

ABSTRACT

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METHYLMERCURY UPTAKE AND
MATERNAL TRANSFER IN ESTUARINE
FORAGE FISH

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Dietary methylmercury (MeHg) uptake in relation to fish lifestage, species, and level of exposure are poorly understood in lower trophic levels, particularly in estuarine species. Furthermore, little is known about the transfer of accumulated MeHg from female to offspring. Dietary MeHg accumulation, as well as growth and survival, were compared in two species of estuarine forage fish: *Cyprinodon variegatus* and *Menidia beryllina*. Results indicated that *M. beryllina* was more sensitive to dietary MeHg exposure than *C. variegatus*. Growth rate and the level of dietary exposure strongly influenced MeHg tissue concentrations in both species. In a second experiment, the source of maternally transferred MeHg was examined using a stable mercury isotope approach. A significant portion of Hg in eggs was from the burden stored in female tissues, suggesting that historical mercury exposure can be important in the context of maternal transfer.

DYNAMICS OF DIETARY METHYLMERCURY UPTAKE AND MATERNAL
TRANSFER IN ESTUARINE FORAGE FISH

By

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Chapter 1: Introduction

Background

Mercury (Hg) is a widespread contaminant that can undergo several transformations in the environment. Its transformation to an organic molecule, via methylation, makes mercury bioavailable and therefore a concern to ecosystem and human health. Although our understanding of mercury cycling and transport within ecosystems has greatly increased, many aspects of Hg bioaccumulation and trophic transfer are still poorly understood. This is particularly relevant to human consumption, as nearly all seafood contains detectable levels of methylmercury (MeHg). Methylmercury is a neurotoxin and endocrine disruptor, which at high levels can cause health problems, especially in developing fetuses (Fitzgerald & Clarkson 1991). Furthermore, although most Hg is emitted from industrial point sources, the long residence time of mercury in the atmosphere causes even remote areas to be affected by long-range atmospheric transport (Morel et al. 1998). Mercury is therefore a global concern.

Current regulations

Mercury pollution has been broadly regulated in the United States under the Clean Air Act, Clean Water Act, Resource Conservation and Recovery Act, and Safe Drinking Water Act. These regulations set standards for mercury levels in natural waters and drinking water, addressed mercury waste disposal, and established public warnings for mercury levels in fish (U.S. EPA, 2010). However, they failed to regulate the major anthropogenic source of mercury: coal-fired power plants.

In 2005, U.S. EPA implemented the Clean Air Mercury Rule, which is their first attempt to regulate mercury emissions from coal-fired power plants. The main goal was to reduce Hg emissions by 70 %, using a cap-and-trade program similar to that in the Acid Rain Program. An initial cap has already been put into effect for existing plants, and a second cap is planned for the year 2018 (U.S. EPA, 2010). Some mercury regulations have also been passed at the state level. For example, in 2009 Maryland passed the Healthy Air Act, which requires reductions in power plant emissions of mercury and other air pollutants.

Overview of Hg in the environment

Sources and fate of inorganic Hg

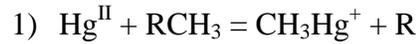
A majority of mercury entering the environment is in an inorganic form, and later transformed into highly bioaccumulative organic compounds, such as MeHg. Therefore, inorganic mercury is important to consider as a precursor to more toxic forms of organic mercury. Inorganic, elemental Hg enters the atmosphere as a byproduct of industrial processes, mainly coal-fired power plants (Wang et al. 2004; Figure 1.1). A large portion of mercury also comes from natural sources, such as volcanic emissions, crustal degassing, and forest fires (Fitzgerald & Clarkson 1991). Approximately 80 percent of atmospheric mercury is gaseous, and exists in its elemental form (Hg^0), while the remaining fraction is either particulate or aqueous (Wang et al. 2004). Elemental mercury is oxidized, usually by ozone, hydroxyl radicals, or halogens in the atmosphere, creating a more reactive form of inorganic mercury: Hg^{II} (Lindberg et al. 2007; Figure 1.1).

Atmospheric mercury is deposited to both aquatic and terrestrial systems (Figure 1.1). Approximately 60 percent of atmospheric mercury is transferred to land, while the other 40 percent is deposited to water (Morel et al. 1998). Both wet and dry depositions contribute to mercury loading. A majority of atmospheric deposition is dissolved Hg^{II} in precipitation (wet deposition), while a smaller fraction adsorbs to aerosols and moves via dry deposition (Morel et al. 1998). Atmospheric deposition is thought to be the main source of mercury contamination in aquatic ecosystems (Wang et al. 2004).

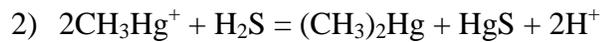
Methylation

In order for mercury to bioaccumulate, it must first be transformed into a bioavailable form of organic mercury. The most common of these compounds is methylmercury. Unlike Hg^0 and Hg^{II} , which predominantly enter watersheds via atmospheric deposition, the main source of MeHg in watersheds is *in situ* production (Munthe et al. 2007). Methylation of mercury occurs under reducing conditions. Sulfate-reducing strains of anaerobic bacteria produce a majority of methylmercury in ecosystems (Gilmour et al. 1992). Photochemical processes, fueled by humic acids, can also methylate mercury. Therefore methylation is common in anoxic environments containing high levels of organic matter. Wetlands and marshes, as well as anoxic bottom waters are sites of high MeHg production. For example, wetlands have been shown to significantly increase MeHg and dissolved organic carbon (DOC) concentrations, while decreasing sulfate in upstream waters (Selvendiran 2008). This causes a net transport of MeHg to downstream aquatic systems.

Mercury must be in its reactive inorganic form (Hg^{II}) in order for methylation to occur (Stein et al. 1996). It can then undergo the following transformation to monomethylmercury:



Additionally, this monomethylmercury can undergo another transformation to dimethylmercury:



The rate of reaction (1) is much faster than that of reaction (2). Therefore, in most systems, monomethylmercury makes up over 95 % of the total methylmercury (Stein et al. 1996).

Bioaccumulation of Hg

Mercury concentrations in the water column, usually on the order of parts per trillion, can translate to concentrations of parts per million in the tissues of high-level consumers. Bioaccumulation of mercury in organisms is affected by a variety of factors, including food web structure, population age structures, and growth rates (Munthe et al. 2007). The transfer of MeHg to higher trophic levels is dependent on bioavailability of mercury to lower trophic levels, such as plankton and benthic invertebrates. In addition, environmental factors play a role in uptake of MeHg. For example, dissolved organic matter (DOM) influences bioavailability. MeHg bound to DOM is less available to phytoplankton (Chen et al. 2008). Additionally, pH and chloride content of natural waters affect speciation of MeHg, and in turn the amount of partitioning to phytoplankton, based on differences in the octanol-water partition

coefficients (K_{ow}) of these compounds (Mason et al. 1996). This is supported by studies that have measured increased levels of MeHg in zooplankton from acidified lakes, compared to reference lakes (Watras & Bloom, 1992).

Bioaccumulation in plankton

Marine diatoms exposed to both inorganic and organic mercury compounds have demonstrated that mercuric compounds with a higher K_{ow} have higher membrane permeabilities and in turn, greater bioaccumulation (Mason et al. 1995). It has also been found that the assimilation efficiency of MeHg from phytoplankton to zooplankton is four times greater than that of inorganic mercury (Mason et al. 1995). Measuring partitioning of mercury within phytoplankton cells showed that a majority of MeHg is sequestered in the cytoplasm, while only 9 % of inorganic Hg is stored in the cytoplasm. This suggests that inorganic mercury is sequestered in cellular membranes, due to its high affinity for the functional groups present, particularly thiols (Mason et al. 1995). Methylmercury compounds are less reactive with membrane functional groups and more are sequestered in the cell cytoplasm, where they can be easily assimilated. This difference in assimilation is due to the fact that zooplankton digest the cytoplasm and excrete the surrounding membrane material. Therefore, the high biomagnification of organic mercury compounds, relative to inorganic species, can be partially explained by differences in their partitioning within cells (Mason et al. 1995).

Studies focused on MeHg uptake in algae have also found species-specific differences (Miles et al. 2001). Phytoplankton-water partition coefficients for methylmercury are significantly higher in eukaryotic species than in the prokaryotic

species. It is hypothesized that the presence of membrane-bound organelles in eukaryotic phytoplankton allowed for additional binding of MeHg within the cell (Miles et al. 2001). This demonstrates that partitioning of methylmercury from water to biota is not only influenced by environmental conditions, but also by physiological characteristics of the organism itself.

Bioaccumulation in fish

As with zooplankton, the amount of MeHg stored in the cytoplasm of ingested algae is directly related to assimilation efficiency in herbivorous fish. This is also true for other food sources. In general, metals bound to the soluble organic portion of prey organisms are easily assimilated (Meyer et al. 2005). Diet is the major source of MeHg exposure in fish (Phillips & Buhler 1978; Hall et al. 1997). A portion of ingested Hg is transferred across the gut wall to the bloodstream and circulated throughout the body (Leaner & Mason 2004). A majority of this assimilated MeHg is stored in muscle tissues (Leaner & Mason 2004).

Many field-based studies have measured Hg concentrations in wild fish populations, to determine the effect of fish age, size, and environmental conditions on Hg accumulation. Mercury concentrations can vary widely among species and over time. For example, in Maryland Reservoirs young of the year white perch (*Morone americana*) accumulate Hg rapidly, achieving whole-body concentrations of 10 ng g⁻¹ ww in less than 3 months (Heyes 2011). Young of the year largemouth bass (*Micropterus salmoides*) can achieve concentrations of 250 ng g⁻¹ ww in the same time period (Heyes 2011). These concentrations can be an order of magnitude higher upon reaching reproductive age. However, neither the larval mercury concentration

(maternally transferred Hg), nor how subsequent accumulation proceeds through development is well known.

Much research has focused on the physical, chemical, and biological factors influencing methylmercury partitioning and bioaccumulation at the base of the food chain. These controls are important, as they ultimately determine MeHg concentrations in higher trophic levels. Many studies have also focused on high-level consumers, such as piscivorous fish, aquatic birds, and marine mammals. However, there is relatively little knowledge of Hg accumulation dynamics and thresholds in fish of lower trophic status.

