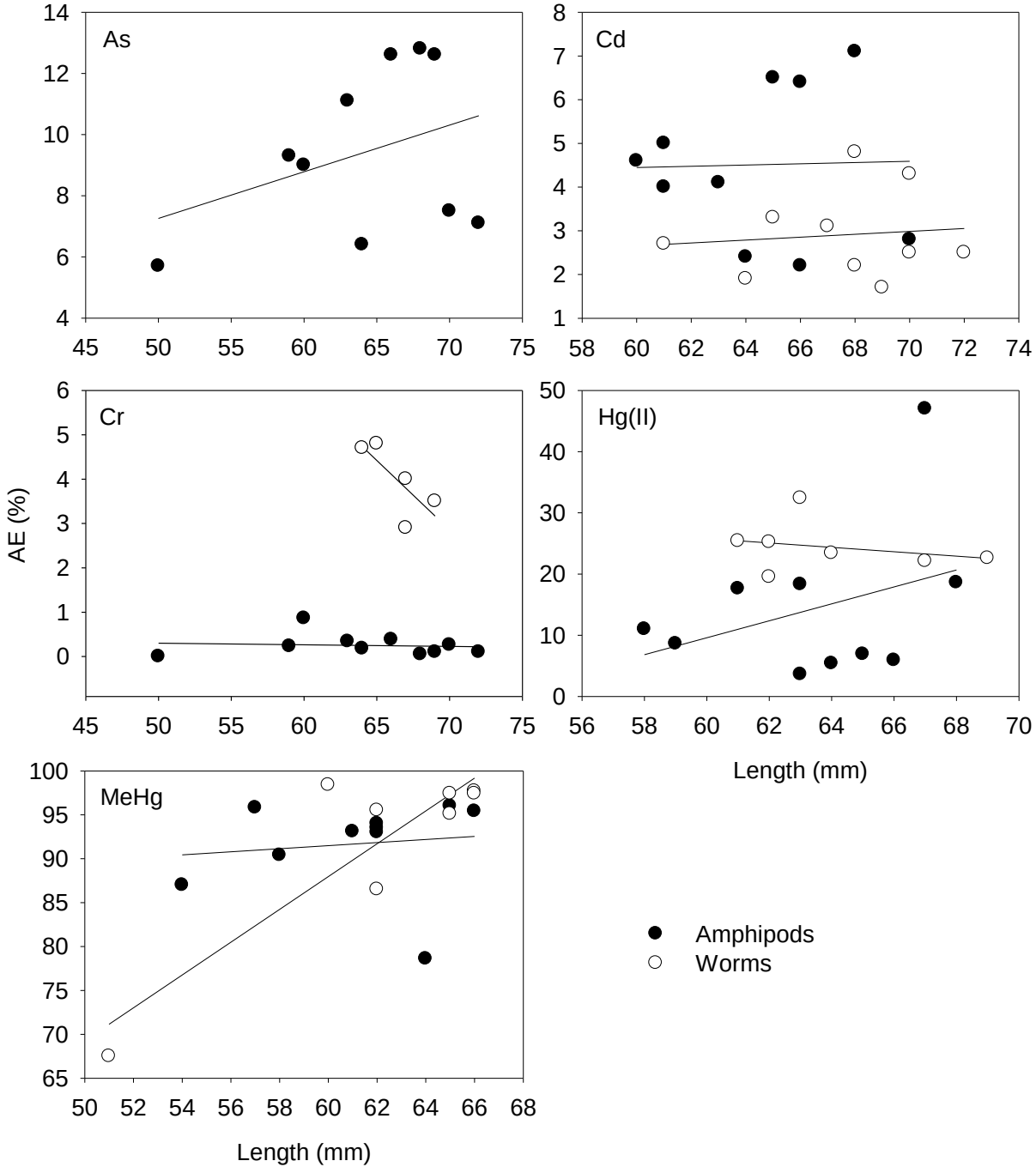


Fig. 2. Metal retention in killifish (*Fundulus heteroclitus*) over 9 days after feeding on radiolabeled amphipod (*Leptocheirus plumulosus*) or worm (*Lumbriculus variegatus*) prey. Values represent means ± 1 SE; n = 5–25 for amphipods and 5–10 for worms.

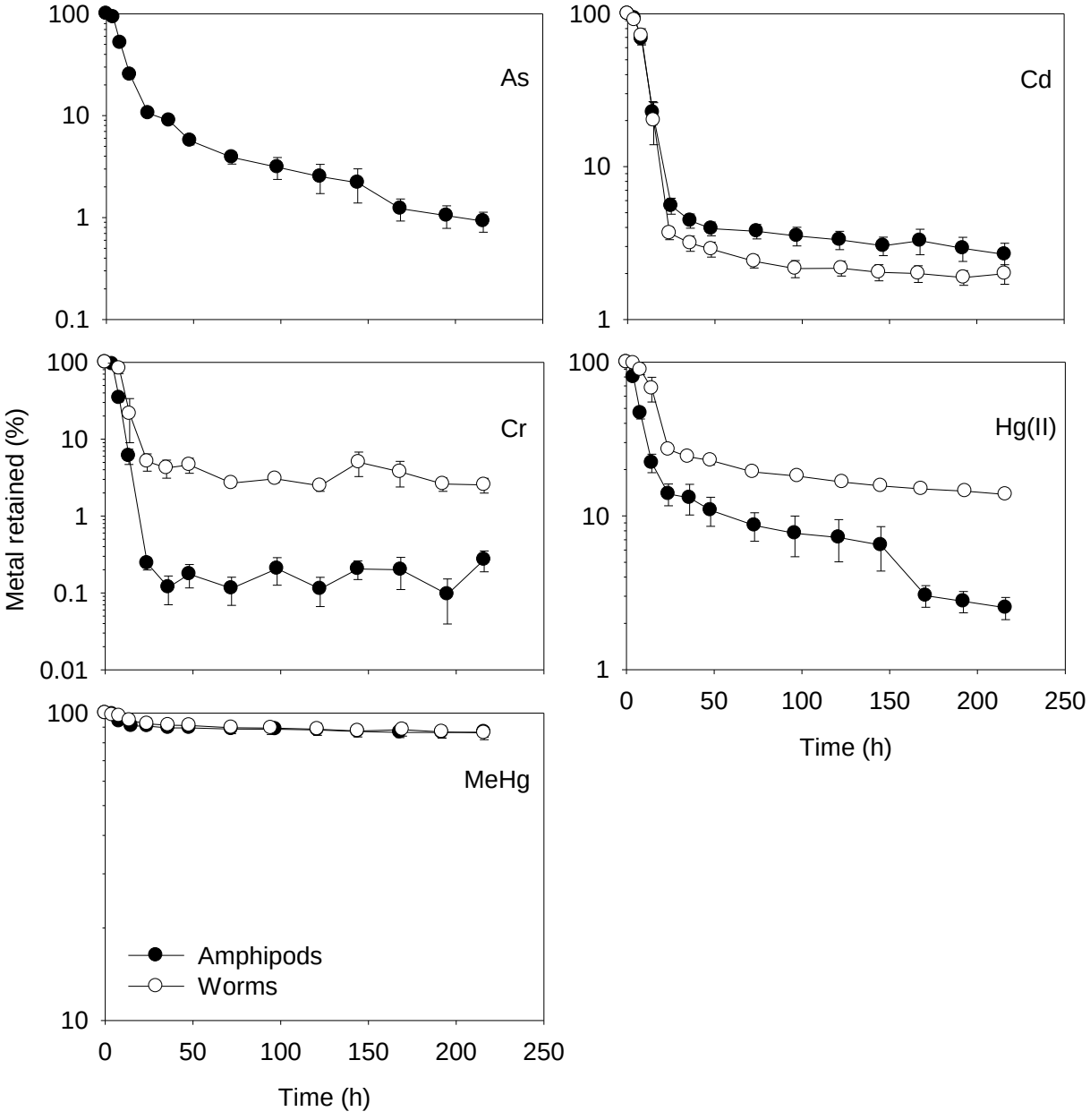


Fig. 3. Tissue distribution of Cd, Hg(II), and MeHg at the end of the 9 day depuration period after feeding on radiolabeled amphipods and worms. Bars represent the percentage of total body burden associated with each tissue compartment. Values represent means ± 1 SE; n = 5 for amphipods and 5–10 for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

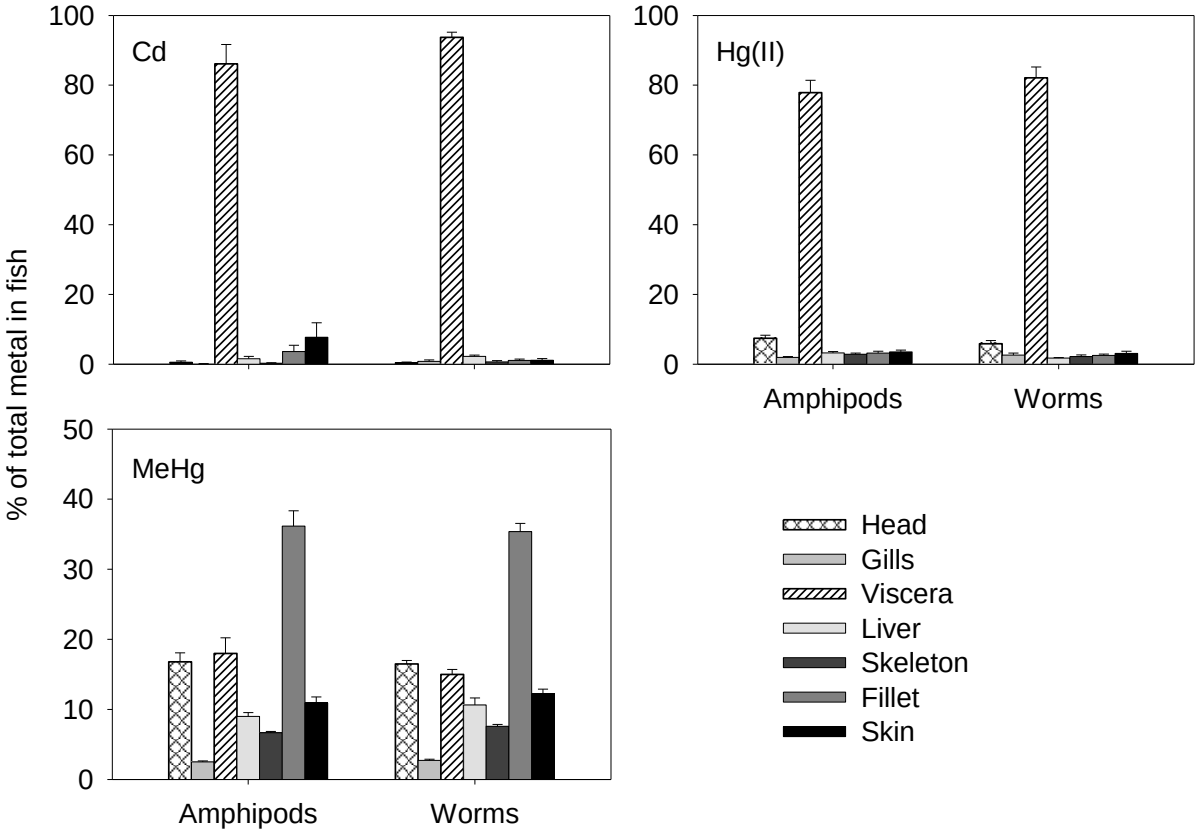
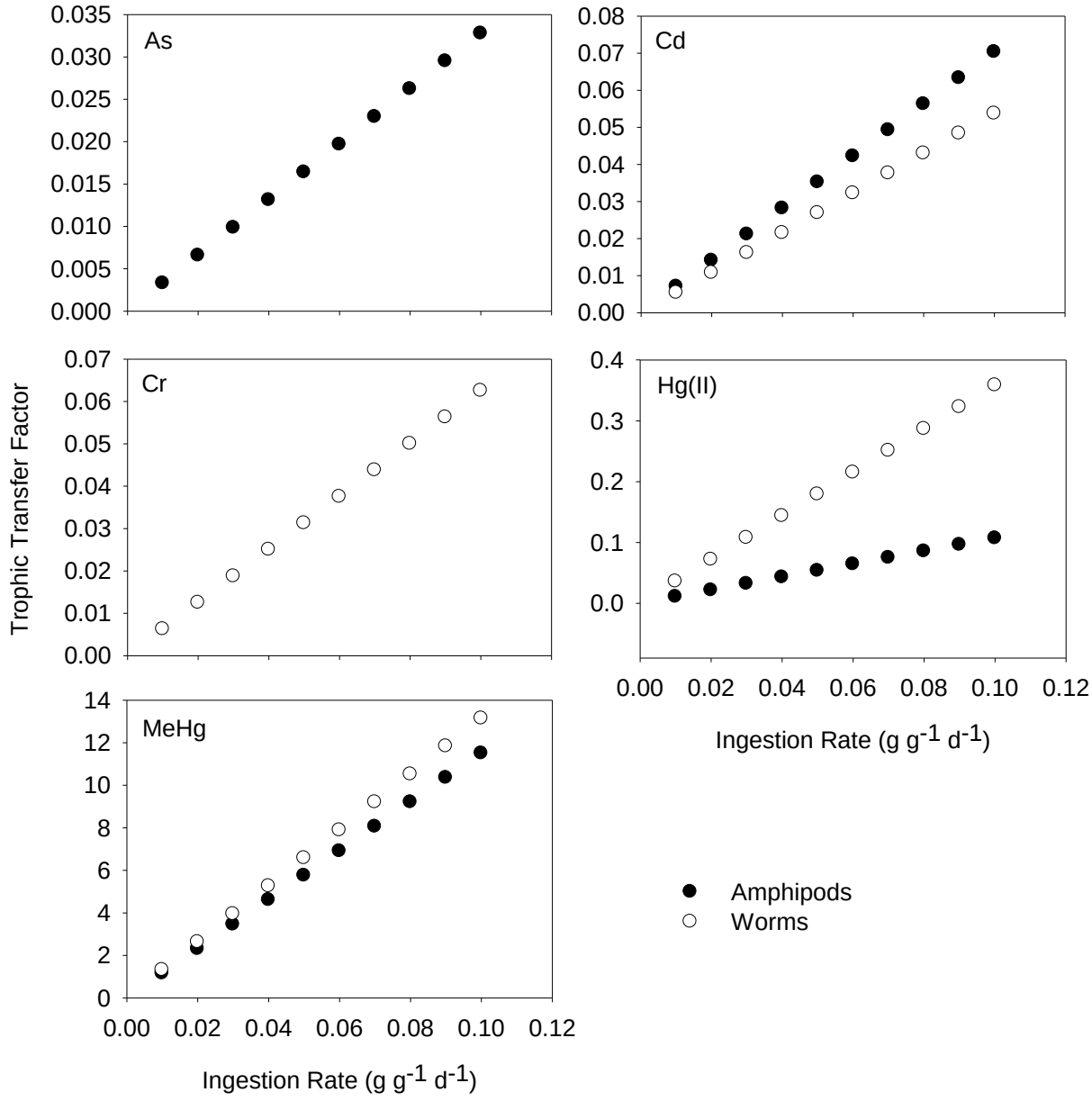


Fig. 4. Model-predicted As, Cd, Cr, Hg(II), and MeHg trophic transfer factors (TTFs) as a function of ingestion rate (IR) for killifish after feeding on amphipod and worm prey. AE and k_{ef} values used are in Table 1. Cr from amphipods was below detection.



Chapter V

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

ABSTRACT

We used a radiotracer technique to experimentally assess whether As(V), Cd, Cr(III), Hg(II), and methylmercury (MeHg) bound to sediment from 3 contaminated field sites (Baltimore Harbor, Elizabeth River, and Mare Island) and the green alga *Dunaliella tertiolecta* are bioavailable to killifish *Fundulus heteroclitus*. Algae are a component of the killifish diet in salt marshes, and although killifish do not actively consume sediment, some is accidently ingested due to attachment to benthic prey. For both sediment and algae, assimilation efficiencies (AE) of ingested metals were highest for MeHg, followed by Hg(II), As, and Cd, and lowest for Cr. Following sediment intubations, AE values 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}) were similar for As, Cd, and Hg(II) and lowest for MeHg, whereas following algal intubation, the k_{ef} values were highest for As, followed by Cr, Hg(II), and 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 (TTF) 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 that the risk of exposure to killifish from ingesting contaminated sediment is minimal.

Dutton J, Fisher NS (2012) Bioavailability of sediment and algal metals to killifish *Fundulus heteroclitus*. Aquatic Biology 16: 85-96

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 enrichment provides a large source of metals to benthic organisms, potentially impacting 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 and Fisher 2011), and the time of sediment exposure to the metal (Wang et al. 1999; Griscom et al. 2000; Baumann and Fisher 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 and 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 and Reinfelder 1995). Sinking planktonic debris enriched in metals can settle to sediments and serve as a source of metal for benthic animals in addition to the metals that sorb directly to the sediments themselves (Baumann and 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 and 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 3 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 that 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 that a metal will be transferred from food to fish at this trophic step. The 3 sediment locations were chosen because they are all contaminated, have differing organic carbon content, grain size distribution, and geochemical properties (Baumann and 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 3 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 and Richardson 1980). These metals are commonly found at elevated concentrations in estuarine sediments, particularly those near industrial areas (Kennish 1997), and their bioavailability is of interest for management of coastal ecosystems.

MATERIALS AND METHODS

Study locations and sediment collection

Sediment was collected by box coring from 2 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. The grain size distribution (described as the percentage of the coarse fraction (> 63µm)), organic carbon content, and background metal concentrations for each location are shown in Table 1. For grain size analysis, ~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 percentage difference between the before and after dry weights was the percentage of organic carbon. Background metal concentrations were analyzed by inductively coupled plasma-mass spectrometry (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 also analyzed at Dartmouth College, and As, Cd, and Cr were analyzed at 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) before use. The 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 with a mean wet weight of 2.1 ± 0.3 g, were purchased from Aquatic Research Organisms and acclimated to experimental conditions for at least 4 wk prior to the start of the experiments. The fish were fed a diet of TetraCichlid™ cichlid flakes (Tetra Holding) and frozen bloodworms daily prior to the start of the experiments and were fed 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, ~6 to 6.5g of sediment from each of the 3 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 ~56% of the weight of sediment from BH, 63% from ER, and 52% from MI.

Radioisotope additions per field location were 104 kilobecquerel (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 cm^3 syringe with a 16G1½ PrecisionGlide needle attached (Becton Dickinson), and the needle was carefully slid into intramedic non-radiopaque polyethylene tubing (internal diameter 1.57 mm, 5 cm long; Clay Adams). Fish were removed from the water, and the tubing was passed through the mouth and esophagus into the start of the intestine, where ~0.06 to 0.1 g of radiolabeled sediment was added. The 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) due to 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 the samples were dried at 60°C for 48 h to determine dry weights.

Statistical analyses were conducted using IBM SPSS statistics software (v. 20). One-way ANOVA and Tukey post-hoc tests were conducted to identify 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) of BH water, amended with nutrients at f/2 concentrations (Guillard and Ryther 1962) but modified so no Cu, Zn, or 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 and 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 x 10^5 for ^{73}As and ^{51}Cr, 6.4 x 10^5 for ^{109}Cd, 3.9 x 10^5 for ^{203}Hg(II), and 7.6 x 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. The algal suspensions were filtered through 0.2 µm polycarbonate membranes, and rinsed 4 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 cm^3 tuberculin syringe (Becton Dickinson) was used), and the fish were intubated with ~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 the present 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). The ^{73}As (half-life ($t_{½}$) = 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), ^{51}Cr ($t_{½}$ = 27.7 d, as Cr(III), dissolved in 0.5 M HCl) was purchased from PerkinElmer, and ^{203}Hg(II) ($t_{½}$ = 46.6 d, as Hg(II), dissolved in 1 M HCl)

was purchased from Eckert & Ziegler Isotope Products. MeHg ($CH_3{}^{203}Hg(II)$) was synthesized in our laboratory using a method described in Rouleau and 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 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 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 estimates 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:

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

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}.

The 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 AE after sediment intubation was calculated as the percentage remaining after 48 h of depuration, due to nearly 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 as follows:

$$tb_{½} = \ln 2/k_{ef} \qquad (2)$$

RESULTS

Assimilation and retention of metals after sediment intubation

After intubation with radiolabeled sediment AE values were highest for MeHg (10 to 14%), followed by Hg(II) (1.9 to 4.1%), As (0.8 to 1.7%), and Cd (0.04 to 0.3%), and lowest for Cr (0.01 to 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 the values 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 of the 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 to 0.9% As, 0.03 to 2% Cd, 0.05 to 0.06% Cr, 1.1 to 1.9% Hg(II), and 9.1 to

14% MeHg. Loss rate constants (k_{ef}) for Hg(II) (0.074 to 0.113 d^{-1}), As (0.057 to 0.097 d^{-1}), and Cd (0.074 to 0.089 d^{-1}) were similar to one another and significantly higher than for MeHg (0.004 to 0.020 d^{-1}) (Table 3). The k_{ef} values 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 AE values were highest for MeHg (82%), followed by Hg(II) (18%), As (15%), and Cd (10%), and lowest for Cr (0.7%). For all metals, AEs were higher after intubation with algae than with sediment. If the sediment AE values for the 3 field locations are averaged, the 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. The k_{ef} values were highest for As (0.223 d^{-1}), followed by Cr (0.119 d^{-1}), Hg(II) (0.085 d^{-1}), and 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 weight) associated with each tissue compartment at the end of depuration after intubation with radiolabeled sediment and algae. The tissue distribution for Cd at 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 of the body

burden associated with each tissue compartment did not vary among field locations, except for Hg(II) in the head (4-22%) and viscera (68-91%). 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 among metals and between sediment and algae due to exposure to different metal concentrations.

Modeling metal bioaccumulation in killifish

The $tb_{½}$ 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 to 1213 d, As for 50 to 85 d, Cd for 55 to 66 d, and Hg(II) for 43 to 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), and 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 TTF <1, indicating that these metals would not be expected to biomagnify (Table 5).