Physiological effects of MeHg

MeHg as a neurotoxin

Hg primarily affects the central nervous system, causing degeneration of neurons. In severe cases of human exposure, specific areas of the brain are damaged, causing loss of sensory and balance, as well as decreased motor function (Fitzgerald & Clarkson 1991). Hg exposure has also been shown to cause more subtle changes in the neurological function of vertebrates. These include impaired behaviors related to foraging, predator avoidance, and reproduction in species of amphibians and fish (Burke et al. 2001; Webber & Haines 2003; Alvarez et al. 2006).

MeHg as an endocrine disruptor

The endocrine system regulates physiological processes, such as reproduction, metabolism, development, and digestion via hormones and enzymes. Recent studies have shown endocrine disruption in fish as a result of MeHg exposure. Several

laboratory dosing experiments have reported decreases in fish growth (Friedman et al. 1996; Houck & Cech 2004; Lee et al. 2011), possibly due to decreased levels of cortisol and thyroid hormone (Friedman et al. 1996) or interference with gastrointestinal function and decreased nutrient absorption (Lee et al. 2011).

Changes in reproductive success related to dietary MeHg exposure have also been found in laboratory dosing studies. After exposure to dietary MeHg, male fathead minnows showed significantly suppressed levels of testosterone, while females showed decreased levels of 17 β -estradiol and lower gonadosomatic index (Drevnick and Sandheinrich 2003). Additionally, spawning success was significantly lower in pairs fed MeHg diets. These changes in sex hormone levels are evidence that methylmercury can act as an endocrine disruptor, and in turn decrease reproductive fitness.

Vitellogenin gene expression has been shown to significantly decrease in female fathead minnows following dietary MeHg exposure (Klaper et al. 2006). Since vitellogenin is an essential yolk precursor protein, these changes likely have downstream effects on egg production. This coincides well with suppressed 17 β -estradiol levels (Drevnick & Sandheinrich 2003) and decreased reproductive effort (Hammerschmidt et al. 2002) found in exposed female minnows. It has been hypothesized that MeHg may bind to estrogen receptors, acting as an estrogen mimic that decreases natural hormone levels (Klaper et al. 2006). A subsequent study by Drevnick et al. (2006) showed that dietary MeHg exposure in fathead minnows significantly increased the occurrence of apoptosis in ovarian follicular cells. These cells are responsible for producing 17 β -estradiol and other sex steroid hormones.

Therefore increased apoptosis by MeHg may also be a mechanism of reduced hormone production (Drevnick et al. 2006).

Maternal transfer of MeHg

While diet is the major source of MeHg in adult fish (Phillips & Buhler 1978; Hall et al. 1997), maternal transfer has been shown to be a significant route of exposure for larval and juvenile fish (Latif et al. 2001; Alvarez et al. 2006). Latif et al. (2001) compared walleye populations from a contaminated and relatively uncontaminated lake. Walleye eggs from both lakes were exposed to varying aqueous MeHg concentrations. Larvae from the contaminated lake had much higher MeHg concentrations, regardless of waterborne mercury exposure, suggesting that MeHg in fish larvae is significantly affected by maternal exposure.

The source of this maternally transferred Hg is still poorly understood. It was originally thought that MeHg partitioned from stores in female tissues into developing oocytes (Niimi 1983). However, a more recent study by Hammerschmidt and Sandheinrich (2005) indicated that egg mercury content was a reflection of the maternal diet during oogenesis, rather than Hg stored in female tissues. Understanding the mechanism and source of this maternally transferred Hg is necessary in order to more accurately assess offspring exposure during sensitive early lifestages.

Rationale

There is limited research on the response of fish occupying lower trophic levels to MeHg exposure. These organisms are important links between producers at the base of the food chain and high-level consumers. The effects of variables such as

fish age, species, and dietary history on MeHg uptake is poorly understood, particularly in estuarine systems. Furthermore, we know very little about the transfer of this accumulated Hg to offspring. It is important to evaluate the resiliency of species to MeHg exposure based upon both lethal and sublethal endpoints, such as growth. Despite the importance of marine fisheries to human society, a majority of research has focused on freshwater fish. There is a need to study other species, with varying life histories, particularly those that inhabit saltwater environments.

This research consisted of two separate dosing studies. Chapter 2 presents findings from an experiment which sought to compare the sensitivity of two estuarine forage fish species (*Cyprinodon variegatus* and *Menidia beryllina*) to dietary MeHg exposure, as well as characterize uptake from juvenile to adult life stages. Chapter 3 presents a second dosing study which used a stable mercury isotope approach to determine the source of maternally transferred MeHg in the sheepshead minnow (*Cyprinodon variegatus*).

Terminology

In the following chapters, several terms are used to distinguish among types of mercury. T-Hg refers to total mercury, which includes all inorganic and organic forms. MeHg refers to monomethylmercury. In Chapter 3, “ambient mercury” is used to describe the mercury fed to fish during stage 1 of dosing. ^{199}Hg is the enriched stable isotope fed to fish during the second period of dosing. Both ambient Hg and ^{199}Hg were measured as T-Hg, however it is assumed that a majority of this is methylmercury, as this was the only form of mercury added to fish diets.

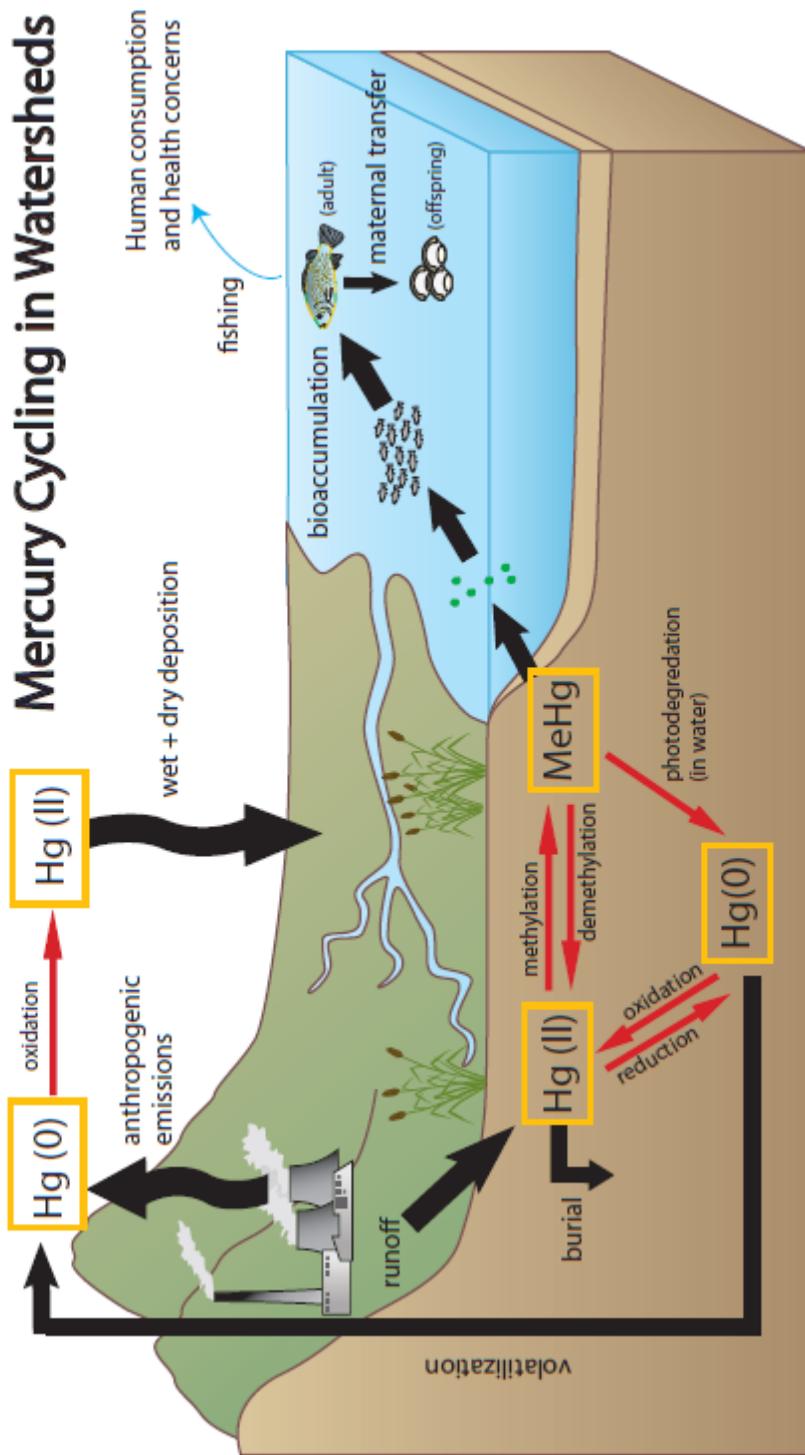


Figure 1.1. Conceptual diagram of mercury cycling in the environment.

Chapter 2: Uptake of dietary methylmercury by two estuarine fish: the sheepshead minnow (*Cyprinodon variegatus*) and inland silverside (*Menidia beryllina*)

Introduction

Background

Mercury is a widespread contaminant that is of concern to both ecosystem and human health. Natural and anthropogenic sources release elemental Hg into the atmosphere (Fitzgerald & Clarkson 1991; Wang et al. 2004), which is oxidized and deposited to terrestrial and aquatic systems as Hg^{II} (Lindberg et al. 2007). This reactive form of mercury can then undergo several transformations in the environment, including methylation by bacteria. Sulfate-reducing strains of anaerobic bacteria produce a majority of methylmercury (MeHg) in ecosystems (Gilmour et al. 1992). This transformation is most prevalent in anoxic sediments having high organic matter contents, such as in wetlands and marshes. As marshes comprise a substantial component of many estuarine ecosystems and serve as nursery habitat, small fish may be exposed to significant concentrations of MeHg (Mitchell and Gilmour 2008).

Methylmercury accumulates in food chains, reaching potentially harmful concentrations in upper trophic levels. Aside from humans, animals occupying high trophic positions in aquatic food webs, such as piscivorous birds, are highly susceptible (DesGranges et al. 1998; Adams and Frederick 2008). Although it is important to understand mercury accumulation and toxicity in species of high trophic position, Hg exposure in these organisms is ultimately determined by their prey

(Phillips & Buhler 1978; Hall et al. 1997). Yet the behavior of mercury in the base of the food chain is poorly understood, particularly in fish of low trophic status.

Many field-based studies have measured Hg concentrations in wild fish to determine the effect of fish age, size, and environmental conditions on Hg accumulation. Mercury concentrations can vary widely among species and over time. For example, in Maryland Reservoirs young of the year white perch (*Morone americana*) accumulate Hg rapidly, achieving whole-body concentrations of 10 ng g⁻¹ ww in less than 3 months (Heyes 2011). Young of the year largemouth bass (*Micropterus salmoides*) can achieve concentrations of 250 ng g⁻¹ ww in the same time period (Heyes 2011). These concentrations can be an order of magnitude higher upon reaching reproductive age. However, neither the larval mercury concentration (maternally transferred Hg), nor how subsequent accumulation proceeds through development is well known.

It is difficult to tease apart processes that control Hg accumulation since the dietary history of field-collected fish is unknown. This is a particularly important variable to consider, as previous studies have shown that diet is the primary pathway of Hg uptake (Phillips & Buhler 1978; Hall et al. 1997). It is difficult to accurately model or predict the level of Hg contamination in prey items. While some studies have examined mercury exposures based on Hg in fish stomach contents (Ward et al. 2010), such studies only address the proximate diet at the time of sampling, and do not necessarily reflect the diet over an individual's entire lifetime.

Laboratory dosing studies provide the opportunity to control and monitor dietary Hg exposure over time. Fish growth and development can also be easily

measured, allowing for evaluation of physiological effects. However, many laboratory dosing studies have measured mercury accumulation over only short time periods, on the order of hours or days (Leaner & Mason 2001; Leaner & Mason 2004), rather than addressing chronic exposure as would occur in natural habitats. Furthermore, most of these laboratory studies have used freshwater species as test organisms, and thus relatively little is known about mercury dynamics in estuarine and marine systems, where the majority of fisheries exploited for human consumption occur.

Study rationale

The relationship between dietary exposure and MeHg accumulation is poorly understood in fish of lower trophic levels, particularly in estuarine ecosystems. It is also unknown how this relationship varies among species. Therefore, two estuarine forage fish species were chosen as test organisms: the sheepshead minnow (*Cyprinodon variegatus*) and the inland silverside (*Menidia beryllina*).

Cyprinodon variegatus and *M. beryllina* inhabit shallow coastal waters along the Atlantic coast of North America from Massachusetts to Mexico (Murdy et al. 1997). Both species are abundant in estuarine marshes and serve as important prey for other vertebrates and macroinvertebrates. Sheepshead minnows are ideal for laboratory studies because of their small size, rapid maturation, and overall tolerance of laboratory conditions. Inland silversides also have rapid development, yet, relative to sheepshead minnows, are much more sensitive to abiotic conditions.

Objectives

The objectives of this study were to:

1. Compare species sensitivity to dietary MeHg with both lethal and sublethal endpoints (growth)
2. Compare accumulation of dietary MeHg between species, among levels of dietary exposure, and over different life stages

Methods

Experimental protocol

Food preparation

Methylmercury diets were prepared with methylmercury (II) chloride (Alfa Aesar). MeHg was incorporated into flake food via an agar/gelatin matrix. Gelatin, agar, flake food, and deionized water were combined in a mass ratio of 0.7:1:20:100 respectively. Agar and gelatin were added to boiling deionized water and stirred for one minute. This mixture was poured into a shallow Pyrex baking dish and mixed with pre-ground flake food. Aqueous MeHg was then added to the mixture at calculated volumes to achieve the following nominal concentrations: 1, 5, 10, and 20 $\mu\text{g g}^{-1}$ dw. After setting in a refrigerator overnight, food was cut into small slices and frozen at -80°C . Food was then freeze-dried and ground with a mortar and pestle to produce a fine, dry, flake mixture. Fish diets were stored at -4°C between feedings to minimize degradation of Hg concentrations. This storage did not significantly decrease food Hg content over the course of the experiment. Actual Hg concentrations averaged 65 % of the target concentrations (Table 2.1).

Husbandry

Fish were housed in 76 L aquaria at Chesapeake Biological Laboratory (Solomons, MD). All tanks were equipped with individual filters, heaters, and aeration to maintain water quality. Water temperature was maintained at approximately 26° C for the duration of the experiment. Filtered ambient river water (Patuxent River, MD, USA) was used, thus salinity varied naturally between 11 and 20 ppt over the course of the experiment. A 14:10 hour light:dark cycle was maintained throughout. Each tank received 1 g of food daily, which was confirmed to be an *ad libitum* regimen, as excess food remained prior to subsequent food additions. Tanks were cleaned weekly and water quality was monitored by measuring dissolved oxygen, temperature, salinity, conductivity, and pH weekly. Ammonia levels were also measured colorimetrically (A.P.I.) in a random subset of tanks periodically. Note that this husbandry protocol was approved by the University of Maryland Center for Environmental Science IACUC (protocol #S-CBL-10-03).

Fish were acquired from Aquatic Biosystems (Denver, CO, USA) at 14 days old and distributed to aquaria at a density of 12 fish per tank (0.16 individuals / L). All individuals were fed control food and acclimated to laboratory conditions for two weeks prior to the start of dosing. The 70-day dosing period spanned the juvenile to adult life stages of both species, beginning at 28 days old and ending at 98 days old.

Treatments and sampling design

Treatments consisted of a control diet and four diets with varying concentrations of methylmercury (see above). Each treatment contained 3 replicate

tanks for each species, totaling 30 aquaria. Treatments and species were randomly distributed among tank positions.

Fifteen individuals of each species were sacrificed at the beginning of the experiment to measure background Hg concentrations, as well as initial length and weight. During the dosing period, 3 individuals were sub-sampled from each tank at three time points: days 23, 46, and 70. Fish were held for 48 hours in separate aerated buckets, to clear gut contents. Each individual was then euthanized via cervical dislocation, measured for total length and wet mass, and frozen at -80° C for later T-Hg analysis. After 70 days of dosing, the experiment was terminated.

Water samples were collected from each tank every two weeks and analyzed for both T-Hg and MeHg to track partitioning of Hg within aquaria over time (see Appendix). Food was sub-sampled every two weeks and analyzed for MeHg to monitor any changes in concentrations due to storage. To extract MeHg from food, triplicate 1 g sub-samples of food were distilled in a solution of 20 mL Milli-Q water, 1 mL 50 % sulfuric acid, and 0.5 mL 20 % potassium chloride (Horvat et al. 1993). The distillate was then analyzed for MeHg (see below).

Sample preparation and analyses

Digestions

Fish carcasses were freeze-dried for 24 hours prior to digestion. Fish were then digested on a hot plate at 120 - 150° C for 6-9 hours, using 5 mL of 50:50 concentrated nitric acid:sulfuric acid. Digestions were done in 50 mL Erlenmeyer flasks with watch glass covers for ventilation. Digestions were considered complete when flasks were free of brown gas. Samples were cooled and diluted to 50 mL with

Milli-Q water. Samples were then oxidized with 1 mL of bromine monochloride (BrCl) and analyzed the following day. Exact dilution volumes were calculated by weight difference.

T-Hg analysis

Analysis of total mercury (T-Hg) was conducted on a Tekran Model 2600 Mercury Analyzer with a Model 2620 Autosampler (Tekran Instruments, Canada). The instrument measures T-Hg via cold vapor atomic fluorescence spectrometry (CVAFS). Briefly, all mercury species within the sample were reduced to elemental mercury (Hg^0) by stannous chloride (SnCl_2). Hg^0 was then concentrated on a gold trap, thermally desorbed, analyzed by CVAFS, and quantified according to EPA method 1631 (US EPA, 1996).

Prior to analysis, excess oxidant was neutralized in samples with 10 μl of hydroxylamine hydrochloride. T-Hg standards were prepared from a NIST stock solution in concentrations of 5, 10, 25, 50, 75, 100, 150, and 200 ng L^{-1} . A matrix blank was prepared, according to the type of samples being analyzed. When analyzing fish samples, the matrix blank consisted of 0.2 % digest acid and 0.08 % BrCl and when analyzing water samples, the matrix blank consisted of 0.5 % BrCl. Quality control included calibration blanks, replicate standards and samples, duplicate dilutions, and duplicate SRMs (DORM-2, National Research Council Canada).

MeHg analysis

Methylmercury analysis was conducted on a Tekran Model 2500 Mercury Analyzer (Tekran Instruments, Canada). Water samples were acidified with 0.5 % sulfuric acid one day prior to analysis and KOH was used to adjust pH between 3 and

9 the following morning. No additional preparation was necessary for distilled food samples. Aliquots of sample were added to bubblers containing a citrate buffer (pH = 4.8). The solution was ethylated with sodium tetraethylborate, converting MeHg to gaseous methylethylmercury (Bloom 1989). This was purged from solution and concentrated on Tenax traps. Methylethylmercury was then thermally desorbed from traps, separated by gas chromatography, and detected by CVAFS. Each run included a set of standards (25-500 pg in volume), as well as blanks and sample replicates.

Statistical analyses

Statistical analyses were conducted in Minitab (Version 13.1, Minitab Inc., State College, PA). Mean values for each replicate were calculated for treatment comparisons by analysis of variance (ANOVA). Assumptions of normality and homoscedasticity were tested prior to each analysis and data were transformed if necessary. Statistical significance was evaluated at $\alpha = 0.05$ in all cases. When factors were significant in ANOVA, Tukey's multiple pairwise comparisons were used to separate specific differences in levels.

Mortality was analyzed by calculating the proportion of individuals that died in each tank over the course of the experiment. Data were arcsine square root transformed to meet normality assumptions and two-way ANOVA was used to evaluate species and treatment differences. In this study, mass and length at a given time period are representative of growth, as all individuals were the same age at the beginning of the experiment (Ward et al. 2010). Mass and length data were analyzed with one-way ANOVA to assess treatment differences for each species. Additionally, mass and length-specific growth rates were calculated for three equivalent time

periods (Day 0-23, 23-46, and 46-70) using the equation:

$$(m_2 - m_1) / m_1$$

Where m_1 represents the initial measurement and m_2 represents the final measurement. These values were then compared with one-way ANOVA to detect treatment differences for each species.

T-Hg tissue concentrations were \log_{10} transformed to meet normality assumptions. Two-way ANOVA was used to determine differences in mass specific and total body burdens between treatments, species, and days. Because no data were available for *M. beryllina* in the highest treatment on day 70 due to mortality, the 14 $\mu\text{g g}^{-1}$ treatment was eliminated from the analysis. Additionally, analysis of covariance (ANCOVA) was performed to evaluate species differences in body burdens, with fish mass as a covariate. To assess the relationship between mercury in the diet and fish body burdens, linear regression analysis was performed on \log_{10} transformed fish and dietary Hg concentrations. This analysis was conducted separately for each species. Pearson product moment correlation analysis was used to examine correlation between fish mass and tissue concentrations in both species.