DISCUSSION

Assimilation of metals after sediment intubation

To our knowledge, the present study is the first to investigate the bioavailability of several metals associated with contaminated sediment for fish. Because no significant differences were noted in the TTF of metals among the 3 sediments (except ER MeHg), it is apparent that sediment geochemistry differences among the 3 sediment sites (Baumann and Fisher 2011) did not influence the AE and k_{ef} of each metal in killifish. The AE values calculated for killifish in the present study are much lower than those calculated for deposit-feeding polychaetes and marine bivalves that ingested radiolabeled sediment. AE values in polychaetes ranged from 43 to 83% for MeHg, 7 to 30% for Hg(II), 1.5 to 59% for Cd, 1.2 to 12% for As and 0.7 to 4.6% for Cr (Wang et al. 1998, 1999; Baumann and Fisher 2011). AE values in bivalves ranged from 5 to 87% (generally >30%) for MeHg, 6 to 35% for Cd, <1 to 20% for Cr, and 1 to 9% for Hg(II) (Gagnon and 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 the present study. For all of the metals investigated in the present study, the sediment AE values are lower than the algal AE values; this observation was also noted in another study using the clam *Macoma balthica* (Griscom et al. 2002a). The difference in AE 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 AE values may be underestimated because the sediment was forced into the intestine during the intubation procedure.

The lower AE values noted for killifish compared to deposit-feeding polychaetes and bivalves could be a result of the difference in gut physiology among 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 and 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 to 3 h 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 acid-volatile sulphide (AVS) 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 and Fisher (2011) calculated that 13 to 42% of As, 33 to 50% of Cd, and 75 to 91% of Cr was bound to the AVS and iron- and manganese-oxide fractions 2 d after the sediment from the 3 field locations used in the present 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 and 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 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 and Mason 2002b).

Assimilation of metals after algal intubation

The wide range of AE values observed after killifish were intubated with radiolabeled algae (0.7 % for Cr to 82% for MeHg) indicates there is large variability in AE among the metals. The general ranking of AE (MeHg > Hg(II) > As > Cd > Cr) is identical to the ranking observed after sediment intubation. To our knowledge, the present study is the first to calculate AE values after fish have 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 the present study were intubated. The Cd, Hg(II) and MeHg AE values calculated from the present 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 and Fisher 1994a, Ni et al. 2000; Xu and Wang 2002; Wang and Wong 2003; Pickhardt et al. 2006; Mathews and Fisher 2008a; Dutton and Fisher 2010, 2011b). Our calculated As AE (15%) is higher than that calculated for killifish fed amphipod prey (9.4%; Dutton and Fisher 2011b), 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 to 19%; Ni et al. 2000, Dutton and Fisher 2011b).

The large range in AE values from algae observed among the metals could be due to varying cellular distributions of the metals in *Dunaliella tertiolecta* cells. Numerous studies have found that the AE of ingested elements in herbivores is related to the cytoplasmic content of these elements in algae, first noted for diatoms by Reinfelder and 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 AE may vary. This relationship between cellular distribution of metals and AE in herbivores may explain our findings, where the MeHg AE is 4.6-fold higher than that for Hg(II). Pickhardt and Fisher (2007) found that 59 to 64% of MeHg is associated with the cytoplasm in freshwater phytoplankton, whereas only 9 to 16% of Hg(II) is associated with the 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*, the AE ranged between 0.2 and 1.3% (Wang and Fisher 1996), comparable to our low Cr AE value of 0.7%. This relationship has also been observed when fish were fed zooplankton; AE values 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 and Fisher 1994a). This was also noted when killifish were fed radiolabeled amphipods and worms, with higher AE values for Cr and Hg(II) after the fish were fed soft-bodied worms (Dutton and Fisher 2011b).

Loss of metals after sediment and algal intubations

The k_{ef} values 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} did not vary between the 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 the k_{ef} of each metal was principally related to the loss of metal from the viscera.

The algal As k_{ef} value calculated in the present 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} values were ~3-fold lower (Dutton and Fisher 2011b). The calculated algal k_{ef} for Cd (0.041 d^{-1}) falls within the range calculated in other studies in which fish were fed worm and zooplankton prey (0.03-0.073 d^{-1}; Xu and Wang 2002; Mathews and Fisher 2008a; Dutton and Fisher 2010, 2011b). The Cr algal k_{ef} value calculated in the present study (0.119 d^{-1}) is 1.9-fold higher than after killifish were fed radiolabeled worms (0.064 d^{-1}; Dutton and Fisher 2011b). The sediment and algal k_{ef} values calculated in the present study for Hg(II) and MeHg (0.074 to 0.113 and 0.004 to 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 to 0.194 d^{-1} for Hg(II) and 0.007 to 0.018 d^{-1} for MeHg; Pickhardt et al. 2006; Mathews and Fisher 2008a, Dutton and Fisher 2010, 2011b).

Tissue distribution of metals

The tissue distribution of the metals investigated in the present 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 to 97% of Cd remains associated with the viscera, which is similar to values calculated in another killifish study (85% associated with the intestine; Dutton and Fisher 2011b) and mangrove snapper *Lutjanus argentimaculatus* (81%; Xu and Wang 2002) but much higher than the percentage calculated for the Atlantic silverside *Menidia menidia* (13-16%; Dutton and Fisher 2010), striped bass *Morone saxatilis* (20%; Baines et al. 2002), and killifish (50%; Mathews and Fisher 2008a). 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, after which it is redistributed around the body via the

blood and accumulates in the fillet which is sulfur-rich. A study by Leaner and Mason (2002a) 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 and Brandt 1995), and therefore potentially human consumers. The tissue distribution of Hg(II) and MeHg presented in our study are comparable to other literature values. For Hg(II), Pickhardt et al. (2006) found that 92 to 96% of Hg(II) was associated with the viscera in mosquitofish *Gambusia affinis* and 67.5 to 73% in redear sunfish *Lepomis microlophus*, 28 to 72% was associated with the viscera in the Atlantic silverside (Dutton and Fisher 2010), and 81 to 84% was associated with the viscera in another killifish study (Dutton and Fisher 2011b). For MeHg, 54% of MeHg was associated with the body in the redear sunfish and 68% in mosquitofish (Pickhardt et al. 2006), 51 to 57% in the Atlantic silverside (Dutton and Fisher 2010), and 51 to 58% in 2 other killifish studies (Mathews and Fisher 2008a, Dutton and Fisher 2011b), which are comparable, but slightly higher than our values of 42 to 49%.

Like MeHg, As was also redistributed around the body, where 48% was associated with the body after algal intubation and 70 to 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 and Fisher 2011b). Another study analyzed field-collected herring, cod, and flounder and found that As accumulates in the fillet (Larsen and Francesconi 2003). Arsenate is known to behave as a phosphate analog in phytoplankton, sharing the same uptake pathway (Sanders and 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 to 100% of As in the muscle tissue was present as arsenobetaine, whereas arsenate accounted for 0%; however 0 to 38% of the As in the intestine was present as arsenate (Kirby and Maher 2002). We did not speciate As in the fish tissue in the present study, so we cannot conclude if this is the case for killifish. The tissue distribution data from the present 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

The TTF values were <1 after sediment intubation for As, Cd, and Hg(II) at all 3 field locations and for MeHg at BH and MI, indicating that these metals are not expected to biomagnify from sediment to killifish. Killifish intubated with sediment from ER had a TTF >1, indicating that MeHg will biomagnify in this field location. TTF values 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 the present study. Therefore, the TTF 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, the present study is the first to calculate TTF values 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 TTF values are similar to those calculated for killifish which consumed radiolabeled amphipods and worms, while the TTF values are higher for As and Cd, and lower for Cr (Dutton and Fisher 2011b). The TTF values following exposure to radiolabeled sediment compared to amphipods and worms were lower for Cd at all 3 locations and MeHg at BH and MI but were similar for As at all 3 locations and MeHg at ER . The Hg(II) TTF values were similar to values calculated after killifish consumed amphipods but higher than that calculated after killifish consumed worms (Dutton and Fisher 2011b).

Sediment and algae as a source of metals to killifish

Metals can accumulate to high concentrations in industrialized coastal regions. The present 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, and 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 and Stiven 1978; Kneib 1986), and can be a significant source of metal, especially MeHg.

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 are means ± 1 SD; n = 3

	Coarse fraction (%)	Organic carbon content (%)	Background conc. (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, and total Hg) for water collected from Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI). For DOC concentration, values are means ± 1 SD; n =3. For background metal concentrations, units are μg l^{-1} for As, Cd, and Cr, and ng l^{-1} for Hg

	Salinity (ppt)	DOC conc. (μM)	Background conc.			
			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 from 3 sites 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 are the percentage of total body burden and radioactivity concentrations (Bq g^{-1} dry weight) associated with each tissue compartment (head, viscera, or body). Tissue partitioning of Cr and BH Cd could not be determined due to nearly complete elimination of the radioisotope. Values are 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 (TTF) 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

Fig. 1. *Fundulus heteroclitus*. Mean (± 1 SE) 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. n = 5 per location

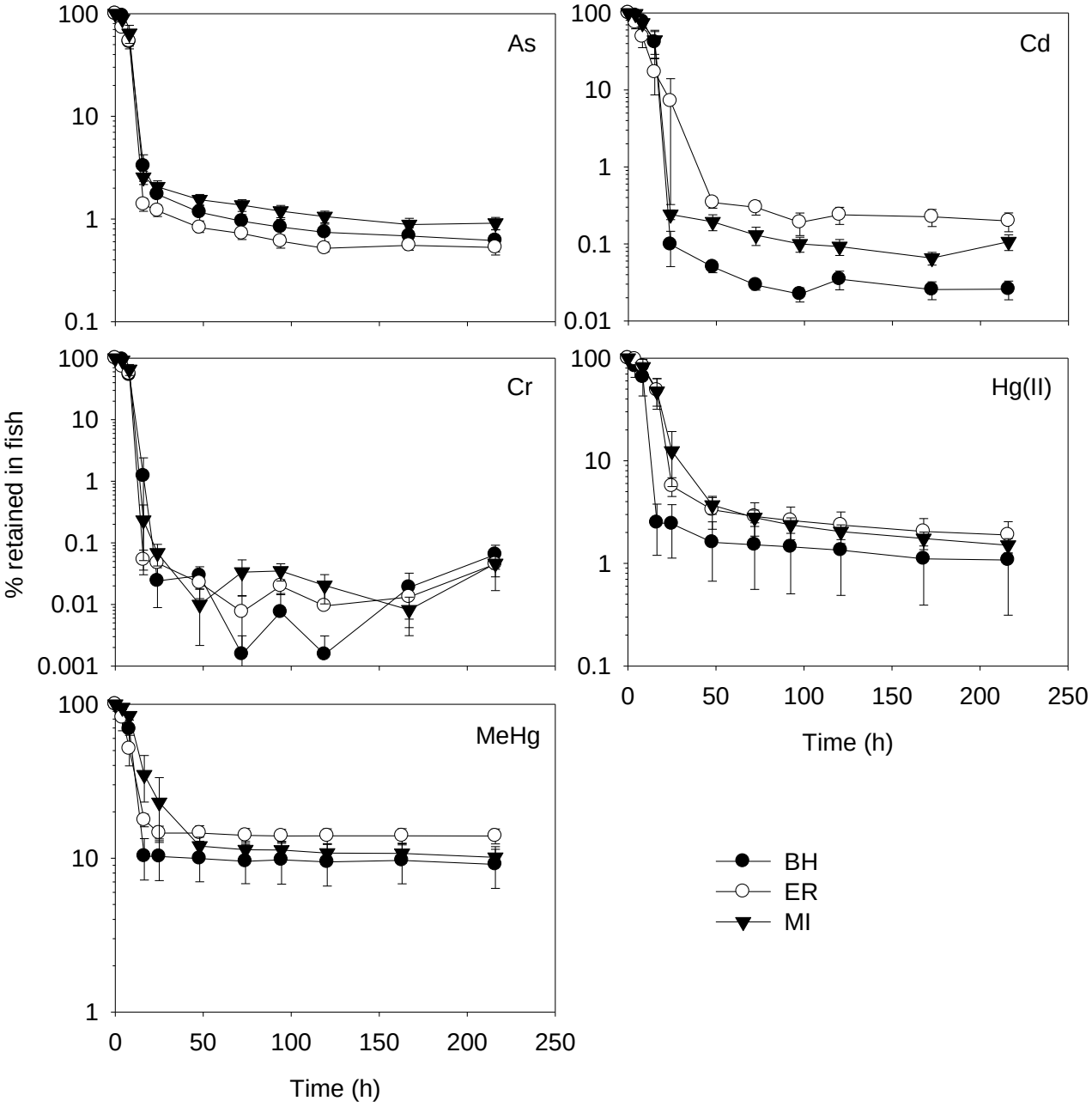
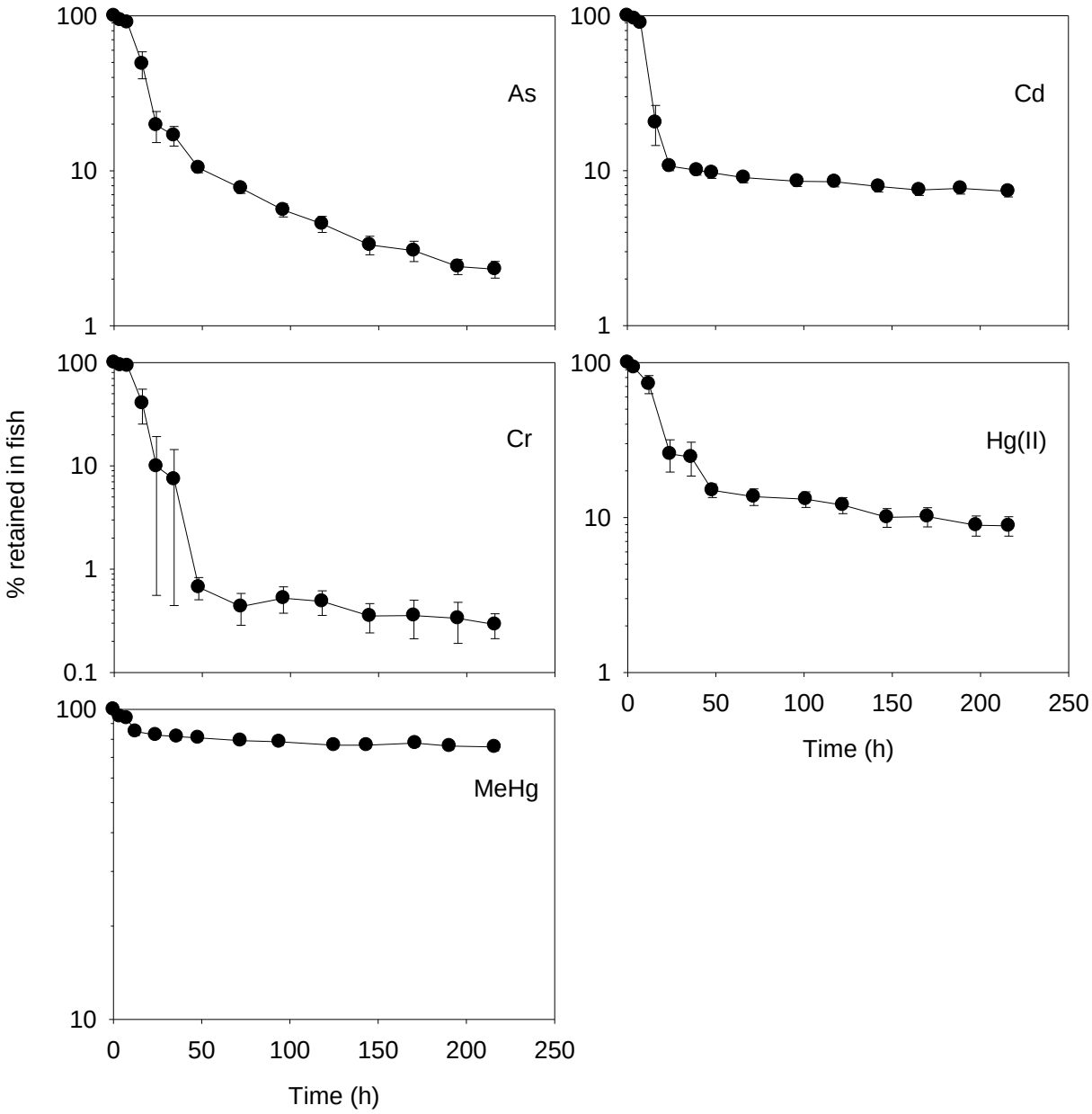


Fig. 2. *Fundulus heteroclitus*. Mean (± 1 SE) loss of As, Cd, Cr, Hg(II), and MeHg from killifish over 9 d following intubation with radiolabeled algae. n = 9-10



Chapter VI

Modeling metal bioaccumulation and tissue distribution in killifish (*Fundulus heteroclitus*) in three contaminated estuaries

ABSTRACT

We experimentally assessed the uptake, loss, and resulting tissue distribution of As(V), Cd, Cr(III), Hg(II), and methylmercury (MeHg) in killifish (*Fundulus heteroclitus*) following aqueous exposure in water collected from three contaminated field sites (Baltimore Harbor, Elizabeth River, and Mare Island) using a radiotracer technique. Uptake rate constants (k_u; L g^{-1} d^{-1}) were highest for MeHg (0.370-0.781) and lowest for As (0.0003-0.0006). Loss rate constants (k_{ew}; d^{-1}) were highest for As (0.046-0.096) and lowest for MeHg (0.006-0.009). The k_u of each metal, except Cr, varied between field sites and was influenced by salinity, whereas the k_{ew} was controlled by the physiological turnover rate of each metal within the fish and for most metals did not vary between field site. Tissue distribution data showed that the highest concentration of As, Cd, Cr, and Hg(II) was in the gills. MeHg was redistributed around the body and increased in concentration in the brain, eyes, and fillet. The kinetic parameters calculated in the present study were entered into a bioaccumulation model together with dietary kinetic parameters determined in a prior study to calculate the predicted body burden of each metal at steady-state and the percentage body burden attributable to dietary exposure on a site-specific basis. Calculated body burdens varied between field sites for As, Cd, and Hg(II). The predicted values for Cd, Hg(II), and MeHg matched independent field data from contaminated estuaries, indicating that the model can account for the major processes governing metal concentration in killifish. Diet accounted for >99% of the body burden of Cd and MeHg, and was the predominant exposure route for As, Cd, and Cr.

INTRODUCTION

Industrialized coastal estuaries have elevated levels of heavy metals in sediments and water due to anthropogenic activities, resulting in increased heavy metal body burdens in aquatic organisms (Kennish 1997). Methylmercury (MeHg) is of particular concern because it is highly toxic, can biomagnify in aquatic food chains (Baeyens et al. 2003), and potentially cause health effects in human consumers of contaminated seafood (Guallar et al. 2002; Chang et al. 2008). MeHg is found at elevated concentrations in a broad spectrum of marine and estuarine fish and shellfish that are regularly consumed as seafood (Karimi et al. 2012).

Fish are exposed to metals through dietary and aqueous exposure pathways, although studies have shown that diet is the dominant uptake route for most metals (Pickhardt et al. 2006; Mathews and Fisher 2009; Dutton and Fisher 2010). Various factors including prey choice, ingestion rate, and growth rate influence the assimilation efficiency of ingested metal (AE; defined as the percentage of ingested metal that crosses the gut lining) and therefore dietary uptake of a metal in fish, but the importance of the aqueous uptake pathway should not be overlooked (Hrenchuk et al. 2012). If the fish is an infrequent feeder, the AE of prey in the fish is low, or the concentration of metal in the prey is low, then the aqueous phase could become an important uptake route. The bioavailability of aqueous metals to fish is predominantly influenced by the salinity and dissolved organic matter concentration (DOM) in the water. Salinity influences metal bioavailability due to chloro-complexation of the metal ion (Dutton and Fisher 2011a), whereas DOM can either decrease or enhance metal uptake into fish, depending on the composition of the DOM and the metal (Dutton and Fisher 2012).

The distribution of metals in fish tissues following dietary or aqueous exposures remains poorly understood. Previous studies have attempted to characterize the tissue distribution of metals following uptake or after a depuration period (Pickhardt et al. 2006; Dutton and Fisher 2010, 2011a, 2012), and these distributions are normally non-tissue specific (e.g., head, viscera, and body). To provide a better understanding of the dynamics of metal redistribution between fish tissues, dissections need to be tissue-specific and carried out throughout the depuration period to enable calculations of metal efflux or influx rates for different tissues.

Killifish (*Fundulus heteroclitus*), a ubiquitous small fish found in estuaries, bays, and salt marshes along the eastern U.S. seaboard, from the Gulf of St. Lawrence to northeastern Florida

(Abraham 1985) can be used as a model organism to investigate metal accumulation and tissue distribution. Killifish are a useful regional bioindicator of heavy metal contamination because they are euryhaline, have a limited swimming range, and varying susceptibilities to aquatic contaminants (Abraham 1985).

To further understand the influence of salinity and dissolved organic carbon (DOC) on metal uptake and loss from the aqueous phase in natural waters and model metal bioaccumulation, we exposed killifish to three metals (Cd, Cr(III), and Hg (as Hg(II) and MeHg)) and a metalloid (As(V)) in water collected from three contaminated field sites with varying salinity and DOC concentrations: two in the Chesapeake Bay (Baltimore Harbor and Elizabeth River) and one in San Francisco Bay (Mare Island). The uptake and loss of each metal was monitored using gamma-emitting radioisotopes, and uptake rate constants (k_us) and loss rate constants (k_{ew}s) were calculated for each metal on a site-specific basis. The tissue distribution of each metal was determined at the end of depuration for all three field sites; however, to investigate how each metal is redistributed around the body over a 9 d period following aqueous exposure, fish in Baltimore Harbor water were dissected regularly throughout depuration to calculate the efflux or influx rate constant from each tissue compartment and the corresponding biological half-life of each metal. Calculated kinetic parameters describing metal bioaccumulation from water (k_u, k_{ew}; this study) were entered into a well-developed bioaccumulation model (Wang et al. 1996) with the AE and loss rate constant after dietary exposure (k_{ef}) from Dutton and Fisher (2011b) to calculate the steady-state metal concentration in killifish and the primary uptake route for each metal at each field site.

All metals were chosen because they are on the list of EPA priority pollutants and are found at elevated concentrations in coastal waters, particularly those near large population centers and industrial activity. All of these metals are of environmental interest due to their potential impact on the health of aquatic ecosystems and the associated health risk to humans from consuming contaminated seafood. Cd and Hg are known to chloro-complex in seawater, whereas As and Cr do not (Bruland 1983).