Lastly, dietary bioaccumulation factors (BAFs) were calculated for each treatment and species, using the equation:

$$[\text{Hg}]_{\text{fish}} / [\text{Hg}]_{\text{diet}}$$

Because BAFs were not statistically different between sampling dates, values were averaged over the entire dosing period, allowing the inclusion of the highest treatment in statistical analyses. Values were \log_{10} transformed and compared between treatments and species using two-way ANOVA.

Results

Survival

The proportion of individuals that died differed significantly between species ($p=0.043$) and among treatments ($p < 0.001$). *M. beryllina* had significantly higher mortality than *C. variegatus* ($p = 0.0428$). Both species in the $14 \mu\text{g g}^{-1}$ treatment had significantly higher mortality than all lower treatments (Figure 2.1). *Cyprinodon variegatus* also displayed delayed mortality, compared to *M. beryllina*, in both the $7 \mu\text{g g}^{-1}$ and $14 \mu\text{g g}^{-1}$ treatments. For example, in the $14 \mu\text{g g}^{-1}$ treatment, mortality was first observed in *M. beryllina* after 24 days of dosing, while *C. variegatus* did not display mortality until day 34.

Growth

Menidia beryllina showed significant differences in mass among treatments on Day 46 ($p=0.035$). Individuals in the highest treatment ($14 \mu\text{g g}^{-1}$) had significantly lower wet mass than individuals in both the control ($p=0.0397$) and $3 \mu\text{g g}^{-1}$ ($p=0.0456$) treatments (Figure 2.2). *Cyprinodon variegatus* showed significant differences in mass specific growth rate between Days 23 – 46 ($p=0.005$). The highest treatment had a significantly lower mass specific growth rate than the $0.6 \mu\text{g g}^{-1}$ ($p=0.0284$) and $3 \mu\text{g g}^{-1}$ ($p=0.0029$) treatments (Figure 2.3). *Cyprinodon variegatus* also showed significant differences in length specific growth rate during the same time period. Individuals in both the $7 \mu\text{g g}^{-1}$ treatment ($p=0.0497$) and the $14 \mu\text{g g}^{-1}$ treatment ($p=0.0057$) had significantly lower length specific growth rates than the $3 \mu\text{g g}^{-1}$ treatment (Figure 2.3).

Mercury accumulation

Accumulation curves for each treatment and species (Figure 2.4) show a large increase in mass-specific T-Hg body burden between Day 1 and Day 23 of dosing. Tissue concentrations between Day 23 and 46 did not differ, however body burdens at Day 70 were significantly higher than those at Day 23 ($p = 0.0435$). T-Hg body burdens were significantly different among treatments ($p < 0.001$) and between species ($p < 0.001$) over the 70-day dosing period. *Menidia beryllina* had significantly higher mass specific body burdens than *C. variegatus* in all treatments. There was also a significant interaction between treatment and species ($p < 0.001$). T-Hg tissue concentrations increased linearly with dietary Hg concentration in both *M. beryllina* and *C. variegatus* ($p < 0.001$, adj. $R^2 > 0.99$; Figure 2.5).

Examining mercury accumulation in terms of total body burden (μg) showed increasing Hg content over time (Figure 2.6). While *M. beryllina* had higher mass-specific tissue concentrations of Hg, *C. variegatus* had significantly higher total body burdens ($p < 0.0001$). Total body burdens differed significantly among treatments ($p < 0.001$) and days ($p < 0.001$), with each sampling point having significantly higher Hg content than the previous. When fish mass (dry wt.) was used as a covariate, no differences were detected between species, and body burden significantly varied with mass ($p = 0.023$). Mass was significantly negatively correlated with mass specific tissue concentrations in all treatments (Figure 2.7).

Dietary bioaccumulation factors (BAFs) were similar among days, but differed significantly between species ($p < 0.001$) and among treatments ($p < 0.001$) (Figure 2.8). There was also a significant interaction between species and treatments

($p = 0.01$). *Menidia beryllina* had significantly larger BAFs than *C. variegatus*. Among *M. beryllina* individuals, the $0.6 \mu\text{g g}^{-1}$ treatment had significantly higher BAFs than the $3 \mu\text{g g}^{-1}$ treatment ($p = 0.0185$) and $7 \mu\text{g g}^{-1}$ treatment ($p = 0.0481$). *Cyprinodon variegatus* individuals showed a similar trend in BAFs. The $0.6 \mu\text{g g}^{-1}$ treatment had significantly higher BAFs than the control treatment ($p = 0.0064$), $7 \mu\text{g g}^{-1}$ treatment ($p = 0.0016$), and $14 \mu\text{g g}^{-1}$ treatment ($p = 0.0062$). The $3 \mu\text{g g}^{-1}$ treatment also had significantly higher BAFs than the $7 \mu\text{g g}^{-1}$ treatment ($p = 0.0057$) and the $14 \mu\text{g g}^{-1}$ treatment ($p = 0.0208$).

Discussion

Survival

Our study suggests that *M. beryllina* is more sensitive to dietary MeHg exposure than *C. variegatus*, based on higher mortality, as well as more rapid onset of mortality. Although most mortality was seen in the highest treatments, there are likely species-specific differences in sublethal responses at lower levels of mercury exposure, which should be investigated in future studies. Species comparisons are important when assessing toxicological effects of a contaminant, however there are limited examples of this in mercury literature.

Growth

Both species had reduced growth in the highest treatments ($14 \mu\text{g g}^{-1}$ and $7 \mu\text{g g}^{-1}$), between days 23 and 46. This suggests an effect of dietary MeHg on growth. However, these reductions did not show consistent treatment effects. For example, growth in the $14 \mu\text{g g}^{-1}$ diet was lower than some treatments, but not always the

control. Further studies with higher replication might reveal stronger treatment effects. No significant effects of MeHg on growth were detected in the other time periods. However, statistical power was very low between days 0 and 23 (power = 0.17) and between days 46 and 70 (power = 0.2). Therefore, we cannot rule out effects of dietary MeHg on growth during these periods.

Previous studies have found similar negative effects of dietary methylmercury on fish growth (Houck & Cech 2004), particularly in larger fish such as walleye (Friedman et al. 1996) and sturgeon (Lee et al. 2011). Hypothesized mechanisms for this include decreased levels of cortisol and thyroid hormone (Friedman et al. 1996), interference with gastrointestinal function and decreased nutrient absorption (Lee et al. 2011), as well as reallocation of energy to MeHg detoxification (Lee et al. 2011). Since fish in this study were fed *ad libitum* and could consume as much food as needed, the last mechanism is an unlikely explanation. The fact that growth reductions were seen even when fish could compensate for increased energy needs, suggests that a physiological impairment may have caused the observed decreases in growth.

Mercury accumulation

Mercury tissue concentrations in *C. variegatus* were very similar to those measured in fathead minnows fed the same dietary MeHg concentrations (Hammerschmidt et al. 2002). Tissue concentrations in both *C. variegatus* and *M. beryllina* were also similar to Hg concentrations measured in sturgeon during a laboratory dosing study (Lee et al. 2011). Although sturgeon (Lee et al. 2011) were exposed to higher dietary concentrations, their final tissue concentrations were similar

to those in our study. Interestingly, sturgeon exhibited much higher mortality in response to these mercury body burdens. Tissue concentrations that showed little mortality in this study were higher than many of those reported in fish from contaminated areas. For example, red drum (*Sciaenops ocellatus*) from Lavaca Bay (TX) contained a maximum of $5.7 \mu\text{g g}^{-1}$ ww, while fish in this study accumulated over $7 \mu\text{g g}^{-1}$ ww with no increase in mortality. This suggests that small forage fish, such as *C. variegatus* and *M. beryllina*, have a high tolerance for dietary MeHg exposure and could be resilient in highly contaminated ecosystems. If fish occupying low trophic levels are capable of surviving with high Hg body burdens, they may serve as mercury reservoirs within food webs. This has important implications for the Hg exposure of higher trophic levels.

The largest change in mercury tissue concentrations occurred within the first 23 days of dosing, even though fish growth during this time period was comparable to the other time periods. We are not aware of any studies that have found differences in MeHg depuration mechanisms during the juvenile lifestage, relative to the adult lifestage. However, this is certainly possible. There may also be differences in the way mercury is assimilated in young fish, when energy demands are high and there is a strong priority on producing new somatic tissue. Additionally, we found significant increases in mass specific Hg concentrations between days 23 and 70, indicating that mercury accumulation occurred during the sub-adult lifestage as well.

We observed a significant linear relationship between dietary Hg exposure and T-Hg body burden, suggesting that fish tissue concentrations respond strongly to the level of Hg contamination in the diet. This coincides with the idea that diet is the

primary pathway of MeHg uptake in fish (Phillips & Buhler 1978; Hall et al. 1997). This strong relationship was observed after only a few weeks of dosing, which also shows that tissue concentrations respond rapidly to concentrations in the diet. This finding is similar to observed in an ecosystem-level stable isotope study (Paterson et al. 2006), which found that newly added mercury in zooplankton and benthic invertebrates was quickly assimilated in wild fish. A study of MeHg uptake kinetics in *C. variegatus* showed that ingested Hg is transferred to the bloodstream and subsequently to tissues at a relatively fast rate, particularly in small individuals (Leaner & Mason 2004), further explaining the rapid responses observed.

Cyprinodon variegatus had significantly higher growth rates than *M. beryllina* throughout the study, which is a factor that should be considered when comparing Hg accumulation between species. We found that mass was negatively correlated with tissue concentrations in both species. This was supported by the lack of species differences in Hg body burdens when mass was used as a covariate. Therefore it is likely that differences in the tissue concentrations of *C. variegatus* and *M. beryllina* were due to species differences in growth over the dosing period, rather than physiological differences in mercury assimilation and depuration. This correlation has been observed in field studies (Harris & Bodaly 1998; Simoneau et al. 2005; Ward et al. 2010), however it is complicated by the variable physical conditions and unknown dietary history of field-collected fish. A bioenergetics-based model comparing fish sampled from two different lakes, with differing mercury concentrations, found that a majority of the variation was explained by differences in dietary MeHg (Harris & Bodaly 1998). However, a significant portion of the variation among lakes was

explained by growth rates. By controlling variables such as dietary MeHg exposure and water chemistry, our laboratory experiment gives additional evidence of the significant effects of fish growth rate on Hg tissue concentrations.

Based on these findings, growth rate is an important variable to consider when comparing tissue concentrations among species. This has ecological implications for consumers. Larger, faster-growing prey items may have reduced mercury content, on a per gram basis. Additionally, an ecosystem that is dominated by smaller, slow-growing prey species may be more susceptible to Hg bioaccumulation in higher trophic levels (Ward et al. 2010). Previous studies have shown that productive systems can experience mercury dilution via algal blooms at the bottom of the food chain (Chen et al. 2005). This effect can be enhanced by subsequent high consumption and growth dilution in consumers (Karimi et al. 2007). Overall, growth is an important factor to consider when assessing mercury accumulation in both individuals and ecosystems.

Lastly, we found that dietary bioaccumulation factors decreased with increased dietary mercury exposure. This pattern was evident for both species. Other laboratory dosing studies have also found decreased assimilation of mercury at higher levels of exposure in both fish (Houck & Cech 2004) and larval amphibians (Unrine et al. 2004). This suggests that MeHg accumulated is not a constant fraction of MeHg ingested, but rather, accumulation is dependent on the level of contamination in the diet. This has implications for bioaccumulation models, which often use a constant term for contaminant uptake. In the case of mercury, it appears that this model parameter should vary with dietary exposure.

Table 2.1. Target MeHg concentrations* and corresponding measured concentrations \pm SE for each treatment. Measured concentrations were averaged over the experiment.

Target MeHg concentration ($\mu\text{g g}^{-1}$ dw)	Actual MeHg concentration ($\mu\text{g g}^{-1}$ dw)
0.06	0.04 ± 0.003
1.00	0.56 ± 0.05
5.00	3.33 ± 0.30
10.00	6.78 ± 0.63
20.00	13.96 ± 1.45

* The target MeHg concentration for control food was not zero because background mercury in flake food was unavoidable (ingredients include shrimp and fish meal, which contain MeHg). The target concentration was based on literature values.

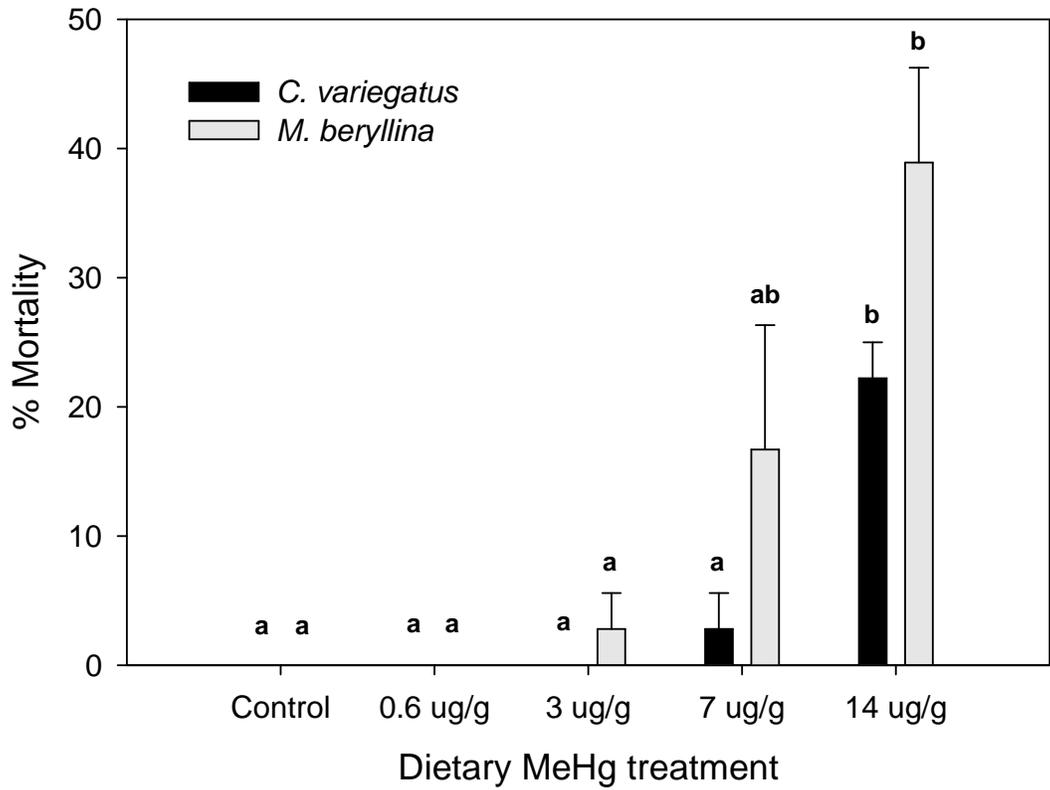


Figure 2.1. Mean percent mortality \pm 1 SE for each treatment, over the 70-day dosing period (n=3). Different letters indicate significantly different values.

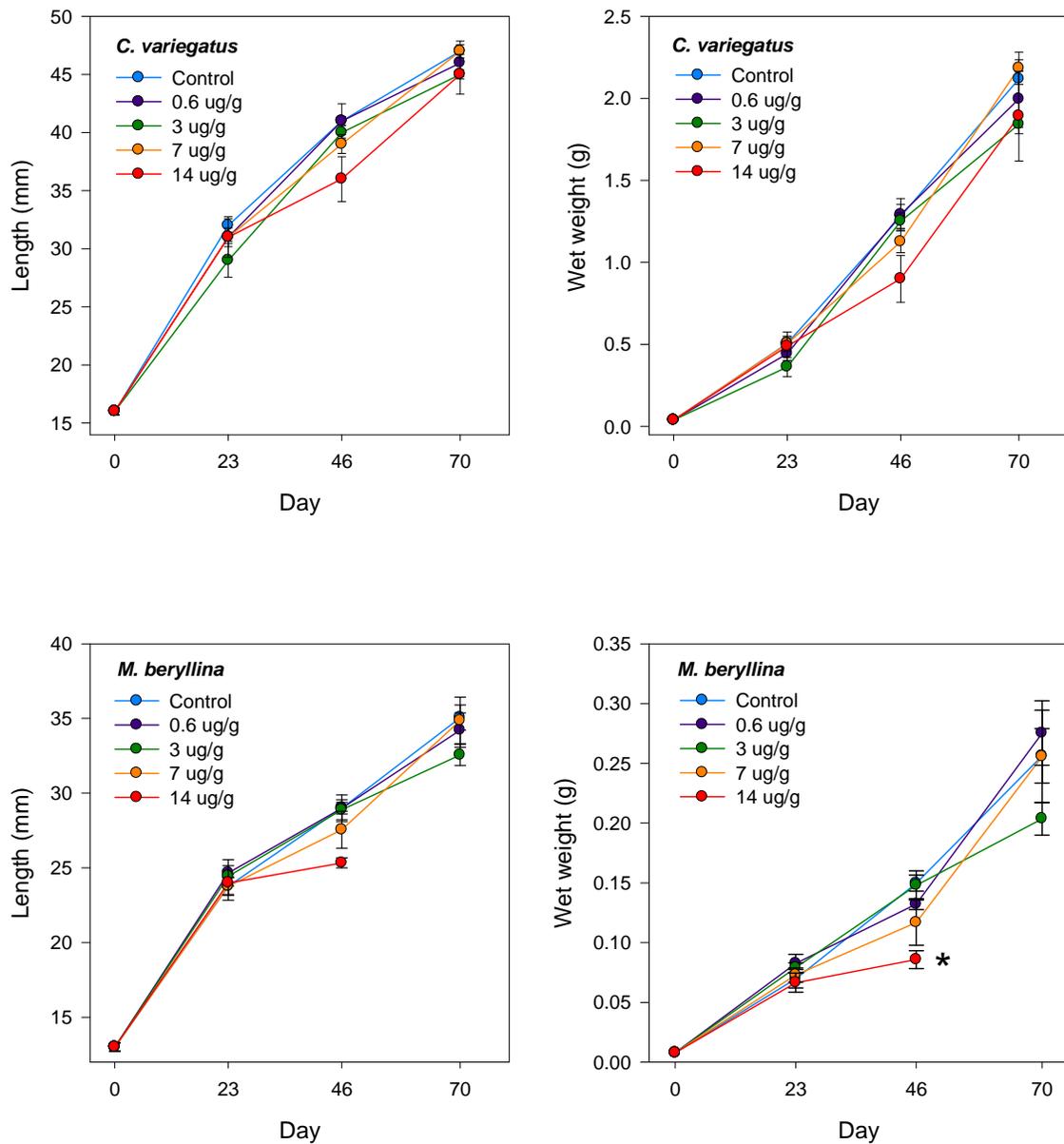


Figure 2.2. Mean length and weight \pm 1 SE over time for both *C. variegatus* (top panel) and *M. beryllina* (bottom panel). $n=3$ for each point. Asterisks indicate significantly different values.