MATERIALS AND METHODS

Water and experimental conditions

Experiments were conducted using 0.2 μm sterile-filtered (Millipak 200, Millipore, Bedford, MA, USA) water collected from three contaminated field sites: Baltimore Harbor (BH; Baltimore, MD; 39°12'25"N, 76°31'41"W), Elizabeth River (ER; Norfolk, VA; 36°12'32"N, 76°20'09"W), and Mare Island naval complex (MI; Vallejo, CA; 38°04'23"N, 122°14'91"W). Water was collected from 2 m depth using a trace metal clean pump. Water parameters (salinity, chloride ion concentration, DOC concentration, and pH) are shown in Table 1. 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), and the DOC concentration was measured using a Shimadzu TOC-5000 total organic carbon analyzer (Columbia, MD, USA). Filtered water from each field site was analyzed for background metal levels by the Trace Element Analysis Core Laboratory at Dartmouth College (total Hg) and Rutgers Inorganic Analytical Core Laboratory (Rutgers University, New Brunswick, NJ, USA; As, Cd, Cr) using ICP-MS and the results are shown in Table 1. All experiments were held at 18 ± 0.5°C on a 14:10 h light:dark cycle.

Fish

Field collected killifish, *Fundulus heteroclitus*, (Taylor River (Hampton, NH, USA); salinity = 25-30 ppt; mean wet weight 1.95 g ± 0.35 SD) purchased from Aquatic Research Organisms (Hampton, NH, USA) were used in this study. Fish were acclimated to experimental conditions for at least 2 weeks prior to the start of experiments and fed a daily diet of bloodworms or brine shrimp and TetraCichlid™ cichlid flakes (TetraHolding Inc., Blacksburg, VA, USA). Prior to the start of experiments fish were starved for 36 h to purge their gut of any remaining food and the fish were not fed during the metal uptake portion of the experiment; therefore metal uptake was only from the aqueous phase.

Metal uptake and tissue distribution from the aqueous phase

Radiolabeled water (250 ml) from each field site was poured into individual containers and left to equilibrate for several hours. Per 250 ml, each fish was exposed to 23-38 kBq ^{73}As, 10-14 kBq ^{109}Cd, 33-38 kBq ^{51}Cr, 1.9-9.6 kBq ^{203}Hg(II), and 1.1-1.3 kBq MeHg. This equates to the addition of the following metal concentrations: 0.27-1.06 nM ^{73}As (BH = 0.27 nM, ER = 1.06 nM, MI = 0.34 nM), 0.45 nM ^{109}Cd, 0.27-0.85 nM ^{51}Cr (BH = 0.27 nM, ER = 0.43 nM, MI = 0.85 nM), 0.01-0.03 nM ^{203}Hg(II), and 0.13 nM MeHg. ^{109}Cd, ^{203}Hg(II), and MeHg were single-labeled, whereas ^{73}As and ^{51}Cr were added together because the gamma emissions of these isotopes did not interfere with each other.

After radioisotope equilibration, one fish was added per container and uptake was monitored at regular intervals for 36-37 h (n = 8 for ER and MI, n = 25 for BH). This time period was chosen to allow sufficient time to radiolabel the fish while minimizing the risk of excessive metal loss from the fish throughout the uptake period. At each sample time throughout the uptake period fish were removed from their container, rinsed twice with unlabeled site water to remove excess radioisotope adhering to the body surface, and radioassayed. A 1 ml water sample was also collected at each sample time and radioassayed to allow for calculations of concentration factors and uptake rate constants in the fish for each metal and field location. No fish showed adverse health effects (death, abnormal swimming behavior, excess mucus production) from the metal exposure.

At the end of metal uptake, fish were radioassayed and returned to individual containers with 600 ml unlabeled water from the same field location, fed unlabeled bloodworms, and allowed to depurate for 9 days. To monitor metal loss, fish were regularly radioassayed for the first 2 days of depuration and then once a day for the remaining 7 days. Water was changed after 1 day and then every other day. At the end of the 9 day depuration period fish in ER and MI water were euthanized using MS-222, dissected into head, gill, viscera, and body (skeleton, fillet, and skin), radioassayed, and dried at 60°C for 48 h to calculate dry tissue weights. To investigate how the body distribution of each metal changed over 9 days following exposure to aqueous metals, 5 fish were euthanized at the end of uptake, and after 1, 3, 6, and 9 days of depuration, and dissected into head, gills, brain, eyes, viscera (excl. liver), liver, skeleton (incl. fins), fillet, and skin, radioassayed, and dried to obtain dry weights. Efflux or influx rates of metals in individual fish tissues were calculated by converting the mean radioactivity concentration in each

fish tissue between day 1 to 9 of depuration into percentage retained and conducting a linear regression analysis; the resulting slope was the efflux or influx rate.

One-way ANOVA and Tukey post-hocs tests were used to determine significant differences ($p<0.05$ or $p<0.01$) between kinetic parameters (k_u, k_{ew}) and field site, and water chemistry (salinity, DOC concentration) and concentration factor in fish. All statistical analyses were conducted using IBM SPSS version 20 statistics software (Armonk, NY, USA).

Radioisotopes and radioanalyses

We used microliter quantities of high specific activity radioisotopes (1221-7844 kBq μg^{-1} ^{73}As, 814-1110 kBq μg^{-1} ^{109}Cd, 3071-6808 kBq μg^{-1} ^{51}Cr, 1369-15,318 kBq μg^{-1} $^{203}Hg(II)$, and 163-196 kBq μg^{-1} MeHg) in the present study. ^{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 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 ($t_{½}$ = 46.6 d, as Hg(II), dissolved in 1 N HCl) was obtained from Georgia State University (Atlanta, GA, USA). MeHg ($CH_3{}^{203}Hg(II)$) was synthesized in our lab using a procedure described elsewhere (Imura et al. 1971; Rouleau and Block 1997; Bancon-Montigny et al. 2004) and held in deionized water. After adding the radioisotopes to experimental water, an equimolar concentration of sodium hydroxide was added to neutralize the acid, after which the pH was checked and remained unchanged.

Killifish were radioassayed non-invasively using a Canberra (Meriden, CT, USA) deep-well NaI(Tl) γ-detector for no longer than 5 minutes to reduce stress on the fish; this allows the same fish to be monitored throughout metal uptake and depuration, therefore reducing biological variability. The propagated counting errors were ≤ 5%, except for As and Cr where propagated counting errors could reach 25% due to low uptake of the radioisotopes. Water and dissected fish tissue were radioassayed for 5 minutes using an intercalibrated LKB Pharmacia-Wallac 1282-CompuGamma CS gamma counter (Turku, Finland). All sample counts were corrected for background radioactivity and radioactive decay. The γ-emission of ^{109}Cd, ^{73}As, ^{203}Hg, and ^{51}Cr was detected at 22, 53, 279, and 320 keV respectively.

Modeling metal bioaccumulation in killifish

Metal bioaccumulation in killifish can be evaluated using a well-developed biokinetic model (Wang et al. 1996; Reinfelder et al. 1998) which takes into account metal uptake and loss from aqueous and dietary sources, and has been successfully tested using fish (Pickhardt et al. 2006; Mathews and Fisher 2009; Dutton and Fisher 2010). Under steady-state conditions:

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

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

Concentration factors (CFs) were calculated by dividing the dpm g^{-1} dry wt in fish by the dpm in 1 ml of water. Dry weight CFs can be converted to wet weight CFs by dividing by 4 (dry weight is approximately 25% of the wet weight). k_u values were calculated as the slopes of regressions for each metal and field site relating dpm g^{-1} dry wt in fish divided by dpm in 1 L of water against time. For the model, k_u values were calculated between the 1 to 13 h uptake time points for As, 1 to 25 h for Cr, 10.5 to 37 h for Hg(II), and 1 to 37 h for Cd and MeHg. k_{ew} values were calculated by fitting an exponential regression between the 2 d and 9 d depuration time points; the k_{ew} was the slope of the curve. For BH, k_{ew}s were only calculated for fish which were sacrificed at the end of 9 d.

In our model calculations, metal AE and k_{ef} values for killifish after feeding on amphipods in Baltimore Harbor water were taken from Dutton and Fisher (2011b) and are as follows: AE (%), As = 9.4, Cd = 4.5, Cr = 0.2, Hg(II) = 14, MeHg = 92; k_{ef} (d^{-1}), As = 0.287, Cd = 0.064, Hg(II) = 0.131, MeHg = 0.008. The Cr k_{ef} after feeding on amphipods was not determined due to near complete elimination of the radioisotope, so we applied a k_{ef} value for Cr in killifish after feeding on worms (0.064 d^{-1}; Dutton and Fisher 2011b). A laboratory study has shown that the IR can be up to 14% dry weight per day; so we applied an average IR value of 7%

(Prinslow et al. 1974). The fish used in this study are 45-65 mm total body length; a field study in Nova Scotia investigating age-length correlation calculated these fish are either in their first or second growing season (Fritz and Garside 1975). A study investigating growth rates calculated a g of 0.05 d^{-1} for Year 1 and 0.014 d^{-1} for Year 2 fish in mid-summer (Kneib and Stiven 1978); we averaged these values and applied a g of 0.032 d^{-1} to the model.

C_w values are shown in Table 1. The total Hg background value (ng L^{-1}) is 3.4 for BH, 2.9 for ER, and 2.7 for MI; speciation of total Hg into Hg(II) and MeHg was determined assuming that 3% of Hg in marine waters is present as MeHg (Hammerschmidt and Fitzgerald 2006). C_f values in crustacean zooplankton prey (dry wt) were obtained from the literature for Hg (0.22 µg g^{-1}; IAEA 2004); according to Francesconi and Lenanton (1992), 75% of Hg in crustacean zooplankton is present as MeHg, so a C_f of 0.055 µg g^{-1} for Hg(II) and 0.165 µg g^{-1} for MeHg was used. C_f values for As, Cd, and Cr were calculated by multiplying the C_w by the CF of each metal in zooplankton; for Cd the CF was 310,000 (Fisher et al. 2000), for Cr the CF was 5000 (IAEA 2004; assuming zooplankton are 80% water), and for As the CF was calculated by dividing the average C_{ss} of As in *Leptocheirus plumulosus* after suspension feeding (0.43 µg g^{-1}; Williams et al. 2010) by the C_w for ER water, resulting in a CF of 310.

Eq. (1) can be rearranged to calculate the percentage of C_{ss} which is attributed to dietary exposure (R), to understand the relative importance of dietary and dissolved exposure routes.

$$R = [(AE \cdot IR \cdot C_f)/(g + k_{ef})]/C_{ss} \cdot 100 \qquad (2)$$

To understand how long it takes for each metal to be excreted from the whole fish or individual tissue compartments after exposure to aqueous metal, the biological half-life ($tb_{½}$) can be calculated:

$$tb_{½} = \ln 2/k_e \qquad (3)$$

Assuming it takes 7 half-lives for >99% of the metal to be released from the whole fish or individual tissue compartments, the biological residence time for each metal can be calculated.

RESULTS

Metal uptake from the aqueous phase

Fig. 1 shows the accumulation of As, Cd, Cr, Hg(II), and MeHg in killifish throughout the 37 h uptake period in experimental waters. To facilitate comparisons among metals, uptake over time is expressed as dry weight concentration factors (CF). For all metals and field sites, metal uptake rates were greatest during the first hour of exposure and then slowed throughout the remaining uptake period, except for Hg(II) in MI water which experienced the greatest uptake between 13-37 h of exposure. The uptake of Cd, Hg(II), and MeHg increased throughout the exposure period at all three field sites, whereas As and Cr uptake began to approach steady-state (except As in MI water and Cr in BH water). Cd, Cr, and MeHg accumulation was highest in BH water, whereas As accumulation was highest in ER water, and Hg(II) accumulation in MI water, at the end of 37 h. When comparing the two Hg species, fish in all three field sites accumulated more MeHg than Hg(II) at the end of 37 h (BH = 21.5-times, ER = 15.4-times, and MI = 2.5-times). Less than 1% of dissolved As, Cd, and Cr were removed from the water by the fish at the end of the 37 h exposure, whereas <28% of Hg(II) and 36-83% of MeHg were removed. The fish showed no adverse health effects (increased mucus production, abnormal swimming behavior, or not feeding during depuration) from the experimental procedure.

Throughout the uptake period CFs were highest for MeHg, followed by Hg(II), Cd, Cr, and lowest for As (Fig. 1). Hg(II) and MeHg CFs were >1 throughout the uptake period, indicating the fish are more enriched in metal than the surrounding water, whereas As CFs were <1 for all field sites. Cd ($p<0.01$; BH vs ER, BH vs MI) and MeHg ($p<0.05$; BH vs MI) CFs had an inverse relationship with salinity, whereas As uptake increased with salinity ($p<0.01$; BH vs ER, BH vs MI) and Cr showed no consistent relationship ($p>0.05$). Hg(II) CFs were similar at 7.6 ppt (BH) and 19.5 ppt (ER) (CFs = 40-44), and increased to 247 at 22 ppt (MI); this relationship was statistically significant ($p<0.01$; BH vs MI, ER vs MI) (Fig. 2). CFs between DOC concentration and field site did not vary significantly for Cr ($p>0.05$), but did vary for As, Cd ($p<0.01$; BH vs ER, BH vs MI for both metals), Hg(II) ($p<0.01$; BH vs MI, ER vs MI), and MeHg ($p<0.05$; BH vs MI) (Fig. 2).

Table 2 shows the uptake rate constants (k_u; L g^{-1} d^{-1}) for each metal and field site. k_us were highest for MeHg (0.370-0.781), followed by Hg(II) (0.026-0.030), and lowest, but comparable

for Cd, Cr, and As (0.0004-0.0008, 0.0005-0.0006, and 0.0001-0.0004, respectively). The k_us did not vary significantly between the three field sites for Cr ($p>0.05$), but did vary for As ($p<0.01$; BH vs ER), Cd ($p<0.01$; BH vs MI), Hg(II) ($p<0.01$; BH vs MI, ER vs MI), and MeHg ($p<0.05$; BH vs MI).

Metal retention and elimination after aqueous exposure

Fig. 3 shows the loss of metals from killifish over 9 d following aqueous exposure in water from the three field sites. All metals at all field sites showed the most rapid elimination within the first several h of depuration and a slower physiological turnover throughout the remaining 8 d. Efflux rate constants (k_{ew}; d^{-1}) were highest for As and Cr (0.046-0.096 and 0.045-0.075, respectively) and lowest for MeHg (0.006-0.009) (Table 2). The k_{ew}s did not vary significantly between field sites for Cd and Cr ($p>0.05$), but did vary for As ($p<0.01$; BH vs ER), Hg(II) ($p<0.01$, BH vs ER, ER vs MI; $p<0.05$, BH vs MI), and MeHg ($p<0.05$; BH vs ER).

The biological half-lives ($tb_{½}$; d) of metals in whole fish following aqueous exposure were highest for MeHg (77-115.5), followed by Cd (27.7-46.2), Hg(II) (15.1-30.1), and lowest for Cr (9.2-15.4) and As (7.2-15.1) (Table 2). Assuming it takes seven half-lives for all metal to be excreted from killifish the residence times are as follows: 539-809 days for MeHg, 194-323 days for Cd, 105-211 days for Hg(II), 65-108 days for Cr, and 51-105 days for As.

Tissue distribution and radioactivity concentrations after aqueous exposure

Table 3 shows the tissue distribution, as percentage of total body burden, at the end of 9 d depuration for each field site. Cd was predominantly associated with the gills (45-60%), As, Hg(II) and MeHg with the body (44-66%, 40-47% and 57-58%, respectively), and Cr was split between the head (22-54%) and body (30-45%). Hg(II) and MeHg showed similar tissue distribution between the three field sites, whereas As, Cd, and Cr did not. In higher salinity waters (ER, MI) the percentage of Cd associated with the gills was lower and the percentage of Cd associated with the viscera was higher than in low salinity water (BH). The percentage of Cr associated with the head decreased, and increased in the viscera and body, as the DOC concentration in the water increased. The percentage of As associated with the gills was greatest in the highest DOC water (ER) and the percentage of As associated with the body decreased as

DOC concentration increased. Table 4 shows the radioactivity concentration of each metal in fish tissue at the end of 9 d depuration on a weight normalized basis for each field site. Radioactivity concentrations could not be compared between field sites for each metal because fish were exposed to varying radioactivity concentrations throughout uptake. The concentration of As was highest in the gills in BH and ER water and nearly evenly distributed between the four tissue compartments in MI water. The concentration of Cd was highest in the gills and lowest in the body at all field sites. Cr concentrations were highest in the gills in BH and ER water, and highest in the head in MI water. The concentration of Hg(II) was highest in the gills and lowest in the body at all field sites, whereas the concentration of MeHg was highest in the viscera and lowest in the head at all field sites.

Table 5 shows the movement of metals between tissue compartments throughout the 9 d depuration after exposure to aqueous metals in BH water. Throughout depuration, Cr and Hg(II) were predominantly associated with the head, Cd with the gills, and As with the fillet. The tissue distribution of MeHg changed throughout depuration; the percentage of MeHg associated with the head and gills decreased (25 to 19% and 23 to 4%, respectively), and increased in the fillet (10 to 33%). Table 6 shows the radioactivity concentration in individual tissue compartments throughout depuration after exposure to aqueous metals in BH water. Throughout depuration Cd and Hg(II) had the highest concentration in the gills, whereas Cr had the highest concentration in the gills and brain. The concentration of MeHg was highest in the gills, viscera and liver, whereas As had a varied tissue distribution throughout depuration which showed no consistent trends. Table 7 shows the rate of metal uptake or loss from each tissue compartment and corresponding biological half-life ($tb_{½}$). As had a high efflux, and therefore a short biological half-life in the liver, whereas Cd, Cr, and Hg(II) were transferred into the liver over time. The gills and liver had the highest loss rate of MeHg, whereas the MeHg was taken up over time into the brain, eyes, and fillet.

Modeling metal bioaccumulation in killifish

Model-predicted metal body burdens at steady-state (C_{ss}; ng g^{-1}; Table 8) were highest for Cd (244-2088), followed by MeHg (266-268), As (8.5-23.5), Hg(II) (4.4-11.5), and lowest for Cr (2.5-3.4). Cr and MeHg C_{ss} values did not vary between field sites, whereas As, Cd, and Hg(II) varied. The diet was the dominant source of Cd and MeHg to killifish at all field sites,

accounting for > 99% of accumulated metal, whereas approximately one-half of Cr accumulation was attributed to aqueous exposure. The importance of As and Hg(II) aqueous and dietary sources varied between field site; the diet accounted for 73% of the As C_{ss} for BH, but only 45% for ER, whereas the diet accounted for 75% of the Hg(II) C_{ss} for BH, but only 29% for MI (Table 8).

DISCUSSION

Modeling metal bioaccumulation in killifish

When the aqueous kinetic parameters calculated in the present study were entered into the biokinetic model with the dietary kinetic parameters calculated in a previous study (Dutton and Fisher 2011b) the predicted body burden (C_{ss}) did not vary between the three field sites for MeHg and Cr, but did vary for As, Cd, and Hg(II). The MeHg C_{ss} values did not vary between field sites because the background concentration of MeHg in the three site waters was about equal. In comparison, Cd had the greatest difference in C_{ss} values between field sites with fish in ER and MI water accumulating 8.5 and 7.8 times more Cd, respectively, than fish in BH water, even though the k_u of Cd was highest in BH water. The lower C_{ss} value in BH water can be attributed to the 10 times lower concentration (C_w) of Cd in BH water. The lower As C_{ss} calculated for killifish in BH water is due to fish in BH water having a 1.5 to 2 times higher k_{ew} and therefore a faster physiological turnover of aqueous metal, and a lower C_w compared to the other two field sites. Killifish in MI water had a 2.5-times higher Hg(II) body burden due to the k_u value being 7.7-times higher in MI fish compared to BH and ER fish. The Hg(II) and MeHg C_{ss} values calculated in the present study are similar to those calculated for freshwater fish (Pickhardt et al. 2006), but lower than those calculated for Atlantic silversides (Dutton and Fisher 2010). Our C_{ss} values calculated for killifish in ER and MI water are similar to values calculated for Atlantic silversides, whereas our BH C_{ss} is much lower due to the low concentration of Cd in BH water (Dutton and Fisher 2010). To our knowledge this is the first study to predict the concentration of As and Cr in fish at steady-state.

No literature values could be found to compare our predicted values to field collected fish at any of the three field sites. The predicted Cd, Hg(II), and MeHg values calculated for killifish in

the present study matched independent field data from other contaminated estuaries within the United States, indicating that the model accounts for the major processes governing metal concentration in killifish and that metal parameters (k_u, AE, k_{ew}, k_{ef}) measured in laboratory experiments are applicable to natural waters. Our model-predicted Cd values are within the lower end of the range observed for field collected killifish in contaminated areas off Long Island, NY (Chernoff and Dooley 1979) and our calculated Hg(II) and MeHg values are within the lower end of the range observed for killifish in a contaminated estuary in the Gulf of Maine (Chen et al. 2009). Our values may possibly be at the lower end of the range due to the average IR (7% d^{-1}) we used in the bioaccumulation model which could be an underestimate. If the IR is increased to 10% d^{-1} then our calculated values falls within the range observed in the field. No As and Cr levels in field collected killifish or other small forage fish were found in the literature. The killifish used in the present study were collected from a non-contaminated river; the background concentrations of metals in killifish, measured by ICP-MS (mean ± 1 SD; n =5) are as follows: As = 1332 ± 623 ng g^{-1}, Cd = 9.5 ± 1.6 ng g^{-1}, Cr = 1368 ± 715 ng g^{-1}, total Hg = 84 ± 15 ng g^{-1}. Our model-calculated Cd and total Hg values for killifish in contaminated estuaries are much higher than for pristine waters, however, our calculated As and Cr values are much lower. The latter can be attributed to ICP-MS analysis calculating total As and Cr concentrations in killifish, whereas the present study predicted the body burden based on ambient As(V) and Cr(III). As in fish is predominantly found as organic arsenobetaine which is accumulated via the diet (Kirby and Mayer 2002) and Cr is mainly taken up as hexavalent Cr (Cr(VI)), the dominant form of Cr found in natural waters (Bruland 1983).

Studies measuring the levels of metals in small field-caught fish are limited and in need of further research, because small forage fish, including killifish, are a conduit for the transfer of metals from lower trophic levels to their predators, including blue crab (*Callinectes sapidus*), bluefish (*Pomatomus saltatrix*), striped bass (*Morone saxatilis*), and dogfish (*Mustelus canis*) (Kneib 1986; Hartman and Brandt 1995; Rountree and Able 1996), which if consumed potentially provide a source of metals to humans.