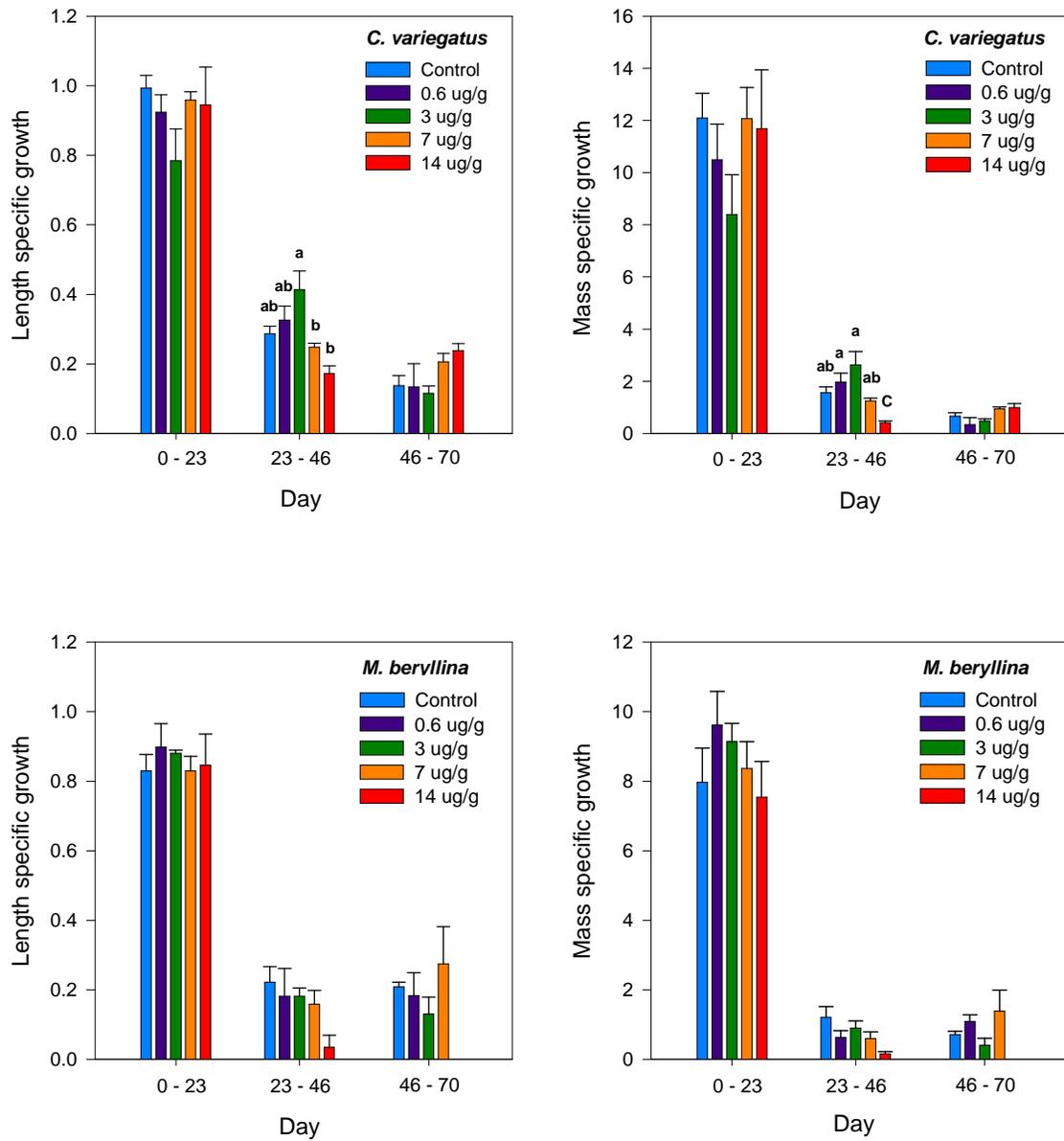


Figure 2.3. Mean length specific and mass specific growth \pm 1 SE for the three experimental time periods (n=3). Different letters indicate significantly different values.

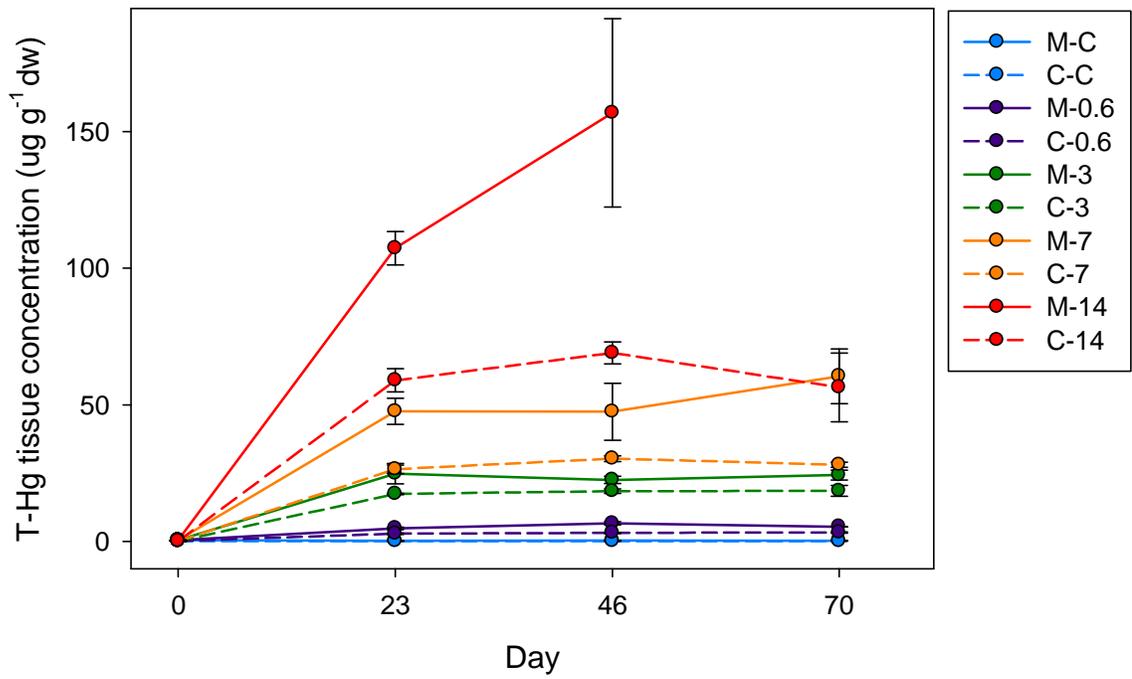


Figure 2.4. Mass specific T-Hg tissue concentrations \pm 1 SE for all treatments over the 70-day dosing period. $n=3$ for each point. In the legend, “M” represents *M. beryllina*, “C” represents *C. variegatus*, and numbers represent dietary Hg concentrations.

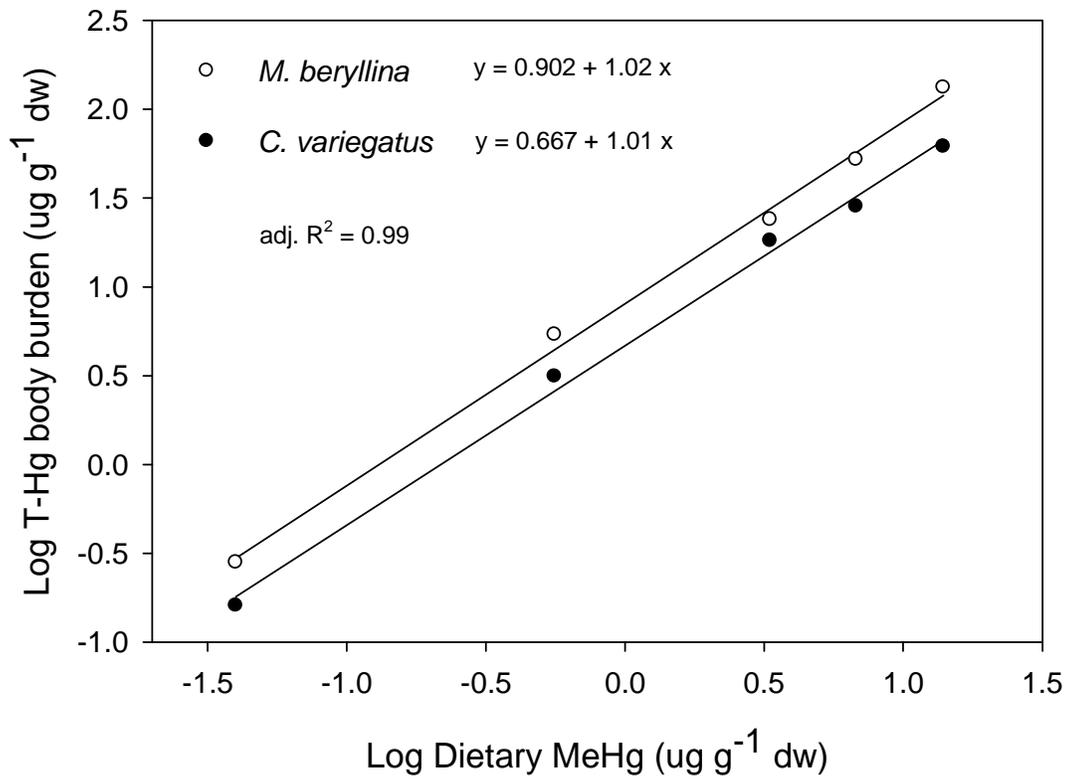


Figure 2.5. Relationship between MeHg concentration in the diet and mass specific T-Hg concentration in fish, for each species. Values were averaged over time for each treatment.

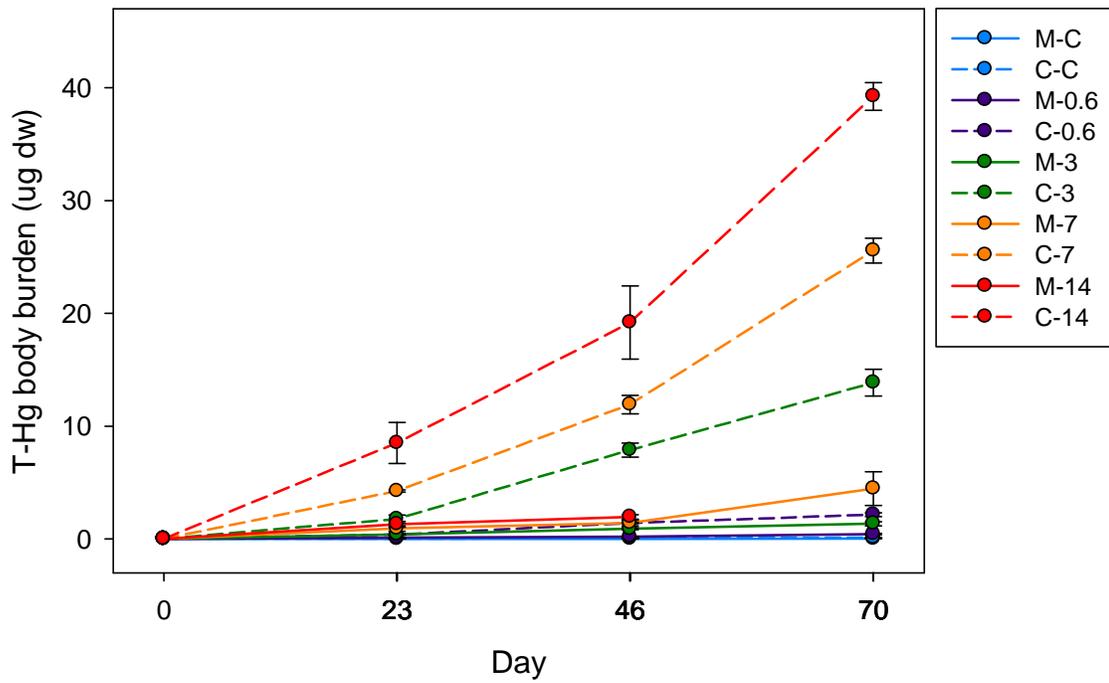


Figure 2.6. T-Hg body burdens \pm 1 SE for all treatments over the 70-day dosing period. $n=3$ for each point. In the legend, “M” represents *M. beryllina*, “C” represents *C. variegatus*, and numbers represent dietary Hg concentrations.

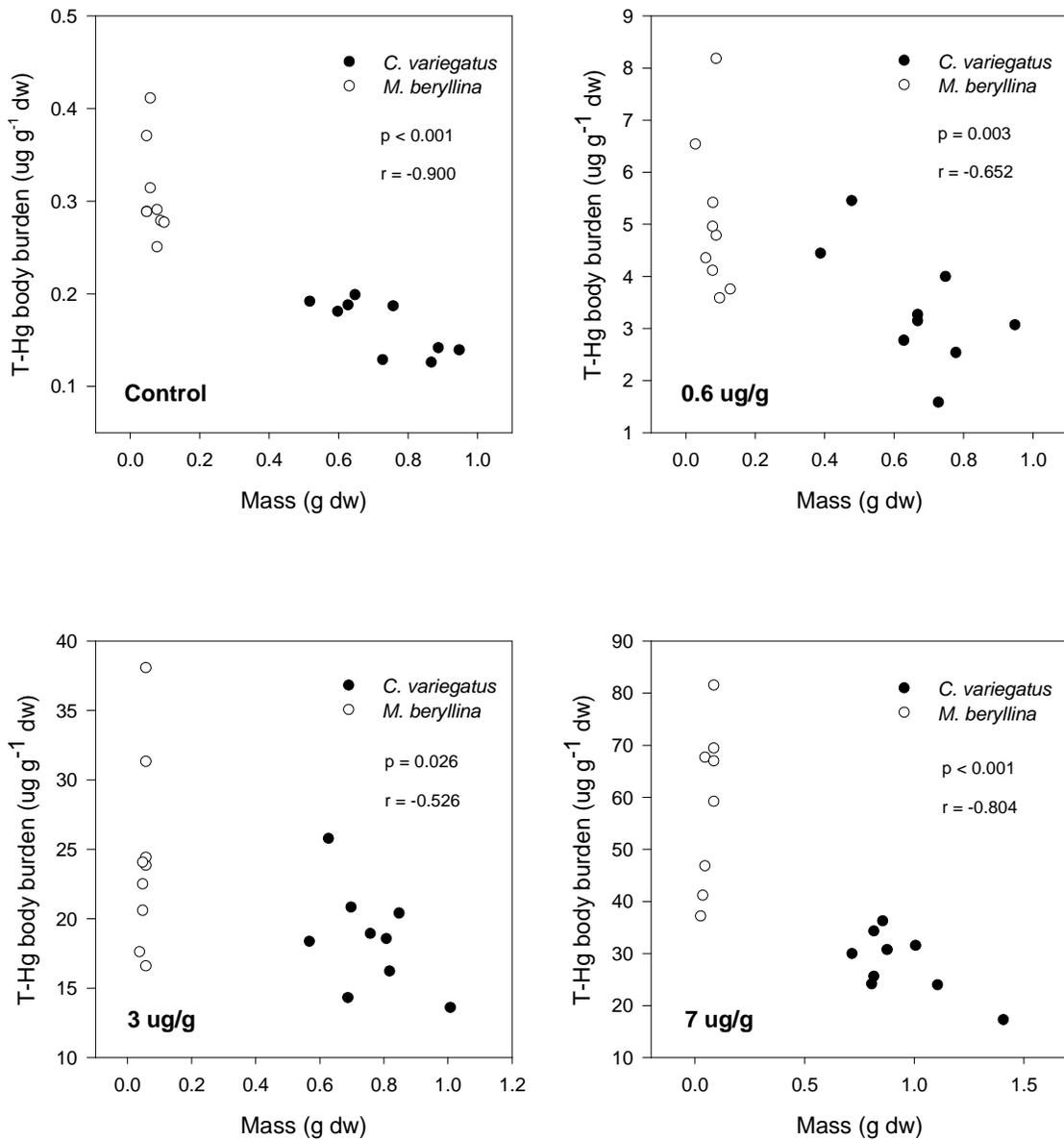


Figure 2.7. Relationship between T- Hg body burden and mass for both species. Each treatment is graphed individually. Points represent individual fish (n=18).

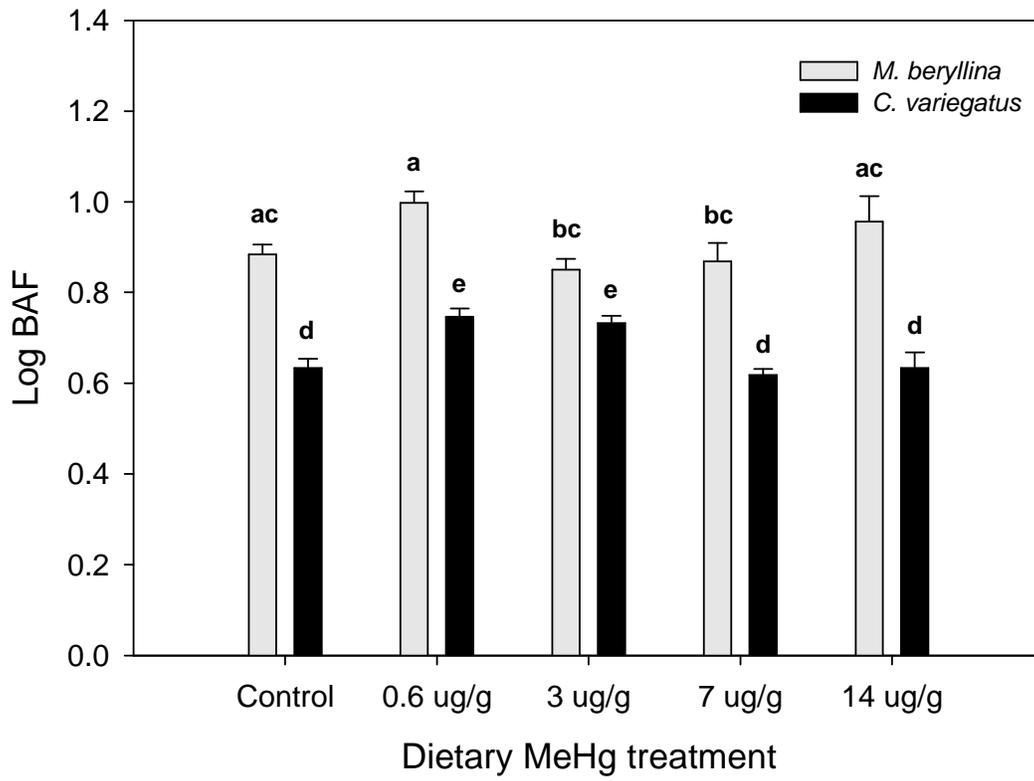


Figure 2.8. Mean Bioaccumulation factor \pm 1 SE for each treatment (n=3). Different letters indicate significantly different values.

Chapter 3: Investigation of maternal transfer of dietary MeHg in the sheephead minnow (*Cyprinodon variegatus*) using a stable mercury isotope

Introduction

Background

Maternal transfer of contaminants is an important exposure pathway as early life stages are often most susceptible to their effects. Pollutants transferred from the mother have the potential to significantly affect survival and development of embryos (Alvarez et al. 2006; Hopkins et al. 2006), as well as adversely affect offspring later in life (Eisenreich et al. 2009; Bergeron et al. 2011). The effects of maternally transferred compounds ultimately have implications for population viability (Hopkins et al. 2006), and are therefore important topics of study.

Mercury is a contaminant of concern in many ecosystems, particularly in its methylated form (MeHg), which readily accumulates in fish and other biota. Recent studies have shown endocrine disruption and reproductive effects in fish as a result of dietary MeHg exposure (Drevnick & Sandheinrich, 2003; Klaper et al., 2006). Effects of MeHg observed in freshwater minnows (*Pimephales promelas*) include changes in sex hormone levels (Drevnick & Sandheinrich, 2003), as well as decreases in the production of vitellogenin, an essential yolk protein (Klaper et al. 2006). As both an endocrine disruptor and potent neurotoxin, MeHg has the potential to impair developing offspring. However, our current understanding of maternal transfer of MeHg and its effects remain limited, particularly in estuarine fish.

While diet is the major source of MeHg for adult fish (Phillips & Buhler 1978; Hall et al. 1997), maternal transfer has been shown to be a significant route of exposure for larval and juvenile fish (Latif et al. 2001; Alvarez et al. 2006). Recent studies on the cellular mechanisms by which MeHg moves through the body have shown that MeHg readily complexes with cysteine (Simmons-Willis et al. 2002). This structure mimics that of methionine and can therefore be transferred across cell membranes to developing oocytes via methionine transporters (Simmons-Willis et al. 2002). It was originally thought that MeHg partitioned from stores in female tissues into developing oocytes. However, a more recent study by Hammerschmidt and Sandheinrich (2005) indicated that egg mercury content was a reflection of the maternal diet during oogenesis, rather than Hg stored in female tissues.

Study rationale

Dynamics of methylmercury transfer from parent to offspring are poorly understood and could significantly affect the reproductive fitness of offspring. This research aims to increase knowledge of maternal MeHg transfer in estuarine fish. By using a stable mercury isotope, we can differentiate between mercury stored in female tissues and mercury assimilated from the maternal diet during oogenesis. Additionally, this approach allows us to quantify assimilation of methylmercury from the diet, and subsequent transfer to offspring. Stable mercury isotopes are powerful tools to trace the fate of mercury species and investigate processes such as methylation, bioaccumulation, and adsorption onto particles (Hintelmann & Ongrianc 2003). Using this technique in the context of maternal transfer is a novel approach.