For Cd and MeHg dietary sources accounted for > 99% of the metal body burden, as shown in other studies (Xu and Wang 2002; Pickhardt et al. 2006; Mathews and Fisher 2009; Dutton and Fisher 2010) when fish were fed zooplankton prey. The present study indicates that aqueous exposure can account for a significant portion of the Hg(II) body burden in killifish, especially in

MI water where aqueous accumulation of Hg(II) accounted for 71% of the C_{ss} value. This is consistent with the findings of another study using freshwater redear sunfish (46-60%; Pickhardt et al. 2006), whereas Dutton and Fisher (2010) calculated that > 96% of Hg(II) in Atlantic silversides is attributed to dietary exposure, significantly higher than our calculated values for killifish. Interspecific differences are presumably attributable to physiological differences between these fish. To our knowledge this is the first study to calculate the relative importance of dietary and aqueous exposure routes on the body burden of As and Cr in fish, and for both metals aqueous exposure accounted for a 27-54% of the total body burden. The present study, as well as prior studies (Pickhardt et al. 2006; Mathews and Fisher 2009; Dutton and Fisher 2010), provide important information to regulatory agencies who determine water quality standards based on the ambient concentration of metals in the water and largely overlook the importance of the diet as a source of metals to fish.

Metal uptake from the aqueous phase

For all three field sites, the greatest accumulation of each metal was observed during the first hour of exposure, due to the bioavailable metal binding to the most reactive site on the fish, which is presumably the gill. It is presumed that after the initial rapid uptake, the rate of metal accumulation decreased due to either saturation of the most reactive binding sites on the gill, or possible internal regulation of the metal in the fish, as observed for As in BH and ER water, and Cr in ER and MI water. The difference in salinity between the three field sites most probably accounted for the difference in the CF and k_u values calculated in this study for most metals, whereas the DOC concentration had less influence. DOC can reduce metal bioavailability for fish, but the three field sites do not differ markedly in their DOC concentration range (2-4.6 mg L^{-1}) which could account for why no clear trend was observed between metal uptake and DOC concentration. It should also be noted that the composition of the DOM presumably varies between field sites, but this was not characterized in this study.

The low uptake of As in waters from all field sites resulted in CFs < 1, indicating the water was more enriched in As than the fish, consistent with earlier findings (Dutton and Fisher 2011a, 2012). This could be attributed to both As(V) and the fish body surface being negatively charged, and therefore repelling each other. As uptake appeared to reach steady-state in killifish in BH and ER water by the end of the 36 h exposure. The high efflux rate (23% d^{-1}) of As from

the liver calculated for killifish in BH water (Table 7) indicates that As may be internally regulated, and therefore part of the reason why steady-state conditions were reached was due to the rapid physiological turnover of As in the liver. As accumulation in killifish increased with increasing salinity; fish in MI water accumulated 2.3 times more As than fish in BH water by the end of 37 h exposure. Marine fish drink to osmoregulate and drinking could be a possible uptake route for As, but this was not supported by the tissue distribution data. This same relationship was observed in another laboratory study where As uptake was investigated as a function of salinity (Dutton and Fisher 2011a) and in a field study, where fish collected from higher salinity waters had a higher body burden of As (Larsen and Francesconi 2003). The k_u values calculated in the present study are comparable to those calculated in other studies (Dutton and Fisher 2011a, 2012).

Cr was the only metal for which salinity and DOC concentration did not influence metal accumulation, as shown by the overlapping error bars in Figure 2. Like As, Cr also binds to oxygen and does not chloro-complex in seawater (Bruland 1983). By the end of uptake, CFs were < 1 in killifish in ER and MI water indicating that the water was more enriched in Cr than the fish at those two sites. Cr(III) is particle-reactive and has low cell membrane permeability which accounts for the observed low uptake at all three field sites. In comparison, Cr(VI) is much less particle-reactive than Cr(III), but has greater cell membrane permeability, and therefore accumulates in marine organisms, as observed in mussels (Wang et al. 1997). Cr accumulation reached steady-state in ER and MI water within the 37 h exposure period; unlike for As, there was not a high efflux rate of Cr from the liver; instead the concentration of Cr increased over time. Another study also concluded that salinity had no influence on the uptake of Cr in fish (Dutton and Fisher 2011a). Cr accumulation was lowest in ER water which had the highest DOC concentration; although the three field sites have a narrow DOC concentration range, this conforms to another study that concluded that as DOM concentration increased from 0 to 5 mg L^{-1}, the uptake of Cr decreased (Dutton and Fisher 2012).

Cd uptake showed an inverse relationship with salinity; CFs in fish in BH water were 2.2 times higher than fish in MI water, similar to other studies (Jackson et al. 2003; Zhang and Wang 2007a; Dutton and Fisher 2011a). As salinity increases the speciation of Cd changes; Cd^{2+} binds to chloride forming $CdCl_2$ which is less chemically reactive and therefore less bioavailable to killifish. The concentration of DOC did not influence Cd accumulation in killifish, supporting

the findings of other studies (Hollis et al. 1996; Dutton and Fisher 2012). This can possibly be attributed to Cd having a lower binding affinity for DOC (log $K_{Cd\text{-}DOC}$ = 7.4) than for fish gills (log $K_{Cd\text{-}gill}$ = 8.6) (Playle et al. 1993). The CF and k_u values calculated in this study are comparable to another study where Cd uptake was investigated as a function of salinity at 6, 12, and 25 ppt (Dutton and Fisher 2011a).

Like Cd, Hg(II) and MeHg chloro-complex in seawater. In freshwater, Hg binds to hydroxide (Hg is found as $Hg(OH)_2$ and MeHg as CH_3HgOH), whereas in seawater Hg(II) and MeHg bind to chloride, forming mercuric-chloride complexes ($HgCl_2$ and CH_3HgCl_2, respectively) (Mason et al. 1996). As salinity increased, Hg(II) and MeHg behaved differently. At the end of uptake, the Hg(II) CF was 5.6 times higher and the k_u value 7.2 times higher in MI water than in ER water, even though there was only a 2.5 ppt salinity difference. The reason for the sudden increase in Hg(II) uptake in MI water after 12 h exposure is not apparent to us. This Hg(II) uptake pattern was also observed in another study where Hg(II) accumulation was investigated as a function of humic acid concentration, where the greatest rate of Hg(II) uptake was noted between 24-47.5 h of exposure (Dutton and Fisher 2012). Furthermore, another study (Dutton and Fisher 2011a) showed a positive relationship between salinity and Hg(II) accumulation, whereas no clear trend was observed in the present study. The inverse relationship between Hg(II) uptake in killifish and DOC concentration was also observed previously (Dutton and Fisher 2012). The Hg(II) k_u values in BH and ER waters calculated in this study are within the range of values calculated for freshwater, estuarine, and marine fish (Pickhardt et al. 2006; Dutton and Fisher 2010, 2011a, 2012), although the k_u of Hg(II) in MI water is higher than most values in other studies.

MeHg uptake showed an inverse relationship with salinity; killifish in BH water accumulated 2 times more MeHg than in MI water. In another study where MeHg accumulation in killifish was investigated as a function of salinity a positive relationship was observed (Dutton and Fisher 2011a). It should be noted that the prior study was strictly controlled so salinity was the only variable, whereas this study was conducted in natural waters. A field study investigating the concentration of Hg in largemouth bass (*Paralichthys lethostigma*) also found an inverse relationship with salinity (Farmer et al. 2010). However, another study using the mangrove clam (*Polymesoda erosa*) concluded that Hg accumulation showed a positive relationship with salinity (Modassir 2000), supporting the prior study by Dutton and Fisher (2011a). Octanol-water

partition coefficients (K_{ow}) calculated for Hg(II) and MeHg favor Hg(II) and MeHg bound to chloride as the most bioavailable species for uptake due to their greater lipophilicity and therefore cell membrane permeability (Mason et al. 1996); as a result Hg(II) and MeHg should be more readily taken up at higher salinities.

Metal retention and elimination after aqueous exposure

The rate of metal uptake in killifish and the resulting body burden due to aqueous exposure is influenced by water chemistry and therefore varied between the three field sites. However, the loss of metals from killifish is controlled by the physiological turnover rate of each metal within the fish. Apart from the higher As and Cr k_{ew} values in BH water, there was little variability in the k_{ew} and therefore the physiological turnover rate between the three field sites for each metal. This could possibly be attributed to the experiments being temperature controlled and therefore the killifish used in each experiment in water from each site had the same routine metabolic rate. As and Cr had the highest k_{ew} at all three field sites, possibly accounting for As and Cr reaching steady-state throughout uptake in BH and ER, and ER and MI water, respectively. To our knowledge this is the first study to calculate k_{ew} values for fish after aqueous exposure to As, whereas our Cd, Hg(II), and MeHg k_{ew} values are within the range calculated in other studies, and our Cr k_{ew} values are higher than those calculated in other studies (Xu and Wang 2002; Wang and Wong 2003; Jeffree et al. 2006; Pickhardt et al. 2006; Dutton and Fisher 2010).

Tissue distribution of aqueous metals

The increase in the percentage of Cd associated with the viscera (presumably the intestine) in higher salinity water (ER, MI) can be attributed to marine fish having to drink to osmoregulate and is therefore an uptake mechanism for Cd, as observed in another study where the tissue distribution of Cd was investigated as a function of salinity (Dutton and Fisher 2011a). A prior study has shown that Cd shares the same uptake pathway in the gill and gut as Ca^{2+} (Franklin et al. 2005). The tissue distribution of As and Cr between the three field sites appeared to be influenced by DOC concentration, for reasons that are not apparent to us. As(V) is known to compete with PO_4 for uptake into cells and the PO_4 transporter was recently identified in zebrafish (Beene et al. 2011). Cr(III) could possibly be complexed to organic compounds which

are selected for by killifish and therefore taken up across the gills as observed in bivalves (Roditi et al. 2000a; Guo et al. 2001), but to our knowledge this is not known to be an uptake mechanism for metals in fish. However, it should be noted that very low radioactivity counts of As and Cr were detected in each tissue compartment, and this may account for some of the larger calculated standard error values. The similar tissue distribution of Hg(II) and MeHg indicates that once inside the body, Hg(II) and MeHg are redistributed around the body in the same way and are not influenced by salinity or DOC concentration. Both Hg species were predominantly associated with the body, presumably the fillet, due to their high binding affinity for sulfur. At all field sites the highest concentration of As (except in MI water), Cd, Cr, and Hg(II) were in the gills, which are presumed to be the dominant uptake site, whereas the highest concentration of MeHg was associated with the viscera, indicating that once MeHg crossed the gills it was effectively redistributed around the body via the blood.

To our knowledge this is the first study to assess the body distribution of As, Cd, Cr, Hg(II), and MeHg in fish throughout a several day depuration period following aqueous exposure and the first study in which metal efflux and influx rates for individual tissue compartments were determined. However, a previous study by Hogstrand et al. (2003) measured organ-specific depuration rates of Ag in two freshwater fish, the rainbow trout (*Oncorhynchus mykiss*) and European eel (*Anguilla anguilla*). Prior studies have focused on factors which influence the AE and k_u values of metals in fish, and the k_{ew} and k_{ef} values have received less attention. It is easier to understand what influences the calculated k_u and AE values because they result from uptake at essentially one site (gills and gut, respectively), however, the whole fish k_{ew} and k_{ef} is essentially an integration of the efflux rate constants from different tissue compartments in fish, each with their own turnover rates. To date, efflux and influx rate constants in different tissue compartments have not been well described in animals, partially because it is difficult to do, but some exceptions exist (Fisher et al. 1996). A radiotracer technique can be used to overcome this issue and provide further insight into what determines whole fish k_{ew} and k_{ef} values, as determined in a prior study using fish (Oliveira Ribeiro et al. 1999) following dietary exposure to MeHg. However, it should be noted that between the metals examined only four tissue compartments had calculated efflux rate constants that were significantly different than zero ($p < 0.05$; Table 7); this can be attributed to the mean radioactivity concentration values being used in

the regression analysis, so for each tissue compartment and metal only four data points were used.

Cd and Hg(II) were not redistributed around the body throughout the 9 d depuration. The large decrease in Cd and Hg(II) radioactivity concentrations in individual tissue compartments, especially the head, gills, and brain (48%, 47%, and 86% for Cd, and 42%, 68%, and 72% for Hg(II), respectively) accounted for most of the metal loss observed from the whole fish and the resulting k_{ew} values. The high k_{ew} (7.5% d^{-1}) for Cr in killifish is attributed to the 70% decrease in radioactivity concentration in the gills and 39% decrease in the viscera. The lack of an apparent trend in the tissue partitioning of As throughout depuration could be due to low radioactive counts in each tissue compartment. The loss of As from each tissue compartment, particularly the brain and liver accounts for the highest calculated k_{ew} value (9.6% d^{-1}) and the shortest $tb_{½}$ of any metal in the whole fish. In comparison, MeHg was effectively redistributed around the body throughout depuration, presumably via the blood, instead of being released from the fish (except the liver), resulting in the low calculated k_{ew} value (0.6% d^{-1}).

For all metals, the liver had an important role in the processing and excretion of metals, presumably via the bile. The influx rate of MeHg into the brain, eyes, and fillet indicated that MeHg accumulate in the sulfur-rich portion of the body (fillet) and can readily cross the blood brain barrier in fish. Another study by Oliveira Ribeiro et al. (1999) also accumulated in the brain following dietary exposure to MeHg, supporting our findings. For each metal the calculated efflux rates for the skin (4-9.7% d^{-1}) were surprisingly high; while care was taken when dissecting the fish, a small portion of muscle tissue may have remained attached to the skin. However, the body surface of killifish is covered in scales which can accumulate metals to high concentrations (Coello and Khan 1996), as a result the calculated efflux rate constant maybe a result of metals detaching from the scales.

Table 1. Salinity, chloride ion concentration, dissolved organic carbon (DOC) concentration, pH, and background metal concentrations (C_w) for Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. For chloride ion concentration and DOC concentration values represent means ± 1 SD; n = 3. For C_w, units are µg L^{-1} for As, Cd, and Cr, and ng L^{-1} for Hg(II) and MeHg.

	Salinity (ppt)	Chlorinity (mM)	DOC (mg L)	pH	C_w As	Cd	Cr	Hg(II)	MeHg
BH	7.6	161 ± 0.2	2.6 ± 0.1	7.56	0.97	0.02	0.19	3.30	0.10
ER	19.5	415 ± 0.5	4.6 ± 0.02	7.45	1.38	0.21	0.25	2.81	0.09
MI	22	447 ± 0.6	2.0 ± 0.08	7.76	2.20	0.19	0.20	2.62	0.08

Table 2. Uptake rate constants (k_u), efflux rate constants (k_{ew}) and corresponding biological half-lives ($tb_{½}$) in killifish after aqueous exposure to metals in Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. Values represent means ± 1 SE; n = 8 for ER and MI k_u and k_{ew}, n = 25 for BH k_u and n = 5 for BH k_{ew}.

		k_u (L g^{-1} d^{-1})			k_{ew} (d^{-1})			$tb_{½}$ (d)
Metal	Site	Mean	SE	Range	Mean	SE	Range	
As	BH	0.0003	0.0004	0.00003-0.009	0.096	0.022	0.037-0.147	7.2
	ER	0.0006	0.0001	0.0003-0.0009	0.046	0.003	0.032-0.057	15
	MI	0.0004	0.0001	0.0001-0.0010	0.062	0.005	0.031-0.079	11
Cd	BH	0.0008	0.0001	0.0004-0.0015	0.025	0.009	0.007-0.056	28
	ER	0.0007	0.0001	0.0004-0.0013	0.015	0.002	0.007-0.027	46
	MI	0.0004	0.00004	0.0002-0.0005	0.016	0.001	0.011-0.020	43
Cr	BH	0.0006	0.0002	0.0002-0.0041	0.075	0.008	0.055-0.096	9.2
	ER	0.0005	0.0001	0.0002-0.0013	0.046	0.011	0.007-0.085	15
	MI	0.0006	0.0001	0.0004-0.0016	0.045	0.009	0.015-0.096	15
Hg(II)	BH	0.026	0.002	0.010-0.052	0.046	0.002	0.040-0.052	15
	ER	0.030	0.003	0.019-0.043	0.023	0.001	0.019-0.028	30
	MI	0.215	0.026	0.073-0.307	0.037	0.002	0.025-0.046	19
MeHg	BH	0.781	0.096	0.268-2.025	0.006	0.001	0.003-0.009	116
	ER	0.426	0.023	0.338-0.547	0.009	0.001	0.005-0.013	77
	MI	0.370	0.056	0.201-0.643	0.009	0.001	0.007-0.012	77

Table 3. Tissue distribution of As, Cd, Cr, Hg(II), and MeHg in killifish after 9 day depuration following aqueous exposure in Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. Values (means ± 1 SE) represent the percentage of total body burden associated with each tissue compartment (head, gills, viscera, and body). n = 5 for BH and n = 8 for ER and MI.

Metal	Location	Head	Gills	Viscera	Body
As	BH	23 ± 4	5 ± 3	20 ± 7	52 ± 11
	ER	23 ± 3	17 ± 2	16 ± 2	44 ± 4
	MI	21 ± 3	3 ± 1	10 ± 2	66 ± 5
Cd	BH	26 ± 1	60 ± 2	8 ± 1	6 ± 1
	ER	17 ± 2	46 ± 4	27 ± 2	10 ± 1
	MI	23 ± 2	45 ± 3	25 ± 7	7 ± 1
Cr	BH	41 ± 5	8 ± 3	18 ± 5	33 ± 4
	ER	22 ± 3	12 ± 2	21 ± 3	45 ± 6
	MI	54 ± 13	12 ± 7	4 ± 3	30 ± 12
Hg(II)	BH	31 ± 1	15 ± 0.1	10 ± 0.7	44 ± 2
	ER	26 ± 0.9	16 ± 0.7	11 ± 0.5	47 ± 1
	MI	31 ± 1	16 ± 1	13 ± 0.6	40 ± 1
MeHg	BH	21 ± 0.6	4 ± 0.3	17 ± 1	58 ± 1
	ER	21 ± 0.6	4 ± 0.3	17 ± 0.3	58 ± 0.9
	MI	23 ± 1	4 ± 0.1	16 ± 0.5	57 ± 1

Table 4. Radioactivity concentrations (Bq g^{-1} dry wt) of metals in fish tissues (head, gills, viscera, and body) at the end of 9 days depuration after aqueous exposure in Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. Values represent means ± 1 SE; n = 5 for BH and n = 8 for ER and MI.

Metal	Location	Head	Gills	Viscera	Body
As	BH	10 ± 3	31 ± 16	17 ± 5	10 ± 4
	ER	24 ± 2	68 ± 14	27 ± 5	15 ± 2
	MI	11 ± 2	14 ± 7	15 ± 5	16 ± 3
Cd	BH	58 ± 7	1336 ± 387	57 ± 13	7 ± 1
	ER	58 ± 12	1073 ± 277	250 ± 48	13 ± 4
	MI	29 ± 5	595 ± 107	91 ± 13	4 ± 0.5
Cr	BH	47 ± 6	128 ± 52	53 ± 21	16 ± 3
	ER	28 ± 7	83 ± 26	37 ± 4	33 ± 20
	MI	19 ± 6	14 ± 7	3 ± 2	4 ± 3
Hg(II)	BH	71 ± 10	260 ± 43	65 ± 10	42 ± 5
	ER	720 ± 117	2368 ± 733	825 ± 197	406 ± 41
	MI	354 ± 38	1295 ± 114	457 ± 56	208 ± 22
MeHg	BH	1106 ± 240	1550 ± 263	2267 ± 300	1332 ± 274
	ER	714 ± 30	1267 ± 46	1840 ± 77	902 ± 45
	MI	712 ± 52	1188 ± 95	1835 ± 151	894 ± 66

Table 5. Metal partitioning in killifish at the end of aqueous uptake (Day 0) and after 1, 3, 6, and 9 days of depuration in Baltimore Harbor water. Values (means ± 1 SE) represent the percentage of total body burden of each metal associated with each tissue compartment; n = 5. Values < 0.1% are represented by *.

Metal	Day	Head	Gills	Brain	Eyes	Viscera	Liver	Skeleton	Fillet	Skin
As	0	17 ± 3	5 ± 1	0 ± 0	1 ± 0.5	9 ± 3	5 ± 0.5	10 ± 3	41 ± 4	12 ± 4
	1	16 ± 2	7 ± 2	5 ± 4	5 ± 2	6 ± 2	4 ± 2	6 ± 2	45 ± 4	6 ± 2
	3	13 ± 3	5 ± 0.4	4 ± 2	7 ± 2	6 ± 2	7 ± 1	17 ± 7	29 ± 3	12 ± 1
	6	25 ± 3	5 ± 2	0 ± 0	2 ± 2	4 ± 2	0 ± 0	4 ± 3	56 ± 9	4 ± 2
	9	13 ± 4	5 ± 3	2 ± 2	9 ± 2	18 ± 6	1 ± 1	16 ± 7	30 ± 7	6 ± 4
Cd	0	31 ± 2	57 ± 3	0 ± 0	0 ± 0	6 ± 1	1 ± 0.1	2 ± 0.2	1 ± 0.2	2 ± 0.1
	1	24 ± 1	66 ± 2	0 ± 0	0 ± 0	4 ± 0.8	1 ± 0.1	2 ± 0.2	1 ± 0.1	2 ± 0.1
	3	23 ± 2	66 ± 2	0 ± 0	0 ± 0	3 ± 0.5	2 ± 0.1	2 ± 0.3	1 ± 0.1	3 ± 0.4
	6	27 ± 0.6	58 ± 2	0.5 ± 0.06	0.5 ± 0.04	6 ± 0.9	3 ± 0.7	2 ± 0.6	1 ± 0.2	2 ± 0.4
	9	25 ± 1	60 ± 2	0.5 ± 0.1	0.5 ± 0.04	4 ± 0.5	4 ± 0.9	3 ± 0.9	1 ± 0.2	2 ± 0.3
Cr	0	30 ± 2	15 ± 1	3 ± 0.8	3 ± 0.4	13 ± 2	4 ± 0.9	11 ± 2	12 ± 0.8	9 ± 1
	1	37 ± 4	20 ± 2	4 ± 1	2 ± 2	19 ± 4	2 ± 1	5 ± 1	4 ± 2	7 ± 1
	3	39 ± 9	17 ± 5	0 ± 0*	2 ± 2	9 ± 4	0 ± 0	31 ± 15	0 ± 0*	2 ± 2
	6	38 ± 5	9 ± 4	3 ± 2	3 ± 2	15 ± 5	4 ± 1	13 ± 6	6 ± 2	9 ± 3
	9	31 ± 3	7 ± 3	3 ± 1	7 ± 3	12 ± 6	6 ± 2	13 ± 5	13 ± 2	8 ± 0.5
Hg(II)	0	29 ± 3	21 ± 3	1 ± 0.8	2 ± 0.3	5 ± 0.4	1 ± 0.2	14 ± 1	10 ± 0.9	17 ± 1
	1	26 ± 1	21 ± 2	3 ± 0.1	1 ± 0.1	5 ± 0.3	2 ± 0.3	11 ± 0.9	12 ± 0.8	19 ± 0.8
	3	30 ± 1	17 ± 0.5	1 ± 0.3	2 ± 0.1	5 ± 0.4	3 ± 0.4	14 ± 0.5	13 ± 1	15 ± 1
	6	31 ± 1	17 ± 1	1 ± 0.04	2 ± 0.3	6 ± 0.4	3 ± 0.4	14 ± 0.5	12 ± 1	14 ± 0.4
	9	29 ± 1	15 ± 0.1	1 ± 0.1	1 ± 0.08	6 ± 0.5	4 ± 0.3	13 ± 0.7	15 ± 1	16 ± 0.7
MeHg	0	25 ± 1	23 ± 1	2 ± 0.1	2 ± 0.1	7 ± 0.3	3 ± 0.3	14 ± 1	10 ± 0.5	14 ± 0.5
	1	24 ± 1	10 ± 1	1 ± 0.1	1 ± 0.1	12 ± 1	4 ± 0.4	14 ± 0.6	17 ± 0.7	17 ± 0.7
	3	20 ± 1	6 ± 0.3	1 ± 0.1	1 ± 0.04	13 ± 0.8	5 ± 0.2	12 ± 0.8	25 ± 0.7	17 ± 0.5
	6	21 ± 0.8	4 ± 0.3	1 ± 0.1	1 ± 0.04	12 ± 0.7	6 ± 0.8	10 ± 0.4	32 ± 2.2	13 ± 1
	9	19 ± 0.8	4 ± 0.3	1 ± 0.2	1 ± 0.04	11 ± 0.4	6 ± 0.7	8 ± 0.6	33 ± 0.7	17 ± 1

Table 6. Radioactivity concentrations (means ± 1 SE) of metals in individual tissue compartments at the end of aqueous uptake (Day 0) and after 1, 3, 6, and 9 days of depuration in Baltimore Harbor water. Units are Bq g^{-1} dry wt for As, Cd, Cr, and Hg(II), and kBq g^{-1} dry wt for MeHg; n = 5.

Metal	Day	Head	Gills	Brain	Eyes	Viscera	Liver	Skeleton	Fillet	Skin
As	0	20 ± 7	64 ± 31	0 ± 0	28 ± 28	24 ± 9	27 ± 7	30 ± 10	32 ± 16	23 ± 8
	1	11 ± 5	41 ± 11	106 ± 85	43 ± 14	12 ± 4	8 ± 4	13 ± 7	17 ± 5	9 ± 3
	3	9 ± 2	36 ± 7	104 ± 45	71 ± 21	12 ± 2	25 ± 6	36 ± 16	13 ± 1	17 ± 3
	6	9 ± 4	18 ± 8	0 ± 0	17 ± 12	3 ± 1	0 ± 0	7 ± 6	11 ± 5	3 ± 3
	9	7 ± 3	25 ± 12	14 ± 14	60 ± 14	29 ± 8	2 ± 2	16 ± 6	10 ± 4	7 ± 4
Cd	0	121 ± 15	2617 ± 248	25 ± 6	22 ± 4	79 ± 18	31 ± 5	18 ± 2	3 ± 0.3	11 ± 1
	1	122 ± 9	2523 ± 979	96 ± 74	32 ± 7	58 ± 16	33 ± 4	21 ± 3	2 ± 0.3	21 ± 2
	3	107 ± 10	2467 ± 261	26 ± 22	36 ± 6	43 ± 8	54 ± 9	20 ± 4	3 ± 0.3	23 ± 3
	6	113 ± 14	1934 ± 320	20 ± 5	26 ± 3	72 ± 15	68 ± 22	22 ± 7	3 ± 0.5	18 ± 3
	9	63 ± 7	1336 ± 387	13 ± 5	15 ± 2	52 ± 11	63 ± 20	18 ± 6	2 ± 0.3	10 ± 0.8
Cr	0	122 ± 7	622 ± 81	401 ± 119	199 ± 34	135 ± 25	107 ± 46	116 ± 24	27 ± 3	69 ± 10
	1	68 ± 10	396 ± 32	275 ± 99	50 ± 40	102 ± 23	26 ± 17	24 ± 7	5 ± 2	26 ± 2
	3	53 ± 7	159 ± 30	17 ± 17	46 ± 31	29 ± 12	0 ± 0	857 ± 803	1 ± 1	64 ± 64
	6	40 ± 7	103 ± 45	101 ± 62	55 ± 26	55 ± 26	55 ± 15	20 ± 8	3 ± 1	15 ± 3
	9	46 ± 2	120 ± 46	137 ± 45	167 ± 51	62 ± 39	48 ± 18	47 ± 20	11 ± 1	23 ± 4
Hg(II)	0	168 ± 10	1308 ± 297	129 ± 75	96 ± 18	134 ± 57	43 ± 11	221 ± 25	50 ± 6	186 ± 57
	1	144 ± 12	917 ± 287	217 ± 63	67 ± 6	75 ± 6	56 ± 9	199 ± 69	43 ± 4	142 ± 15
	3	158 ± 23	436 ± 78	105 ± 20	166 ± 25	186 ± 60	119 ± 39	196 ± 14	46 ± 6	135 ± 20
	6	120 ± 15	557 ± 103	41 ± 11	98 ± 10	101 ± 47	67 ± 15	125 ± 19	33 ± 6	86 ± 14
	9	83 ± 12	298 ± 49	60 ± 14	58 ± 9	73 ± 13	77 ± 8	94 ± 13	28 ± 3	71 ± 12
MeHg	0	1.7 ± 0.3	10 ± 1.1	0.7 ± 0.1	1.1 ± 0.2	2.8 ± 0.8	3.6 ± 0.7	2.0 ± 0.2	0.7 ± 0.2	1.5 ± 0.2
	1	1.7 ± 0.1	6.1 ± 0.8	1.1 ± 0.2	1.2 ± 0.1	3.8 ± 0.6	7.9 ± 0.7	2.2 ± 0.2	1.1 ± 0.1	2.0 ± 0.2
	3	1.5 ± 0.2	3.3 ± 0.3	1.5 ± 0.2	1.2 ± 0.1	4.4 ± 0.5	7.7 ± 1.1	2.1 ± 0.1	2.0 ± 0.2	1.9 ± 0.1
	6	1.2 ± 0.1	1.9 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	2.8 ± 0.4	4.1 ± 1.0	1.3 ± 0.1	1.4 ± 0.3	1.4 ± 0.2
	9	1.2 ± 0.3	1.8 ± 0.3	1.8 ± 0.4	1.8 ± 0.9	2.5 ± 0.4	3.0 ± 0.4	1.3 ± 0.2	1.6 ± 0.3	1.5 ± 0.3

Table 7. Rates of metal uptake (positive values) and loss (negative values) in individual tissue compartments (d^{-1}) and the corresponding biological half-lives ($tb_{½}$) following aqueous exposure in Baltimore Harbor water. Rates of metal loss significantly different from zero ($p<0.05$) are represented by *. nd: not determined.

Metal		Head	Gills	Brain	Eyes	Viscera	Liver	Skeleton	Fillet	Skin
As		-0.05	-0.079	-0.272	-0.017	0.055	-0.23	-0.046	-0.063	-0.097
	$tb_{½}$	13.9	8.8	2.5	40.8	nd	3	15.1	11	7.1
Cd		-0.073	-0.081*	-0.222	-0.1	0.009	0.076	-0.014	-0.006	-0.095
	$tb_{½}$	9.5	8.6	3.1	6.9	nd	nd	49.5	115.5	7.3
Cr		-0.051	-0.14	0.018	0.147	-0.019	0.084	-0.099	0.15	-0.076
	$tb_{½}$	13.6	5	nd	nd	36.5	nd	7	nd	9.1
Hg(II)		-0.074	-0.112	-0.171	-0.047	-0.036	0.007	-0.102*	-0.062	-0.095*
	$tb_{½}$	9.4	6.2	4.1	14.7	19.3	nd	6.8	11.2	7.3
MeHg		-0.047	-0.153	0.044	0.037	-0.067	-0.135*	-0.077	0.02	-0.04
	$tb_{½}$	14.7	4.5	nd	nd	10.3	5.1	9	nd	17.3

Table 8. Model-predicted body burden of metals in killifish at steady-state (C_{ss}) and the percentage of body burden attributed to dietary exposure for each metal and field site. C_{ss} values were calculated using C_w values in Table 1, k_u and k_{ew} values in Table 2, and AE, k_{ef}, C_f, IR and g values in the Materials and Methods section. BH: Baltimore Harbor, ER: Elizabeth River, MI: Mare Island.

Metal	Location	C_{ss} (ng g^{-1})	% diet
As	BH	8.5	73
	ER	19	46
	MI	24	60
Cd	BH	244	> 99
	ER	2088	> 99
	MI	1914	> 99
Cr	BH	2.5	57
	ER	3.4	53
	MI	3.0	48
Hg(II)	BH	4.4	75
	ER	4.8	68
	MI	12	29
MeHg	BH	268	> 99
	ER	267	> 99
	MI	266	> 99

Fig. 1. Accumulation of aqueous As, Cd, Cr, Hg(II), and MeHg (as dry-weight concentration factor) over a 37 h uptake period in killifish, *Fundulus heteroclitus*, in Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. Values represent means ± 1 SE; n = 8 for ER and MI, and 25 for BH.

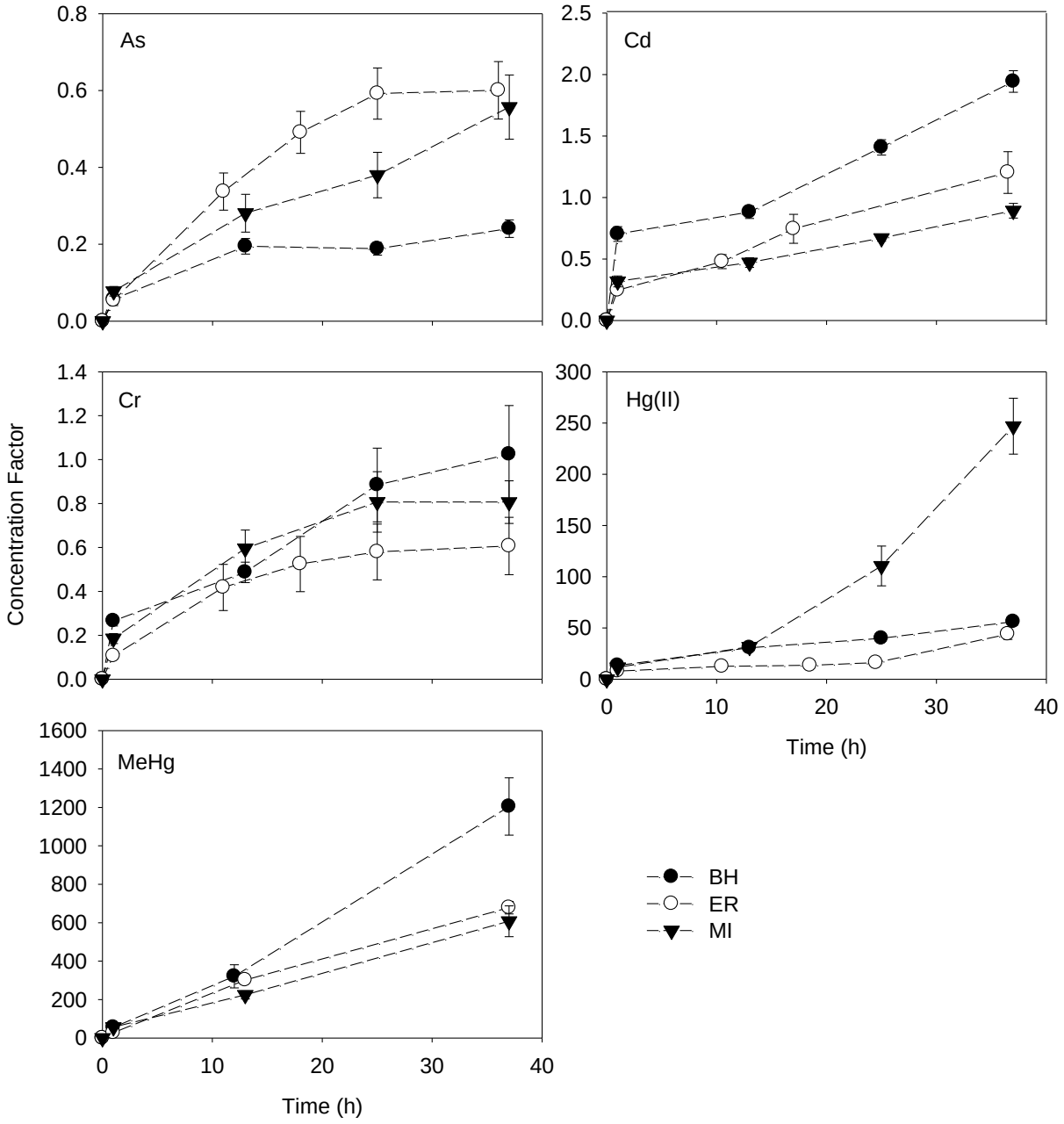
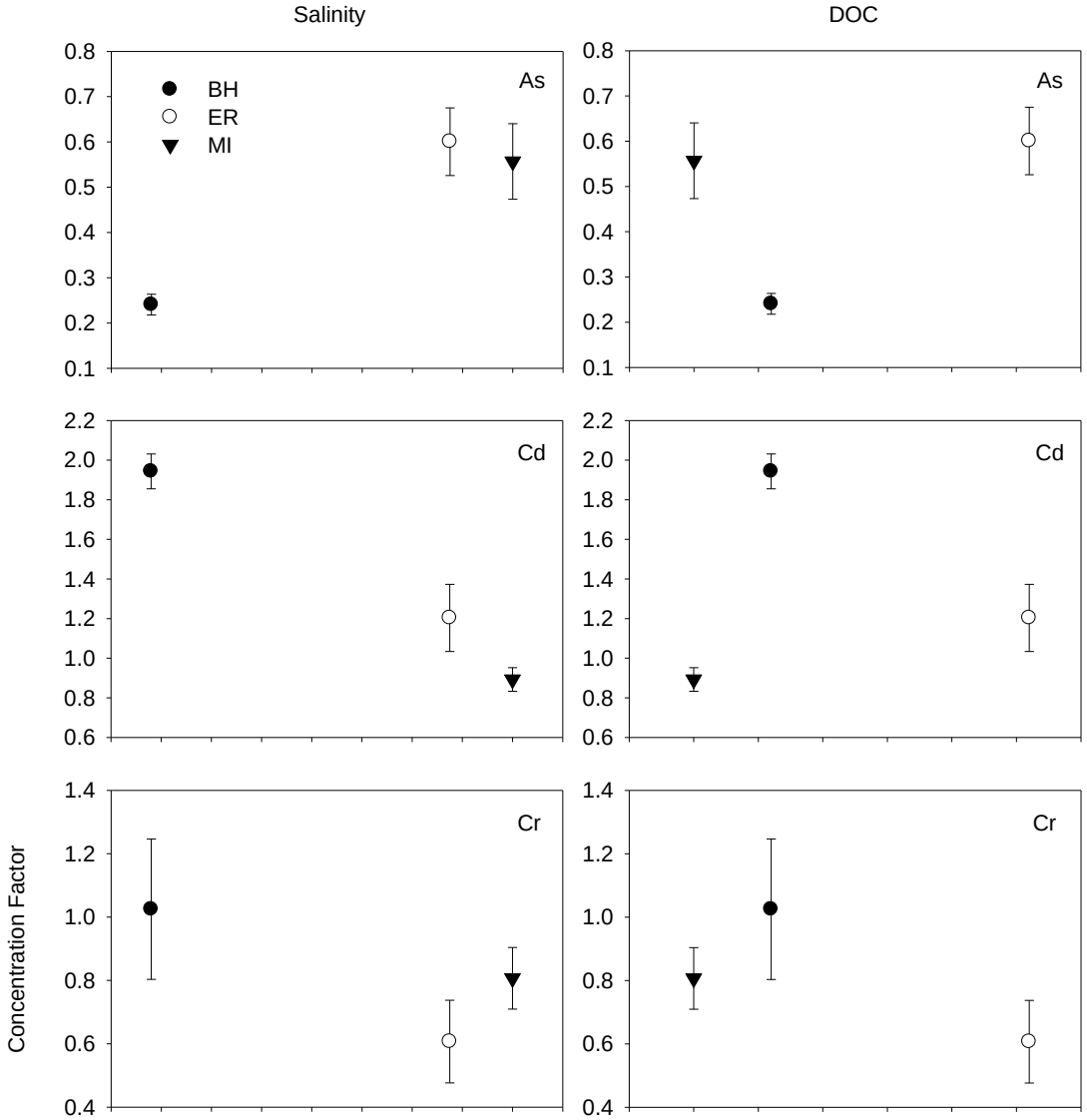


Fig. 2. Metal concentration factors as a function of salinity and dissolved organic carbon (DOC) concentration in killifish (*Fundulus heteroclitus*) after 37 h exposure in Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. Salinity and DOC values for each location are found in Table 1. Values represent means ± 1 SE; n = 8 for ER and MI, and 25 for BH.



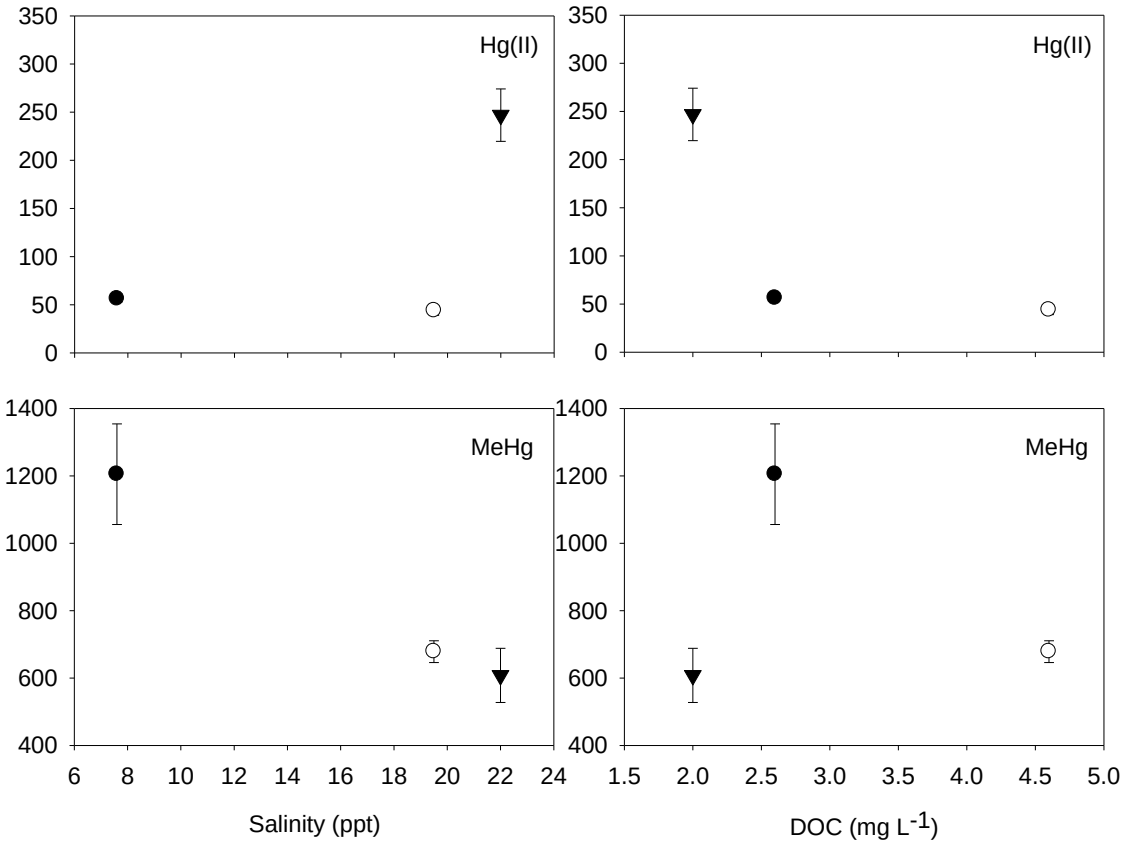
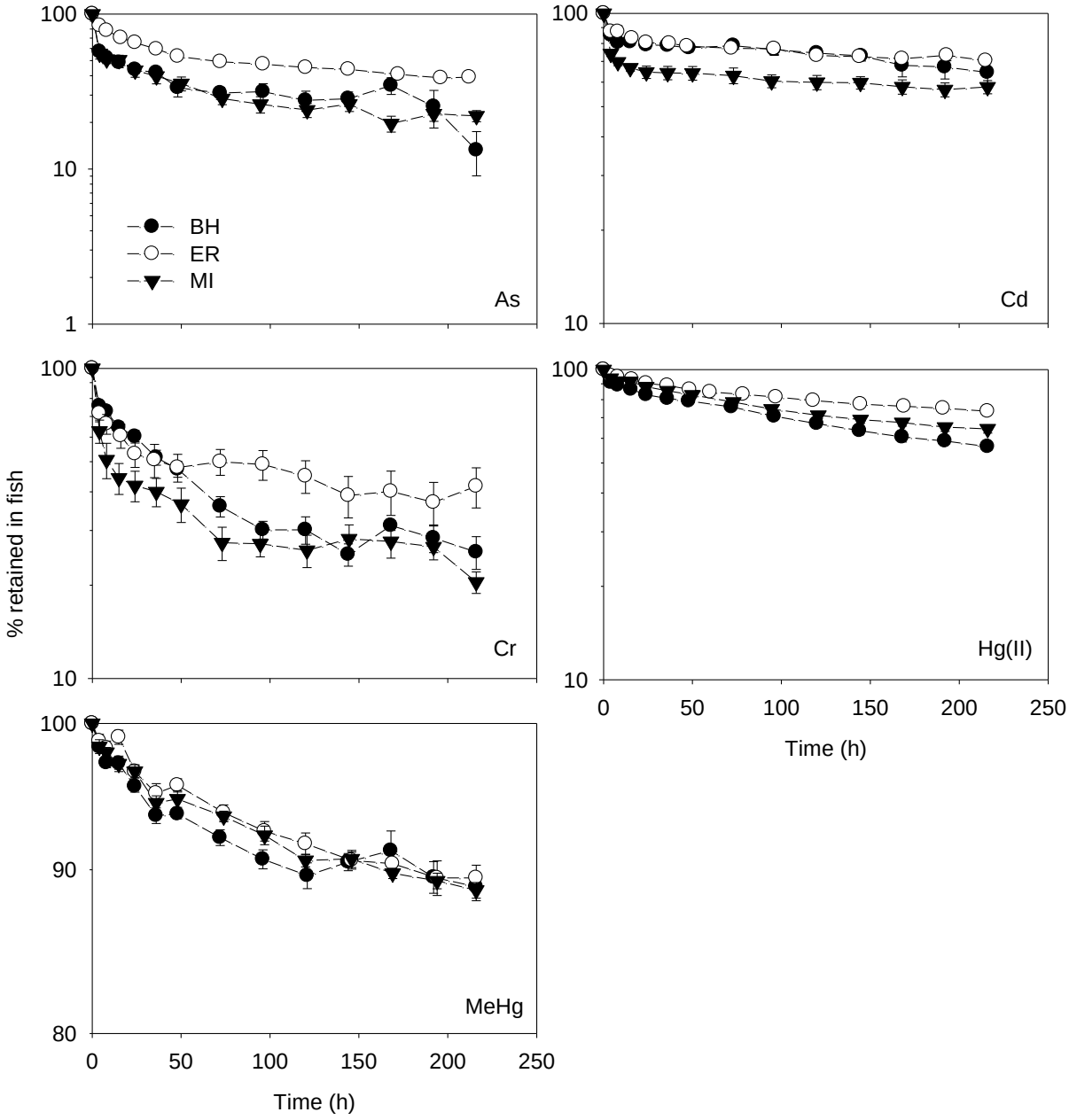

Hg(II)
Hg(II)
MeHg
MeHg
350
300
250
200
150
100
50
0
1400
1200
1000
800
600
400
6
8
10
12
14
16
18
20
22
24
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Salinity (ppt)
DOC (mg L-1)

Fig. 3. Loss of As, Cd, Cr, Hg(II), and MeHg from killifish (*Fundulus heteroclitus*) over 9 d after aqueous exposure in Baltimore Harbor (BH), Elizabeth River (ER), and Mare Island (MI) water. Retention is shown as percent of initial body burden after exposure. Values represent means ± 1 SE; n = 8 for ER and MI, and 5-20 for BH.



Chapter VII

Intraspecific comparisons of metal bioaccumulation in the juvenile Atlantic silverside *Menidia menidia*

ABSTRACT

We experimentally assessed the uptake, loss and resulting tissue distribution of Am, Cd, inorganic Hg(II), methylmercury (MeHg), Se and Zn in two North American populations (Nova Scotia and South Carolina) of juvenile Atlantic silverside (*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 to 89%) and lowest for Am (0.3 to 1.9%). No pronounced difference in metal uptake was noted between the 2 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 metals investigated, MeHg and Zn in both populations and Cd in the South Carolina population are expected to biomagnify at this trophic step.

Dutton J, Fisher NS (2010) Intraspecific comparisons of metal bioaccumulation in the juvenile Atlantic silverside *Menidia menidia*. Aquatic Biology 10: 211-226

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 either the dissolved (Jeffree et al. 2006) or dietary (Reinfelder and Fisher 1994a; Ni et al. 2000; Baines et al. 2002; Mathews and Fisher 2008a,b) exposure route, and few studies combine the 2 exposure routes to calculate total body burdens (Xu and Wang 2002; Wang and Wong 2003; Pickhardt et al. 2006). Studies have shown that diet is the dominant exposure pathway for metals in freshwater and marine fish (Xu and Wang 2002; Pickhardt et al. 2006; Mathews and Fisher 2009). Dietary exposure accounts for 40to 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 and 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 and Wang 2002; Zhang and 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 and Fisher 1996; Xu and 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 and Wang 2002) and grunt, *Terapon jarbua* (Zhang and Wang 2006). No concise relationship has been found for Cd, with higher AEs at lower ingestion rates in *L. argentimaculatus* (Xu and Wang 2002), but no significant relationship between ingestion rate and AE in *T. jarbua* (Zhang and 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 *Salmo salar*, 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 and 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 and Present 1990; Present and Conover 1992). This counter-gradient variation evolved to overcome the shorter growing season, and size-selective winter mortality at higher latitudes (Conover and Present 1990). At the end of the growing season, the body length of *M. menidia* is the same throughout the species range, indicating that the northern fish grow faster throughout the shorter growing season (Conover and 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 2 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 2 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 *Artemia franciscana* nauplii), which were then 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, and Hg 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 and Richardson 1980). These metals have a wide range of AEs in herbivores (Reinfelder and Fisher 1991; Wang and Fisher 1998), which has been correlated with the cytoplasmic distribution of metals in phytoplankton food (Reinfelder and Fisher 1991, 1994b), and this, in turn, can affect the assimilation efficiency of metals in fish when fed crustacean prey (Reinfelder and 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 and 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) Southampton (SHSW) seawater, collected 5 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 (total Hg), and Rutgers Inorganic Analytical Laboratory, Rutgers University (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 under a 14:10 h light:dark cycle.

Fish maintenance and acclimation

Juvenile F1 and F2 generation laboratory-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 mo). Nova Scotia fish were 59 ± 5 mm (mean ± SD) long, with a mean wet weight (wt) of 0.9 ± 0.18 g, while South Carolina fish were 38 ± 5 mm long, with a mean wet wt of 0.32 ± 0.1 g. Fish were spawned and raised at the Flax Pond Marine Lab (Oldfield, New York) from field-collected fish following a protocol described elsewhere (Conover and Present 1990, Present and Conover 1992), and transferred to our lab at least 3 wk before the start of experiments where they were acclimated to SHSW, and fed a daily diet of brine shrimp (*Artemia franciscana*) nauplii. 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 laboratory (Beck and Poston 1980). At least 4 d prior to each experiment, 8 to 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 and 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 to 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 s 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 wt 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 to 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 and 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 (*Artemia franciscana*) nauplii 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 these were 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 = 5to 8 per population).

Radioisotopes and radioanalysis

^{109}Cd ($t_{½}$ = 463.3 d) and ^{65}Zn ($t_{½}$ = 244.1 d), both dissolved in 0.1 N HCl, were purchased from Los Alamos National Laboratory, ^{241}Am ($t_{½}$ = 432.7 y, dissolved in 3 $NHNO_3$) was purchased from Amersham, ^{75}Se ($t_{½}$ = 119.8 d, as selenite, dissolved in 0.1 N HCl) was purchased from Isotope Products, and ^{203}Hg(II) ($t_{½}$ = 46.6 d, dissolved in 1 N HCl) was obtained from Georgia State University. MeHg ($CH_3{}^{203}$Hg(II)), held in deionized water, was synthesized in our laboratory 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 µl 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 (2 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 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 allowed propagated counting errors typically ≤ 5% to be obtained. 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 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 by 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 and Fisher 1998), and more recently freshwater and marine fish (Wang and 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:

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

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 k_u, by dividing radioactivity in individual fish (g^{-1} dry wt) by radioactivity in ml^{-1} water and by time (d). AE and k_e values 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:

$$tb_{½} = \ln 2/k_e \qquad (2)$$

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:

$$W = a \cdot L^b \qquad (3)$$

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:

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

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 and Fitzgerald 2006), and were 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 wt) 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 and 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:

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

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:

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

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-wt concentration factors (CF), defined as dpm (disintegrations per minute) 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 *Menidia menidia*).

After aqueous exposure in SHSW, depuration curves indicated 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 percentages of metal eliminated were 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 percentages of initial metal remaining were 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 constant (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, AE was 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, AE was 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 2 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 2-compartment loss pattern: the rapid loss during the first several h 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 percentages of initial metal remaining were 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 2 silverside populations for each metal. Am and Hg(II) were mainly associated with the head (64 to 69% Am, 49 to 55% Hg(II)), whereas Zn was distributed between the head (37 to 40%) and body (44%). Am was the only metal to show no association with the viscera (0 to 1%). MeHg was predominantly associated with the body (70 to 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 2 populations. Cd and MeHg were predominantly associated with the body (80 to 84% Cd, 51 to 57% MeHg), whereas Zn was mainly associated with the head (49 to 52%). Se was mainly distributed between

the head (44 to 45%) and body (41 to 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 and 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 $tb_{½}$ values

were highest for MeHg (50 to 116 d), followed by Am (17 to 30 d), Zn (11 to 14 d), Cd (9 to 13 d), and lowest for Hg(II) (4 to 10 d). The $tb_{½}$ for Se could only be calculated after a dietary exposure (6 to 7 d). For Cd, Hg(II), and Zn there was little difference in $tb_{½}$ among populations and exposure routes. There was little difference between the $tb_{½}$ values of MeHg between 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 7 $tb_{½}$ 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). After dietary exposure, they were: 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 x 10^{-10} and 1.4 x 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 to 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 *Menidia 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, CFs were >1, indicating fish are more enriched in metal than the surrounding water. CFs and 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 by Zhang and Wang (2007b). 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 2 populations could also account for some of the difference, but respiration rates were not measured during this study.

The MeHg k_u values obtained in this study (1155 and 4375 ml g^{-1} d^{-1}) are similar to those found for the marine sweetlips *Plectorhinchus gibbosus* (4515 ml g^{-1} d^{-1}, Wang and 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 to 78 ml g^{-1} d^{-1}, Pickhardt et al. 2006), and 10-fold lower than that observed in marine fish (195 ml g^{-1} d^{-1}, Wang and 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 in Nova Scotia silversides, and 257-times higher in South Carolina silversides) is consistent with previous reports for other fish (Wang and 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 and Wang 2002). Am is highly particle-reactive (Fisher and 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 and Wang 2002).

Assimilation efficiencies

The wide range of assimilation efficiencies (0.3% for Am to 89% for MeHg) observed in our study indicates that they are highly variable among metals. The AE ranking we noted is consistent with the observations of Reinfelder and Fisher (1991) in copepods, who found 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 2 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 to 95%; Wang and Wong 2003; Pickhardt et al. 2006; Mathews and 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 and Mason 2002a). 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 and 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 to 27%; Wang and Wong 2003; Pickhardt et al. 2006), but much lower than those observed in the freshwater mosquitofish (42 to 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). This allows more time to solubilize the metal in the gut, resulting in a higher AE.

Cd assimilation showed the greatest variation between the 2 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 to 28%; Baines et al. 2002; Mathews and Fisher 2008b). However, our values were higher than those observed for killifish *Fundulus heteroclitus* (7%, Mathews and Fisher 2008a), grunt *Terapon jarbua* (6.3%, Zhang and Wang 2006), and mangrove snapper (9.8%, Xu and 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 to 32%), but when the fish were fed copepods the AEs decreased to 10 to 14%. This was also noted in another study in which uniformly radiolabeled copepods were fed to silversides (*Menidia 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 was 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 to 1.9%) are lower than other values noted in fish (4 to 6%; Baines et al. 2002; Mathews and 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 for copepods (Fisher and 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 and 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 and 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 2 populations. The Zn AEs reported in this study (24% in Nova Scotia, 29% in

South Carolina) are comparable to those obtained for the grunt (23%), striped bass (23 to 40%), and mudskipper (21%) fed zooplankton prey (Ni et al. 2000; Baines et al. 2002; Zhang and Wang 2006). Our values are higher than those reported for glassy (5 to 17%, Ni et al. 2000), mangrove snapper (14.5%, Xu and Wang 2002), sea bream (14%, Mathews and Fisher 2008b), and silversides (6%, Reinfelder and 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 to 77%; Reinfelder and Fisher 1994a; Baines et al. 2002; Xu and Wang 2002; Zhang and 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 and 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 to 7%, 69 to 54%, and 43 to 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 to 8 h after feeding. However, Xu and Wang (2002) noted that at high ingestion rates (as in both our silverside populations: 0.37 and 0.58 g g^{-1} d^{-1}) there is no difference in Cd, Se, and Zn AEs, 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 to 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 and Conover 1992). Nova Scotia silversides also accumulate lipids at a faster rate than South Carolina fish (Schultz and Conover 1997). MeHg was the only

metal which Nova Scotia fish assimilated more than South Carolina fish. Although the difference in AE 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 2 population and exposure routes (except for South Carolina silversides after Hg(II) exposure where the efflux rate was 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 2 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 in the 2 populations. The high efflux rates for all metals except MeHg after dietary exposure indicate that digestive processes aid in the rapid removal of metal. This was particularly evident for Hg(II) in South Carolina silversides, where it was 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 for Am, Cd, Hg(II), MeHg, and Zn are similar to those determined in other studies (Wang and Wong 2003; Pickhardt et al. 2006; Mathews et al. 2008). However, our values for Am, Cd, and Zn are higher than those for turbot (Jeffree et al. 2006). The latter 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 and 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 those noted for 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 through salt removal across gills, and if

Hg(II) is excreted unselectively through the same ion-transport channels used for salt removal, the efflux rate will be higher.

Our k_{ef} values for MeHg are consistent with those obtained in other studies of fish fed zooplankton prey (Wang and Wong 2003; Pickhardt et al. 2006; Mathews and Fisher 2008a). Our k_{ef} values for Cd, Se, and Zn are also within the range noted in other studies where fish were fed zooplankton prey (Xu and Wang 2002; Mathews and Fisher 2008a,b), but unlike for MeHg, there is greater variability in these efflux rates among studies. Our Hg(II) k_{ef} values were higher than those determined for freshwater fish (Pickhardt et al. 2006), but the Nova Scotia silverside k_{ef} was consistent with that of marine sweetlips (Wang and 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 and 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 and 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 those noted for 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 2 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 to 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 and 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 and 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 to 84%), consistent with observations in striped bass (Baines et al. 2002). Other studies have shown that Cd remains associated with the gut (Xu and Wang 2002; Mathews and 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 were 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 was 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 have 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 (authors' unpubl. data). The higher percentage of Cd associated with the viscera in Nova Scotia silversides could be due to a higher drinking rate, but this was not measured in our study.

Our results show that the 2 species of mercury we examined behaved very differently. At the end of depuration after aqueous exposure, 49 to 55% of Hg(II) remained associated with the head, consistent with findings in mosquitofish and redear sunfish (Pickhardt et al. 2006), whereas 70 to 72% of MeHg was associated with the body, which is higher than noted previously (~55%, Pickhardt et al. 2006). After dietary exposure, 51 to 57% of MeHg body burden was associated with the body, consistent with that found in killifish (58%, Mathews and 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 and Mason 2002a, 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 were entered into the biokinetic model, the predicted steady-state body burden (C_{ss}) was 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 2 populations can in part be attributed to higher AEs in South Carolina silversides. In Nova Scotia silversides, the 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 and Present 1990; Present and 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 and 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 and 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 and Wang 2002; Pickhardt et al. 2006; Mathews and Fisher 2009) for Cd, MeHg, and Zn. However, Pickhardt et al. (2006) noted that diet contributed 73 to 88% of the Hg(II) burden in mosquitofish, and 40 to 55% in redear sunfish, significantly lower than our calculated values. Differences between the 2 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 and 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 and 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 and Daborn 1981), and are preyed upon by piscivorous fish and sharks (Hartman & Brandt 1995; Rountree and Able 1996; Buckel et al. 1999). Of the 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). This is consistent with the findings of Xu and Wang (2002), Wang and Wong (2003), Mathews and Fisher (2008a,b), and 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 and Wong 2003; Pickhardt et al. 2006; Mathews and Fisher 2008a). Zn was 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 and Fisher 2008b; Mathews et al. 2008), while others have predicted Zn will if the ingestion rate and assimilation efficiency are high enough (Xu and Wang 2002; Mathews et al. 2008). TTF could also be a function of body size: Zhang and Wang (2007b) 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 and Wang 2003, Mathews and Fisher 2008a,b). Apart from that of 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 *Menidia menidia*.

Table 1. *Menidia* menidia. Uptake rate constants (k_u) and efflux rate constants (k_{ew}) in Atlantic silversides 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 2 populations are represented by * ($p < 0.05$) and ** ($p < 0.01$). n = 4 to 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. *Menidia menidia.* Assimilation efficiencies (AE) and efflux rate constants (k_{ef}) in Atlantic silversides from Nova Scotia (NS) and South Carolina (SC) after feeding on brine shrimp (*Artemia franciscana*) nauplii. Statistically significant differences (by t-test) between kinetic parameters and the 2 populations are represented by * ($p < 0.05$). n = 5 to 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. *Menidia* menidia. 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 to 9 per population for aqueous exposure and 5 to 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. *Menidia* menidia. Biological half-lives ($tb_{½}$; d) of metals in Nova Scotia (NS) and South Carolina (SC) populations of the Atlantic silverside after aqueous and dietary exposure. k_e values used are in Tables 1 and 2. nd: not determined.

	Population	Aqueous $tb_{½}$	Dietary $tb_{½}$
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. *Menidia menidia*. 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 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	C_{ss}	% 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

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

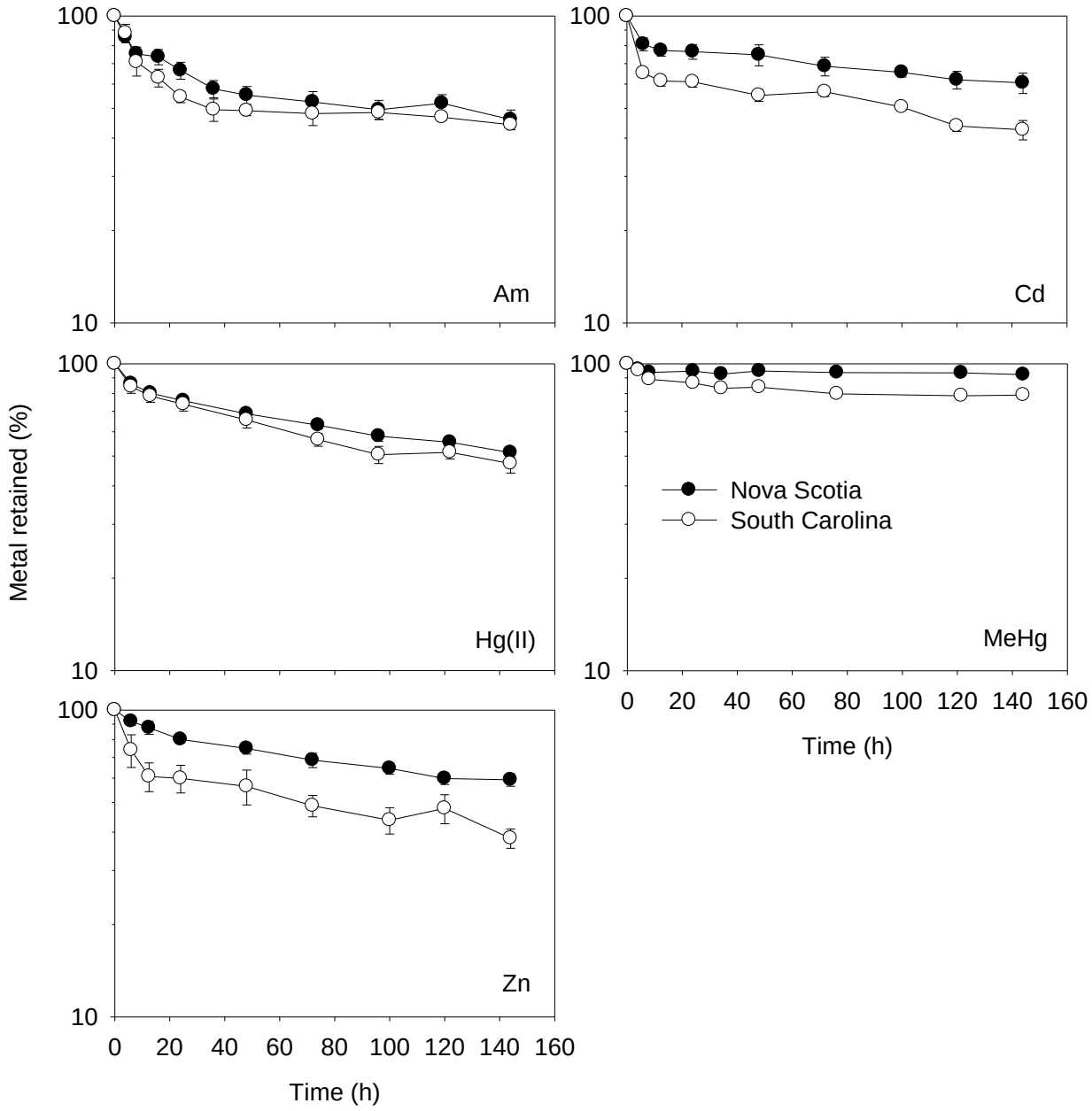


Fig. 2. *Menidia menidia.* Metal retention in Atlantic silversides from Nova Scotia and South Carolina after pulse feeding on brine shrimp (*Artemia franciscana*) nauplii (n = 5 to 8 per population). Retention is expressed as percent of initial body burden after exposure. Values represent means ± 1 SE

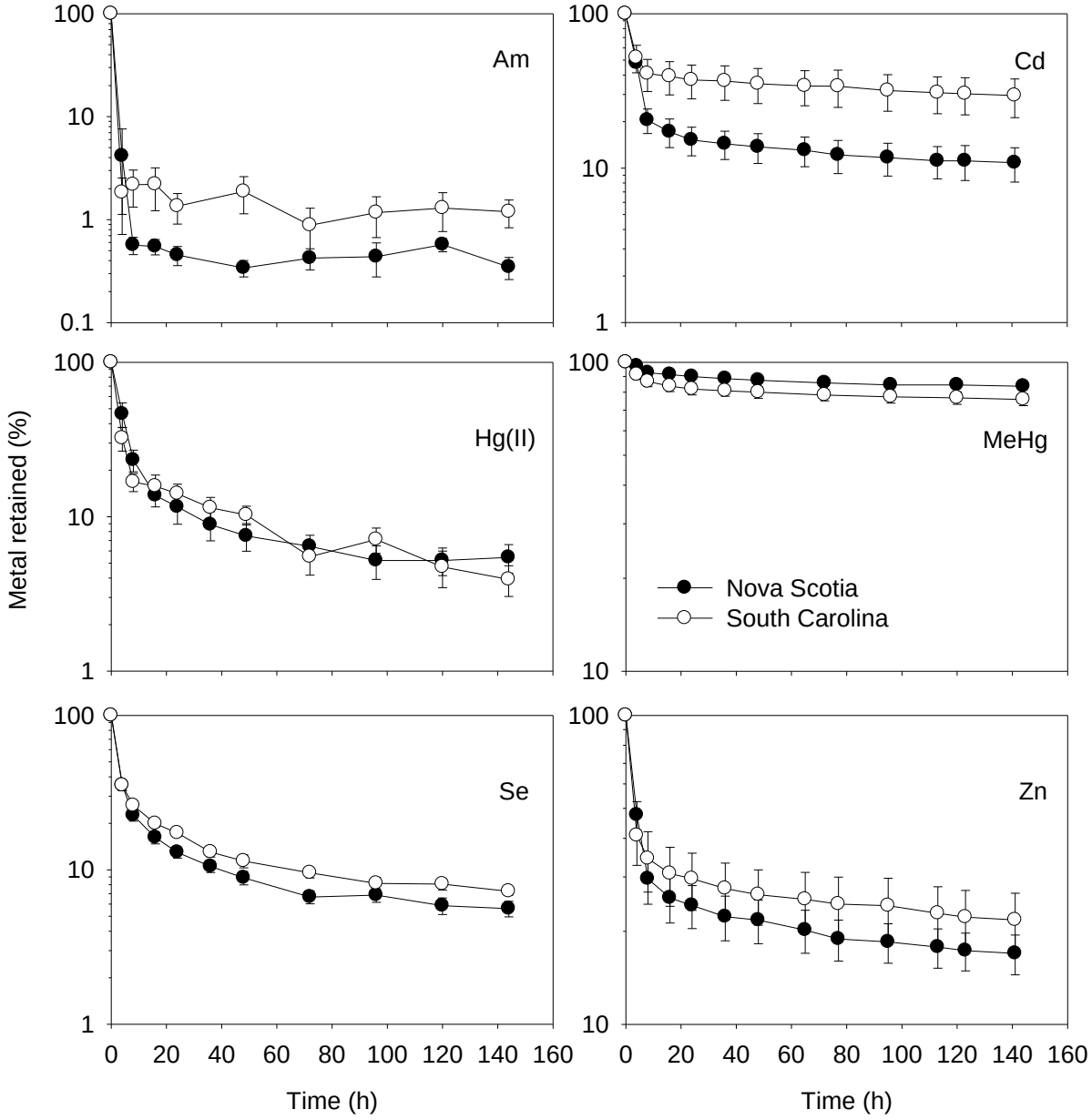


Fig. 3. *Menidia menidia.* Metal partitioning in Atlantic silversides from Nova Scotia (NS) and South Carolina (SC) at the end of depuration after aqueous exposure in Southampton seawater. Bars represent the percentage of total body burden associated with each tissue compartment (head, viscera, body).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

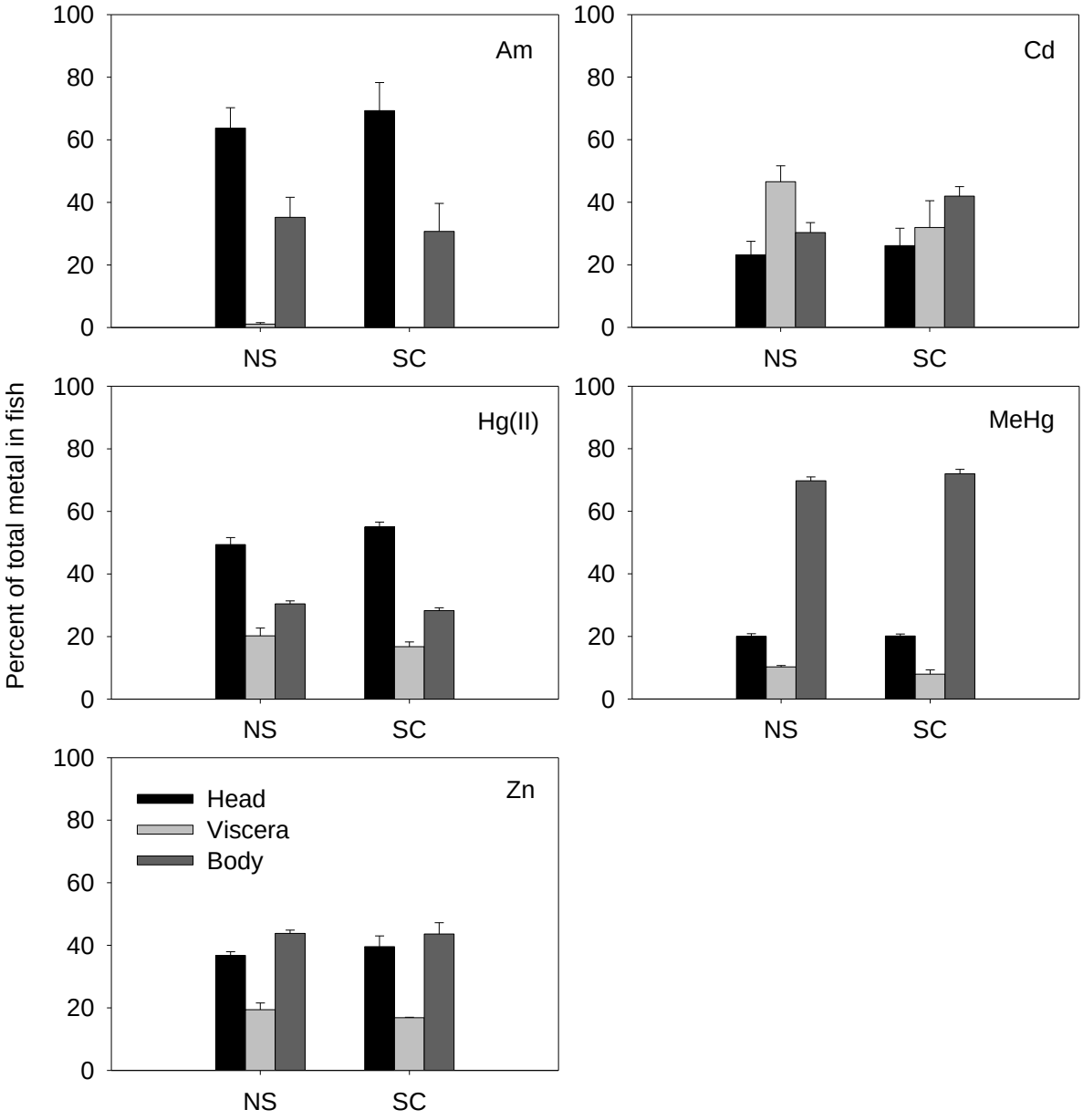
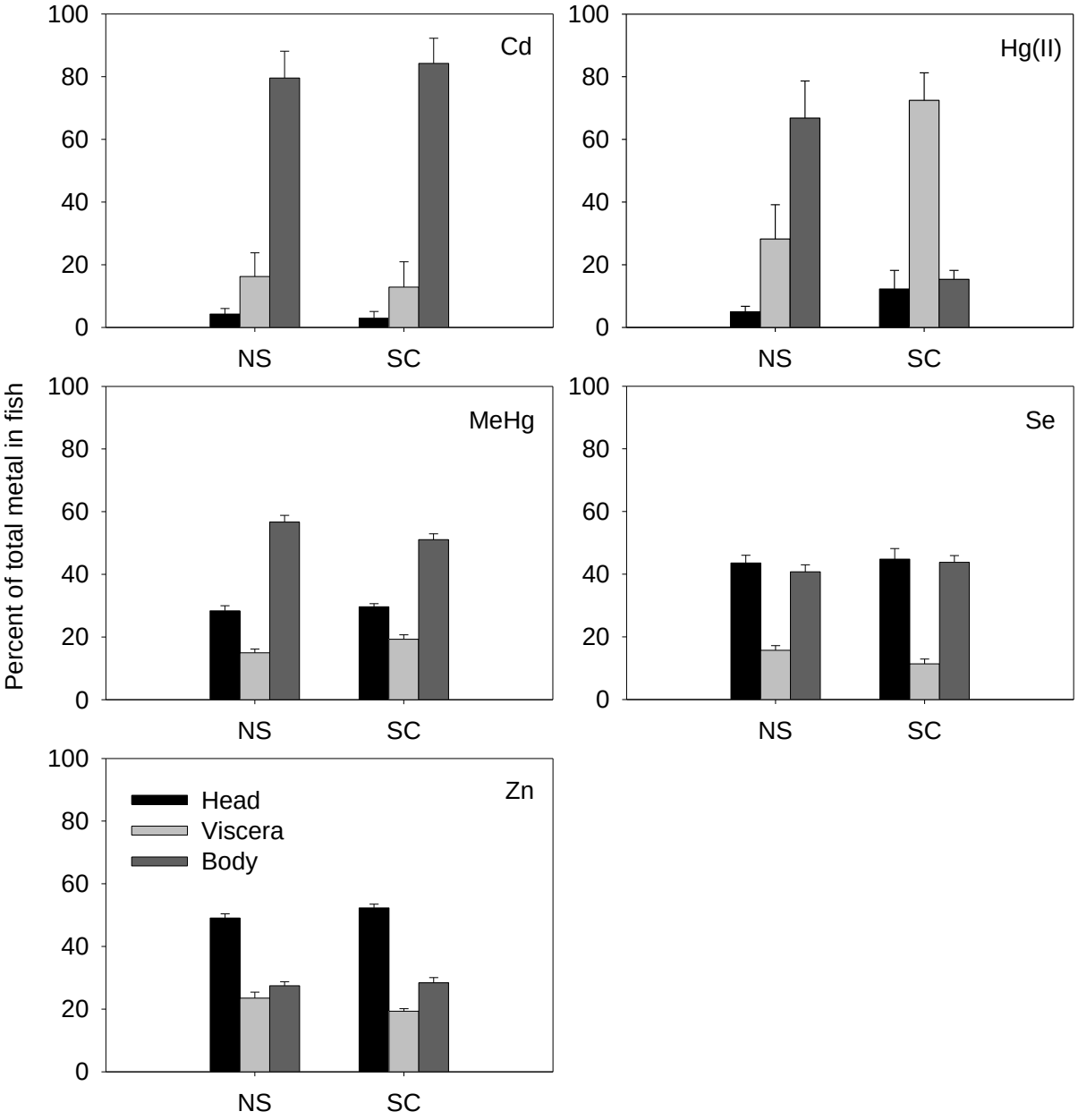


Fig. 4. *Menidia menidia.* Metal partitioning in Atlantic silversides from Nova Scotia (NS) and South Carolina (SC) at the end of depuration after dietary exposure. Bars represent the percentage of total body burden associated with each tissue compartment (head, viscera, body). Values represent means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



Chapter VIII

Conclusions

OVERVIEW OF THE DISSERTATION

The general objective of this dissertation was to investigate the influence of water chemistry (salinity and dissolved organic matter concentration), prey type, and fish physiology (ingestion rate and growth rate) on metal accumulation in two small forage fish, the killifish *Fundulus heteroclitus* and the Atlantic silverside *Menidia menidia*. The kinetic parameters calculated in these laboratory experiments (k_u, AE, k_{ew}, k_{ef}) can be entered into a quantitative biokinetic model to predict steady-state metal concentrations in field-caught fish on a site-specific basis and the percentage of that body burden attributed to dietary exposure. Research on metal accumulation in small forage fish is important because these fish are a conduit for the transfer of metals from lower trophic levels to their predators, which may be consumed by humans, potentially posing a risk to human health.

All of the experiments conducted throughout this dissertation were carried out in the Fisher laboratory at Stony Brook University using a well-developed radiotracer technique. This allowed the uptake and loss of metals following either aqueous or dietary exposure to be monitored over time in the same individual fish, therefore reducing biological variability. Furthermore, because all of the experiments could be carefully controlled, clear results were produced from relatively simple experiments. However, there are two potential problems associated with these laboratory based studies. First, the results are not always applicable to field conditions, as discussed in more detail below. Second, the fish probably behaved differently in a laboratory setting that what they would in their natural environment. Both killifish and Atlantic silversides are schooling species (Fay et al. 1983; Abraham 1985) and may become stressed in a laboratory setting where they are held in individual containers or small fish tanks. It is difficult to investigate the impact of stress on these fish, aside from monitoring their respiration rate which would be very hard to do, so it is not known how stress impacts the rate of metal uptake and loss from aqueous and dietary sources. While killifish showed no signs of stress and no mortality occurred throughout the experiments, Atlantic silversides were very sensitive to handling, resulting in a higher mortality rate.

The work carried out in this dissertation enables a better understanding of the factors influencing metal accumulation in small forage fish, the relative importance of aqueous and dietary exposure pathways, and the resulting tissue distribution from both uptake routes. The metals and metalloids investigated throughout this dissertation were chosen due to their different

chemical characteristics and environmental interest. All of the chosen elements are found at elevated concentration in estuarine waters close to industrial activity and urbanized areas. This dissertation can be broken down into several key findings.

KINETIC MODELING OF METAL ACCUMULATION IN FISH

A simple biokinetic model can be used to predict metal body burdens in field-caught killifish and Atlantic silversides. This model takes into account metal uptake into the fish and metal loss from the fish following exposure to aqueous and dietary metals. To calculate the body burden of metals in fish at steady-state, metal uptake and loss parameters following aqueous and dietary exposure to metals (k_u, AE, k_{ew}, k_{ef}) must be combined with physiological rates (IR, g) obtained from the literature, and measurements of metal concentrations in water and food collected from a specific study area (C_w, C_f). This enables predicted metal concentrations in fish to be calculated on a site-specific basis and comparisons to be made between field sites (Chapter VI). Prior studies using fish (Pickhardt et al. 2006; Mathews and Fisher 2009) and Chapter VI of this dissertation have shown that the model is relatively effective to date. As shown in Chapter VI, the model can effectively account for the major processes governing metal accumulation in killifish on a site-specific basis.

The C_{ss} values calculated for killifish in Chapter VI and Atlantic silversides in Chapter VII could not be compared to fish caught in the field at the same locations (Baltimore Harbor, Elizabeth River, and Mare Island for killifish, and Nova Scotia and South Carolina for Atlantic silversides). During our collection trips to Baltimore Harbor, Elizabeth River, and Mare Island, I was unable to seine for killifish due to logistical constraints, and the Atlantic silversides used were raised in the laboratory and not collected from the field. Furthermore, no values could be found in the literature for other studies measuring metal levels in killifish and Atlantic silversides at the same locations. This is something that warrants further study because metal concentrations in fish are limited for a lot of heavily contaminated estuarine and coastal areas. My calculated C_{ss} values did fall within the range for killifish and other small fish species caught in other heavily contaminated field sites (Chernoff and Dooley 1979; Chen et al. 2009) and were much higher than the levels recorded for fish in pristine sites.

Both dissolved and ingested metals serve as sources of metal uptake for estuarine fish. The relative importance of these routes on the total body burden is dependent on water chemistry, prey type, and the rate of physiological processes. If the aqueous or dietary conditions change, the relative importance of each exposure route may also change as a result. In order to understand the mechanisms which control metal accumulation in estuarine fish, each uptake pathway must be separately quantified and the factors which influence the rate of metal uptake and loss must be understood for both exposure pathways.

Prior studies have concluded that the diet is the dominant source of many metals to fish (Xu and Wang 2002; Wang and Wong 2003; Pickhardt et al. 2006; Dang et al. 2009; Mathews and Fisher 2009; Zhang et al. 2011). Studies using killifish (Chapter VI) and Atlantic silversides (Chapter VII) conclude that the diet accounts for > 95% of the total body burden of Cd, MeHg, Se, and Zn, whereas the aqueous phase should not be overlooked as a source of metal for Am, As, and Cr. The dietary importance of Hg(II) showed interspecies variability; for Atlantic silversides the diet accounted for > 96% of the total body burden, whereas for killifish the diet accounted for 29 to 75% of the total body burden. This has important implications for water quality agencies, including the U.S. Environmental Protection Agency, who base their water quality standards on the ambient concentration of metal in the water. Furthermore, the water quality standards are based upon toxicity studies and not bioaccumulation studies, thus possibly overlooking public health implications of metal concentrations in fish tissues. The findings of this dissertation, as well as prior studies, stresses the need to consider the diet as a major source of metals to fish when conducting risk assessments.

SALINITY AND DISSOLVED ORGANIC MATTER CONCENTRATION INFLUENCES METAL ACCUMULATION FROM THE AQUEOUS PHASE

Since the 1970's it has been well recognized that metal speciation and therefore its chemical reactivity plays a critical role in controlling metal uptake from the aqueous phase by aquatic organisms. Whether a metal is available in the free-ion form, or whether it is complexed to inorganic or organic ligands in the water, not only influences how much metal is taken up into the fish from the dissolved phase, but it also influences how much metal the fish will ingest as well. At the bottom of the food chain, the greatest enrichment step occurs between the water and

the phytoplankton (Fisher and Reinfelder 1995); if the aqueous metal is in a complexed form which is not bioavailable to the phytoplankton, then there will be a lower concentration of metal in the zooplankton, and therefore the fish, compared to an aqueous medium where the metal is present in the free-ion form which is more likely to be bioavailable.

Chapters II and III investigated the influence of salinity and dissolved organic matter concentration (humic acids) on the uptake of As, Cd, Cr, Hg(II), and MeHg to killifish from the aqueous phase. Metal uptake as a function of either salinity or dissolved organic matter concentration varied by metal. It should be noted that the experiments conducted in Chapters II and III do not reproduce the conditions of the natural environment. In these experiments, all water parameters were kept constant and either salinity or the humic acid concentration in the water was the only variable that changed, whereas in natural waters differences in the temperature of the water, pH, salinity, and dissolved organic matter type and concentration all influence metal uptake from the dissolved phase. While the salinity and humic acid concentration experiments do not reproduce the natural conditions of the aquatic environment, it does allow us to understand more clearly the interactions between metals and inorganic and organic ligands in water, and how that influences metal bioavailability and uptake to fish from the aqueous phase.

Prior studies investigating metal uptake across a salinity gradient have not focused on the potentially toxic metals used in this study, except Cd; therefore my dissertation provides new information on this issue. Cd uptake showed an inverse relationship with salinity (as found in prior studies for a range of aquatic organisms; e.g. Hall and Anderson 1995; Jackson et al. 2003), Cr uptake showed no relationship with salinity, and As, Hg(II), and MeHg uptake increased with salinity. The relationship between As accumulation and salinity warrants further study, because laboratory experiments (Chapters II and VI) and field-caught fish (Larsen and Francesconi 2003) both show an increase in As with increasing salinity. As behaves as an oxyanion in seawater and does not chlorocomplex, and the observed relationship remains wholly unexplained. However, dissection data revealed that As and Cd had a greater association with the viscera at higher salinities and this may be associated with marine fish drinking to osmoregulate. While this has been previously noted for Cd, it has not been observed for As. Scott et al. (2006) calculated the drinking rates for killifish in brackish water (10% strength of seawater; 3.5 ppt) and freshwater. The drinking rate decreased from 1.32 ml kg^{-1} d^{-1} in brackish water to 0.4 ml kg^{-1} d^{-1} 12 hours after freshwater transfer and increased slightly to 0.6 ml kg^{-1} d^{-1} 7 days after freshwater transfer.

A subsequent study by Scott et al. (2008) calculated the drinking rate in killifish after transfer from brackish water to seawater (35 ppt). The drinking rate rapidly increased from 1.3 ml kg^{-1} d^{-1} in brackish water to 2.9 ml kg^{-1} d^{-1} 12 hours after transfer to seawater and decreased to 2.25 ml kg^{-1} d^{-1} 7 days after seawater transfer. Water and ion absorption across the killifish intestine (measured *in vitro*) decreased with increasing salinity due to a reduced dietary salt requirement in seawater (Scott et al. 2006, 2008), indicating that intestinal ion exchange and water absorption has a critical role in osmoregulation in fish (Grosell 2006).

Zhang and Wang (2007a) calculated the drinking rates for the black sea bream (*Acanthopagrus schlegi*) at 0 ppt and 35 ppt using ^{14}C-polyethylene glycol as a tracer. The drinking rate in freshwater was 33% of the drinking rate in seawater (0.05 L kg^{-1} d^{-1} and 0.15 L kg^{-1} d^{-1}, respectively), however, the overall water uptake into black sea bream did not vary with salinity (1.1 to 1.3 L kg^{-1} d^{-1}). Zhang and Wang (2007a) determined that a greater proportion of Cd was associated with the viscera when the drinking rate is higher and therefore the importance of the gastrointestinal tract as an uptake site for metals increases at higher salinities, supporting the findings in Chapter II. The fish were only dissected into head, gills, viscera, and body (fins, fillet, and skin) in Chapter II, and future studies should include a more detailed dissection of the viscera to conclude whether the intestine is an uptake site for As and Cd, and rule out the possibility of As and Cd being redistributed from the gill to the viscera (especially the liver) via the blood.

Prior studies investigating the influence of DOM on the aqueous accumulation of metals in fish have focused on Ag, Cd, Cu, and Ni, due to their presence in freshwater systems and the use of the BLM to develop water quality criteria for these metals in freshwater systems. With the exception of Cd, the other potentially toxic metals investigated in Chapter III have largely been overlooked. Futhermore, previous studies have focused on whether metal uptake to fish is reduced in the presence of DOM, and the physiological impacts on the gills. Chapter III adds new information to the literature because As, Cd, Cr, Hg(II), and MeHg uptake to fish has been investigated over a DOM concentration gradient. Cr, Hg(II), and MeHg uptake showed an inverse relationship with humic acid concentration, Cd uptake showed no relationship with humic acid concentration, and As uptake increased with increasing humic acid concentration. Like with salinity, As is the element which remains wholly unexplained, and future studies are needed to understand the observed relationship. Future work should include trying to estimate

metal speciation in the water for each DOM concentration using a well-developed model such as WHAM (Windermere Humic Aqueous Model) or NICA-Donnan (NICA = NonIdeal Competitive Adsorption) to try and link the observed metal uptake relationships with increasing DOM concentration to metal speciation in the water for each metal. This will provide further understanding as to whether the observed relationships are a result of metal speciation in the water, fish physiology, or whether both factors play a role.

Based on the findings of these two sets of experiments, future studies are needed to investigate the combined effect of salinity and DOM concentration, to more realistically calculate uptake rates for waters with different chemical characteristics. This would provide a more realistic representation of metal uptake in natural waters and allow us to see whether one variable has a stronger influence on metal uptake over the other. Based on the findings of Chapters II and III, metal uptake to killifish from the aqueous phase was investigated in three natural waters with varying salinity and DOM concentration in Chapter VI. This study concluded that salinity appeared to have a greater influence on metal uptake than DOM concentration, but as discussed in Chapter V, this may be due in part to the narrow concentration range of DOM in the three site waters.

The biokinetic model uses short-term aqueous exposure data (often a few hours to a day depending on the organism) when calculating k_u. The aqueous uptake experiments carried out in Chapters II, III, and VI, all conclude that metal uptake is rapid during the first several hours of exposure due to metals binding to the most reactive site on the fish, the gills. Metal uptake slows down thereafter, presumably due to either metal binding sites on the fish becoming saturated, or possible internal regulation in the fish, as observed for As and Cr. By using short-term exposure data the k_u value may be overestimated, and therefore the diet is an even greater source of metals. However, if the calculated k_u value does not include the first several hours of metal uptake, then the impact of metal storage within the fish and metal loss from the fish must be considered. As a result, long-term exposures will measure the net accumulation of metal, which is the balance between uptake and loss (Luoma and Rainbow 2005). The biokinetic model also assumes that the k_u calculated from a short-term exposure is constant over time; the sudden decrease in metal uptake after the first several hours of exposure shows this clearly is not the case.

METAL BIOAVAILABILITY FROM THE DIET

When investigating the bioavailability of ingested metals to fish, studies focus on the assimilation efficiency, defined as the percentage of metal which is physiologically taken up across the gut lining during the processing of a single meal. Through the use of radioisotopes scientists can easily monitor metal uptake and loss using a single pulse-chase method. As Chapters IV, V, and VII shows there is a very large interelemental variation in AEs (0.2% for Cr to 92% for MeHg), AEs vary depending on the type of prey consumed, and intraspecies variability in AEs was noted for the Atlantic silverside (Cd, Hg(II)).

The large interelemental variation in AEs and the difference in AEs among the prey types can be explained by the distribution of metal within the prey. In Chapter IV, AEs were calculated for killifish after feeding on amphipod and oligochaete prey. Cd and Hg(II) AEs were higher after feeding on oligochaetes. This can be attributed to the difference in body partitioning of metal within the prey and the ability of the killifish to digest it. A soft-bodied worm is easier to digest, whereas the amphipod has a chitinous exoskeleton which the killifish can't digest, and therefore the metals bound to it are not bioavailable. The chosen prey was radiolabeled using different methods; amphipods were radiolabeled via a dietary exposure and oligochaetes via an aqueous exposure. The deposit-feeding polychaete *Nereis succinea* was originally chosen for these experiments and was radiolabeled via a sediment exposure, however, the killifish would not eat them and if the polychaetes were cut up into smaller pieces the radioisotopes would leach into the water. Due to these logistical constraints oligochaetes were fed to killifish instead. As a result of the different labeling techniques, the AEs in killifish could be influenced by different body partitioning within the prey as observed in another study where grunt (*Terapon jarbua*) were fed different prey items (Zhang and Wang 2006). Ng and Wood (2008) investigated the subcellular distribution of waterborne Cd in oligochaetes and concluded that 72 to 80% of the Cd was associated with the trophically available fraction (metallothionein-like proteins, heat denatured proteins, and organelles) which is bioavailable to fish.

In Chapter V, killifish were intubated with radiolabeled algae, which is an important food source in salt marshes (Kneib 1986; McMahon et al. 2005). The AE of each metal in killifish following algal intubation was related to the percentage of metal associated with the algal cytoplasm. The low algal Cr AE value (0.7%) in killifish can be attributed to >98% of Cr being bound to algal surfaces (Wang and Fisher 1996), which should not be bioavailable to killifish.

The difference in algal AEs for Hg(II) and MeHg is a result of a larger proportion of MeHg being associated with the algal cytoplasm which is assimilable, whereas Hg(II) was predominantly associated with the cell surface (Pickhardt and Fisher 2007). The As AE following algal intubation (15%) was higher than after killifish fed on amphipods (9.4%). This is important for killifish residing in salt marshes for two reasons; first, they assimilate more As when feeding on an algal diet and a study has shown that plant and algal matter accounts for 78% of the gut content of killifish residing in salt marshes (McMahon et al. 2005). Second, AE only takes into account the percentage of As which crosses the gut lining and not the concentration in the prey. Studies have shown that As biodiminishes with increasing trophic level in freshwater and estuarine food webs (Lindsay and Sanders 1990; Chen and Folt 2000), so an algal diet not only results in a higher AE in fish, but also has a much higher concentration of As. Therefore, algae could provide a significant dietary source of As to fish in salt marshes. It is clear from Chapters IV and V, and prior studies using a range of aquatic organisms, that for metals to be assimilated they must first be solubilized from the ingested food in the gut, and secondly the solubilized metal must be present in a biologically available form for transport across the gut lining (Leaner and Mason 2002a, b). For example, MeHg once solubilized in the fish's gut is taken up across the gut lining bound to cysteine (Leaner and Mason 2002a).

Future studies focusing on the influence of prey type on the AE of ingested metals need to consider a wider diet, fish size and therefore age, and whether there is seasonal variability in AE values. Chapters IV and V only calculated AEs after killifish ate amphipods, oligochaetes, and algae. However, killifish consume a varied diet, which includes copepods, polychaetes, gastropods, insects, larval fish, foraminifera, and detritus (Kneib 1986; McMahon et al. 2005), in addition to the prey types investigated throughout this dissertation, and AEs would be expected to vary with the type of prey consumed. To only use one prey item to calculate the AE for the biokinetic model may lead to an over- or underestimation of this kinetic parameter. Ideally, AEs should be calculated for a range of prey items and combined with gut content analysis to accurately predict the AE used in the biokinetic model. Furthermore, the type of prey consumed changes as the fish ages (Kneib 1986) due to different dietary requirements and the ability to catch larger prey as body size increases. Therefore, the AE could vary throughout the life cycle of the fish. Lastly, AEs could show seasonal variability as the food sources change, as demonstrated by stable isotope analysis (Kneib et al. 1980; Perga and Gerdeaux 2005). This may

change the AE value in the fish if the dominant prey type consumed varies throughout the year, and the importance of dietary versus aqueous exposure as a source of metals to killifish as a result. Killifish need to be collected throughout the year for gut content analysis and stable isotope analysis, and the data combined with the AEs calculated for different prey items, to investigate whether the AE changes significantly throughout the year.

SEDIMENT AS A SOURCE OF METALS TO KILLIFISH

One very interesting finding of this dissertation is that sediment-bound metals are not directly bioavailable to killifish (Chapter V), including MeHg (AE = 10 to 14%), even though sediment is a major repository for metals and provides a significant source of metals to other benthic organisms, including polychaetes and bivalves. While killifish do not actively consume sediment, some may accidently be ingested if attached to benthic prey. The AEs calculated for killifish are lower than those calculated for bivalves and polychaetes (Gagnon and Fisher 1997; Wang et al. 1997, 1998, 1999; Griscom et al. 2000, 2002a; Baumann and Fisher 2011). This interspecies variation in the bioavailability of sediment-bound metal may be in part due to differences in the pH and gut fluid composition between these organisms. For metals to be solubilized from sediment the gut fluid needs to be fairly acidic (pH 5-6) and have a high concentration of amino acids or surfactants (Mayer et al. 1996; Ahrens et al. 2001). While this is an interesting finding for killifish, it may not be applicable to all bottom-feeding fish. Killifish do not have a stomach and are therefore lacking glands which secrete pepsin and hydrochloric acid (Babkin and Bowie 1928). Digestion occurs under alkaline conditions in killifish (Babkin and Bowie 1928; Targett 1979). Other fish which do have a stomach and can secrete hydrochloric acid may assimilate more sediment-bound metal due to a lower pH gut fluid.

Due to ocean acidification resulting from global climate change, the pH of the ocean is starting to decrease. This will have a large impact on the oceans including a shift in the seawater carbonate chemistry, a reduced ability for shell-forming planktonic and benthic organisms to secrete their shells, and changes in the chemical speciation of seawater and biogeochemical cycling (Doney et al. 2009). Interestingly, one recent study calculated that marine fish precipitate $CaCO_3$ in their intestines and the excreted $CaCO_3$ accounts for 3 to 15% of the oceanic $CaCO_3$ production (Wilson et al. 2009). The authors predict that future $CaCO_3$ excretion from fish will

increase with increasing seawater temperature and CO_2 concentration associated with global warming. These findings can potentially influence the rate of future ocean acidification. Future studies are needed to investigate whether metals will begin to dissociate from sediment if the pH drops and what impact this potentially new pool of bioavailable metal will have on estuarine organisms. Laboratory experiments are needed to investigate whether the sediment-bound metal will become more bioavailable to amphipods, polychaetes, and bivalves, and if the metals could be released in to the overlying watercolumn where they could be taken up by fish, either from the dissolved phase, or through the planktonic food chain. Studies in freshwater systems investigating how a decrease in water pH influences metal bioavailability for aquatic organisms can provide an indication of what may happen in estuarine and marine environments. Freshwater systems, unlike marine systems, cannot buffer the pH of water due to the low concentration of carbonate. Hg bioavailability in freshwater systems increases as pH decreases (Chen et al. 2005) and studies investigating the influence of decreasing pH on metal accumulation in freshwater organisms have conflicting results; two studies concluded that Hg uptake increased with decreasing pH (Barkay et al. 1997; Watras et al. 1998), while another study concluded that Hg uptake decreased with decreasing pH (Fjeld and Rognerud 1993). A change in pH in the water will influence the pH of pore water in sediment, and potentially the bioavailability of metals from sediment.

Metals can accumulate in sediment in industrialized coastal regions. Prior studies (e.g. Mason et al. 2006) have concluded that metals bind to sediment under hypoxic or anoxic conditions and are released from sediment under oxic conditions. With increasing nutrient concentrations and increasing water temperatures in estuarine waters leading to hypoxia, this could have important implications on the bioavailability of metal-bound sediment. Prior studies using deposit-feeding polychaetes and bivalves have concluded that metal bioavailability to benthic organisms is dependent on whether the sediment is oxidized or reduced. The deposit-feeding polychaete, *Nereis succinea*, assimilated less Ag, Cd, Co, Hg(II), Se, and Zn when fed anoxic sediment than when fed oxic sediment (Wang et al. 1998, 1999). In anoxic sediments, a greater proportion of the metal is bound to the insoluble sulfide fraction, and is therefore less bioavailable to polychaetes. The bioavailability of metals bound to oxic and anoxic sediment varies by species for marine bivalves. The clam (*Macoma balthica*) assimilated less Ag, Cd, and and Co when fed anoxic sediment, whereas the mussel (*Mytilus edulis*) assimilated more Cd, Co,

and Cr when fed anoxic sediment (Griscom et al. 2000). The expected increase in MeHg production associated with climate change is of particular concern; methylation rates are temperature dependent (Downs et al. 1998), potentially leading to higher concentrations of MeHg in the sediment and water column as ocean temperatures rise, and therefore increasing the exposure level of MeHg to pelagic and benthic organisms.

The availability of sediment-bound metal experiments investigated in Chapter V using anoxic sediment from Baltimore Harbor (MD), Elizabeth River (VA), and Mare Island (CA) are representative of natural sediment conditions. Sediment from Baltimore Harbor and Elizabeth River is heavily contaminated with metals (Hall and Alden 1997; Mason and Lawrence 1999; Hall et al. 2002; Conrad and Chisholm-Brause 2004; Mason et al. 2004; Shafer et al. 2004; Graham et al. 2009). At the three field locations, the oxic sediment layer only accounted for the top 1 cm (Mare Island) to 4 cm (Baltimore Harbor) of the surface sediment and all the sediment beneath was anoxic. After the sediment was radiolabeled, it was left to age for 1 week in a sealed container, and the fish were then intubated with the sediment. Benthic organisms, including bivalves and deposit-feeding polychaetes will burrow to depths greater than 4 cm in the natural environment, so the sediment attached to their body surface will be anoxic, and potentially ingested by killifish while consuming benthic prey.

UNDERSTANDING METAL EFFLUX FOLLOWING DISSOLVED AND DIETARY EXPOSURE

Metal efflux rates following aqueous or dietary exposure are relatively consistent among the metals and under different uptake conditions. Prior studies have focused on metal uptake from aqueous and dietary exposure to metals, and little attention has been given to what influences the rate of metal loss from fish. Depuration curves showing metal loss from fish over time normally show a biphasic pattern; the initial rapid loss of metal throughout the first day, and then the slower physiological turnover of metal for the remainder of the depuration period; the latter of which is used to calculate the k_{ew} or k_{ef} values. What influences k_{ew} and k_{ef} is poorly understood compared to k_u and AE for two reasons. Firstly, it is easier to understand what influences k_u and AE because metal uptake essentially occurs at one site (gills and gut, respectively), whereas k_{ew} and k_{ef} is essentially an integration of the efflux rate of metals from

different tissue compartments, each of which have their own turnover rate. Secondly, it is difficult to do, but advances have been made due to the use of a radiotracer technique (e.g. Fisher et al. 1996; Hogstrand et al. 2003). In Chapters IV and VI, I attempted to understand what influences k_{ef} and k_{ew} following dietary and aqueous exposure, respectively. This information helps provide texture to the overall whole-fish efflux rate values. However, the tissue specific efflux rate constants do not take into account reabsorption between different tissue compartments e.g. bilary excretion from the liver to the intestine, reabsorption of metals in the kidneys, and redistribution of metals between different tissues via the blood, which will influence whole-fish k_{ef} and k_{ew}.

Following dietary exposure, Cd and Hg(II) remained primarily associated with the intestine in killifish, whereas As and MeHg readily crossed the intestinal wall and were redistributed around the body. The intestine had a higher efflux rate of As and MeHg compared to Cd and Hg(II) (Chapter IV). Following aqueous exposure, the liver was shown to have an important role in the processing and excretion of metals, presumably via the bile, and MeHg was shown to accumulate in the brain, eyes, and fillet in killifish (Chapter VI).

It is harder to experimentally calculate the efflux rates of metals from different tissue compartments following dietary exposure than following aqueous exposure. To calculate an efflux rate from each tissue compartment, the mean radioactivity concentration must be converted into a percentage at certain time points throughout depuration. Efflux rates from individual tissue compartments following aqueous exposure are easier to calculate because each fish is exposed to the same concentration of metals in the water throughout uptake. During dietary experiments, although the fish are offered the same amount of food, individual fish will eat different amounts, and therefore the total radioactivity concentration in each fish will vary. As a result, the mean radioactivity concentrations cannot be converted into percentages throughout depuration, and hence efflux rates from individual tissue concentrations following dietary exposure could not be calculated in Chapter IV.

THE IMPORTANCE OF INGESTION RATE AND GROWTH RATE

The rates of physiological processes (ingestion rate, growth rate) have a significant influence on metal accumulation in fish. Juvenile fish have a higher ingestion rate to assimilate

more carbon from food, which enables them to grow faster and add more body mass daily. A study by Zhang and Wang (2007b) concluded that ingestion rate was the most important parameter when investigating metal accumulation in fish as a function of size. Ingestion rate is negatively correlated with fish size (Zhang and Wang 2007b) and the AE of metals is negatively correlated with ingestion rate (Xu and Wang 2002) due to a faster gut passage time at higher ingestion rates. Based upon these two correlations, the AE of metals in fish should decrease as the fish ages, which could be attributed to a slower growth rate and less ingested carbon being converted into biomass.

The influence of growth rate is normally not considered in the metal bioaccumulation model because the growth rate is negligible in comparison to the metal efflux rate (k_{ew}, k_{ef}). However, if growth is rapid, particularly in juvenile organisms then it must be considered in the model (Luoma and Rainbow 2005). Rapid growth occurs in response to consuming high quality food (Karimi et al. 2007; Mitra et al. 2007). A rapid increase in tissue biomass can result in growth dilution of metals, as observed in freshwater zooplankton and other aquatic invertebrates (Karimi et al. 2007, 2010), and Atlantic salmon (*Salmo salar*; Ward et al. 2010). Growth dilution has a greater impact on metal bioaccumulation for metals which have a slow efflux rate from organisms, e.g. MeHg, than for metal which have a higher efflux rate, e.g. Cr, because the growth rate exceeds the efflux rate. If the principle of growth dilution is ignored in fast growing juvenile fish then the body concentration of metals will be overestimated.

Chapter VII investigated metal uptake and loss from aqueous and dietary sources in two populations of Atlantic silversides (Nova Scotia and South Carolina) with differing rates of physiological processes. This is a unique situation where intraspecific comparisons can be made within a species because different populations have different rates of ingestion and growth, both variables of which have been fully addressed in bioaccumulation studies. Nova Scotia silversides, found at the northern end of the species range, have higher rates of ingestion and growth due overcome a shorter growing season and size-selective winter mortality, compared to South Carolina silversides which are at the southern end of the species range. For all investigated metals, except MeHg, AEs were higher in fish from South Carolina. This was attributed to a lower ingestion rate which resulted in a longer gut passage time, and therefore greater solubilization of metal from the prey. It was also noted that South Carolina silversides always had a higher k_u compared to Nova Scotia silversides, due to their smaller body size and therefore

higher surface area to volume ratio. When the kinetic parameters calculated in laboratory studies (k_u, AE, k_{ew}, k_{ef}) were entered into the biokinetic model, fish from South Carolina accumulated more Am, Cd, and Zn than fish from Nova Scotia. In Nova Scotia fish, the rapid growth to overcome the shorter growing season also results in a lower bioaccumulation of metals. This study concludes that the importance of ingestion rate and growth rate should not be overlooked in bioaccumulation studies. However, the growth rate is not a fixed value in juvenile fish; as fish age, the rate of growth slows down and therefore growth dilution will have less influence on the body burden of metals.

Water temperature controls the rate of physiological processes and metal accumulation in aquatic organisms, and can therefore be considered an important environmental factor that governs metal body burdens. Two studies investigated the influence of temperature and salinity on the toxicity of metals to estuarine organisms: Bryant et al. (1985) determined that the mean survival time of the amphipod *Corophium volutator*, bivalve *Macoma balthica*, and oligochaete *Tubifex costatus* exposed to aqueous As decreased as temperature and As concentration increased, and Voyer and Modica (1990) determined that the LC50 of Cd in the mysid shrimp *Mysidopsis bahia* decreased with an increase in temperature and decrease in salinity. Baines et al. (2005) investigated the influence of temperature (2°C and 12°C) on the accumulation of dietary metals by the mussel *Mytilus edulis* collected from the Arctic (north coast of Norway) and temperate region (Long Island Sound). Mussels generally assimilated more metal at 2°C than at 12°C and this was attributed to the digestive processing time and metabolic rate of the organism.

The growth rate of juvenile Atlantic silversides increases with latitude to overcome a shorter growing season (Conover and Present 1990). Laboratory experiments investigating the growth rate of Nova Scotia (NS) and South Carolina (SC) silverside populations determined the growth rate is temperature dependent and the greatest difference in growth rate between the two populations is observed at 21°C (0.59 and 0.31 mm d^{-1}, respectively), compared to 15°C and 28°C (0.22 and 0.15 mm d^{-1}, and 0.55 and 0.34 mm d^{-1}, respectively) (Conover and Present 1990). The temperature the experiments were conducted at in Chapter VII will influence the accumulation of metals and the importance of growth dilution between the two populations. Experiments were conducted at 21°C due to this temperature having the greatest difference in growth rate between the two populations and both populations of silversides are exposed to this

water temperature in the wild (Conover and Present 1990). If these experiments were conducted at 28°C, growth dilution would be expected to have a reduced effect on the body burden of metals and less of a difference would be noted between the two populations, whereas at 15°C there would be no significant difference on the body burden of metals as a function of growth rate in the two Atlantic silverside populations because the growth rates are similar.

TISSUE DISTRIBUTION OF METALS FOLLOWING AQUEOUS AND DIETARY EXPOSURE

This dissertation concludes that the tissue partitioning of metal in fish varies by metal and exposure route. Following dietary exposure the primary uptake site is the intestine. Whether a metal is redistributed around the body via the blood to other tissue compartments is determined by whether the metal can cross the intestinal wall. Studies using killifish have shown that Cd and Hg(II) remain associated with the intestine following dietary exposure, whereas As and MeHg is redistributed around the body and is predominantly associated with the fillet (Chapters IV and V). The accumulation of metals in the fillet potentially poses public health consequences if the same tissue distribution is observed in commercial fish. As(V) and MeHg cross the intestinal wall because they bind to or are an analog for other biologically essential compounds; As(V) is a PO_4 analog and shares the same uptake pathway (Sanders and Windom 1980), whereas MeHg binds to cysteine and is taken up as a MeHg-cysteine complex (Leaner and Mason 2002a).

The gills are considered to be the predominant uptake site during an aqueous exposure (Chapter II, III, and VI). However, the head and skin also accumulates metals and should be considered important uptake sites. The head contains the opercular flap, a hardened bony structure which protects the gills. Estuarine fish are hypoosmotic to the surrounding water. There is a passive influx of salt into the body and a passive loss of water. Estuarine fish replace the water that is lost through actively drinking water, whereas the accumulation of salt (NaCl) is actively lost through the secretion of salt at the gills. The opercular epithelium is involved in maintaining the internal salt balance in freshwater and marine fish and therefore has an important role in osmoregulation (Wood and Laurent 2003; Scott et al. 2005). The opercular flap may be an important uptake site for metals as well, but further studies are warranted. The opercular epithelium may account for 50% of the Ca^{2+} uptake in fish, so could possibly be an important

uptake site for Cd as both elements share the same uptake pathway (CM Wood, personal communication). Metals bound to the skin may be attached to the mucus or the scales which have been shown to accumulate metals (Varanasi et al. 1975; Coello and Khan 1996). Mucus is known to be the fish's first protective barrier to metal exposure; in one experiment which was not reported in this dissertation, I accidently added too much MeHg to the water and within 30 minutes the killifish were shedding excessive amounts of mucus to try and reduce their body burden.

FINAL CONCLUSION

The body burden of metals in small forage fish is a result of the rate of metal uptake and loss following aqueous and dietary exposure. This dissertation provides further insight into how water chemistry, fish physiology, and prey type influences the overall metal accumulation in fish and the importance of aqueous and dietary exposure routes as a source of metals to fish.

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