Effects of methylmercury exposure on lower trophic levels have implications for species of higher trophic levels, including important commercial species. Furthermore, compared to freshwater species, accumulation and effects of MeHg in estuarine and marine organisms are understudied. Therefore, we chose to focus on the estuarine minnow *Cyprinodon variegatus*. *Cyprinodon variegatus* inhabits shallow, coastal waters of North America from Massachusetts to Mexico (Murdy et al. 1997). Sheepshead minnows hatch after 4 to 7 days, remain as larvae for approximately 28 days, and metamorphose to the juvenile stage which lasts approximately 35 days prior to sexual maturation (Raimondo et al. 2009). Sheepshead minnows are ideal for laboratory studies because of their small size, rapid development, and tolerance of laboratory conditions. Due to its abundance in estuarine marshes (e.g. Rowe and Dunson, 1995), *C. variegatus* serves as important food source for other vertebrates and macroinvertebrates.

Objectives

The objectives of this study were to:

1. Assess the effect of dietary MeHg on egg production by *C. variegatus*
2. Quantify maternal mercury transfer and determine if it occurs in a dose-dependent nature
3. Use a stable mercury isotope to trace maternal transfer and test recent findings that suggest that transfer is largely from the maternal diet during oogenesis

Methods

Experimental protocol

Food preparation

Methylmercury diets were prepared with methylmercury (II) chloride (Alfa Aesar) for the first period of dosing (28 days), and with laboratory synthesized Me¹⁹⁹Hg for the remainder of dosing (63 days; Figure 3.1). ¹⁹⁹Hg, a stable isotope of mercury, was purchased from Oak Ridge National Laboratory. Me¹⁹⁹Hg was synthesized by methylation of ¹⁹⁹Hg with methylcobalamin and subsequent extraction using methylene chloride. This was based on methods described in Hintelmann and Ogrinc (2003). Me¹⁹⁹Hg solutions were analyzed for both T-Hg and MeHg to confirm that all mercury in solution was MeHg.

MeHg was incorporated into flake food via an agar/gelatin matrix. Gelatin, agar, flake food, and deionized water were combined in a mass ratio of 0.7:1:20:100, respectively. Agar and gelatin were added to boiling deionized water and stirred for one minute. This mixture was poured into a shallow Pyrex baking dish and mixed with pre-ground flake food. Aqueous MeHg was then added to the mixture at calculated volumes to achieve the following nominal concentrations: 1, 5, and 10 μg g⁻¹ dw. After setting in a refrigerator overnight, food was cut into small slices and frozen at -80°C. Food was then freeze-dried and ground with a mortar and pestle to produce a fine, dry, flake mixture. Fish diets were stored at -4 °C between feedings to minimize degradation of Hg concentrations. Actual Hg concentrations averaged 101 % of the target concentrations (Table 3.1).

Husbandry

Fish were housed in 76 L aquaria at Chesapeake Biological Laboratory (Solomons, MD). All tanks were equipped with individual filters, heaters, and aeration to maintain water quality. Water temperature was maintained at approximately 26 ° C for the duration of the experiment. Filtered ambient river water (Patuxent River, MD, USA) was used, thus salinity varied naturally between 7 and 15 ppt over the course of the experiment. A 14:10 hour light:dark cycle was maintained throughout. Each tank received 1 g of food daily, which was confirmed to be an *ad libitum* regimen, as excess food remained prior to subsequent food additions. Tanks were cleaned weekly and water quality was monitored by measuring dissolved oxygen, temperature, salinity, conductivity, and pH weekly. Ammonia levels were also measured colorimetrically in a random subset of tanks periodically. Note that this husbandry protocol was approved by the University of Maryland Center for Environmental Science IACUC (protocol #S-CBL-10-03).

Sheepshead minnows were acquired from Aquatic Biosystems (Denver, CO) at 14 days old. Prior to the study, fish were held in 38 L aquaria for a two-week acclimation period, during which time they were fed control food. At the end of this period, individuals were randomly distributed among 76 L aquaria at a density of 12 fish per tank (0.16 individuals / L), and dosing was initiated.

Treatments and sampling design

Treatments consisted of a control diet and three diets with varying concentrations of methylmercury. Treatments consisted of a control, and nominal MeHg doses of 1 (low), 5 (medium), and 10 $\mu\text{g g}^{-1}$ dw (high). Each treatment

contained 6 replicate tanks, totaling 24 aquaria. Treatment and species were randomly distributed among tank positions.

Juveniles were fed a non-isotopic MeHg diet for 28 days, after which point diets were switched to an isotopic diet containing Me¹⁹⁹Hg (Figure 3.1). This switch was timed to occur before the onset of oogenesis. To confirm this, 3 females were sacrificed from one tank per treatment and dissected to evaluate reproductive status. No developed eggs were found in individuals at the beginning of isotope dosing. Therefore, we assumed that the isotopic diet spanned the period of oogenesis.

Individuals were fed the isotopic diet for 63 days, for a total Hg dosing period of 91 days. At the end of the experiment, fish were transferred to clean aerated water and held unfed for 48-hours. Individuals were then euthanized by cervical dislocation, measured for total length and wet mass, and frozen at -80° C for later mercury analysis. Prior to freezing, all female fish were dissected to remove eggs. Eggs were lightly rinsed with deionized water, counted, and weighed. Eggs from each female were pooled into 5 mL Teflon vials and frozen at -80° C for subsequent digestion and mercury analysis. Because sheepshead minnows have asynchronous ovaries, eggs vary in development and size within the gonad. Therefore, only eggs that were large enough to be accurately removed and counted were included in egg analyses.

Each batch of food was sub-sampled and analyzed once, in order to measure actual mercury doses. Since significant MeHg breakdown in food was not found in the previous experiment, weekly sub-sampling of food was not done in this experiment. To extract MeHg from food, triplicate 1 g sub-samples of food were distilled in a solution of 20 mL Milli-Q water, 1 mL 50 % sulfuric acid, and 0.5 mL

20 % potassium chloride (Horvat et al. 1993). The distillate was then analyzed for MeHg (see below).

Sample preparation and analyses

Digestions

Fish carcasses were freeze-dried for 24 hours prior to digestion. Fish were then digested on a hot plate at 120-150° C for 6-9 hours, using 5 mL of 50:50 concentrated nitric acid:sulfuric acid. Digestions were done in 50 mL Erlenmeyer flasks with watch glass covers for ventilation. Digestions were considered complete when flasks were free of brown gas. Samples were cooled and diluted to 50 mL with Milli-Q water. Samples were then oxidized with 1 mL of bromine monochloride (BrCl) and analyzed the following day. Exact dilution volumes were calculated by weight difference.

Eggs from each female were freeze-dried in 5 mL Teflon vials and digested with 2 mL of 50:50 nitric acid:sulfuric acid for 24 hours in a 60° C oven. Samples were then diluted and oxidized using the same procedure described for fish.

T-Hg analysis

Analysis of total mercury (T-Hg) was conducted on a Tekran Model 2600 Mercury Analyzer with a Model 2620 Autosampler (Tekran Instruments, Canada). The instrument measures T-Hg via cold vapor atomic fluorescence spectrometry (CVAFS). Briefly, all mercury species within the sample are reduced to elemental mercury (Hg^0) by stannous chloride (SnCl_2). Hg^0 is then concentrated on a gold trap, thermally desorbed, analyzed by CVAFS, and quantified according to EPA method 1631 (US EPA, 1996). This instrument was interfaced with an ICP-MS (Hewlett

Packard 4500, Agilent Technologies), in order to separate and quantify individual Hg isotopes after total mercury detection by CVAFS. Concentrations of ^{199}Hg and ambient Hg were calculated based on methods of Hintelmann & Ogrinc (2003).

Prior to analysis, excess oxidant was neutralized in samples with 10 ul of hydroxylamine hydrochloride. T-Hg standards were prepared from a NIST stock solution in concentrations of 5, 10, 25, 50, 75, 100, 150, and 200 ng L⁻¹. A matrix blank was prepared, according to the type of samples being analyzed. When analyzing fish samples, the matrix blank consisted of 0.2 % digest acid and 0.08 % BrCl and when analyzing water samples, the matrix blank consisted of 0.5 % BrCl. Quality control included calibration blanks, replicate standards and samples, duplicate dilutions, and duplicate SRMs (DORM-2, National Research Council Canada).

MeHg analysis

Aliquots of distilled samples were added to bubblers containing a citrate buffer (pH = 4.8). The solution was ethylated with sodium tetraethylborate, converting MeHg to gaseous methylethylmercury (Bloom 1989). This was purged from solution and concentrated on Tenax traps. Methylethylmercury was then thermally desorbed from traps, separated by gas chromatography, and detected by CVAFS (Tekran Model 2500). Each run included a set of standards (25-500 pg in volume), as well as blanks and sample replicates.

Statistical analyses

Statistical analyses were conducted in Minitab (Version 13.1, Minitab Inc., State College, PA). Mean values for each replicate were calculated for treatment comparisons by analysis of variance (ANOVA). However, for linear regressions and

correlation analysis relating females specifically to their eggs, values for individual fish from all replicates combined were used. This was thought to be the most biologically relevant approach. As a result, n values are higher in linear regressions and correlation analyses than in ANOVA. Assumptions of normality and homoscedasticity were tested prior to each analysis and data were transformed if necessary. Statistical significance was evaluated at $\alpha = 0.05$ in all cases. When factors were significant in ANOVA, Tukey's multiple pairwise comparisons were used to separate specific differences in levels.

One-way ANOVA was used to compare the number of eggs per female in each treatment. Total Hg (T-Hg), ambient Hg, and ^{199}Hg concentrations in fish and eggs were log transformed and analyzed for treatment differences using one-way ANOVA. Control fish and eggs were eliminated from these analyses because they were never exposed to ^{199}Hg . Linear regression analysis was used to determine if egg T-Hg concentration was dependent on maternal Hg burden. Pearson product moment correlation analysis was used to determine correlations between egg T-Hg concentration and 1) the number of eggs in the mother and 2) Hg in the maternal diet.

To calculate the percent of Hg transferred from a female to her eggs, a pre-oogenesis fish body burden was estimated by adding fish and egg Hg burdens. The percent of this total found in eggs was then calculated. The percentages of ^{199}Hg and ambient Hg maternally transferred were log transformed prior to correlation analysis (Pearson product moment). These percentages were also compared among treatments with one-way ANOVA. Because the number of eggs per female appeared to differ among treatments but was not statistically significant, we conducted a retrospective

power analysis (target power 0.70) on these data to assess effects of experimental design on this result. Lastly, due to water quality issues, several replicates were lost. Therefore, n values in ANOVA are lower than the original number of replicates stated in the experimental design.

Results

Egg production

Female egg production did not significantly differ between treatments. The control treatment had the highest number of eggs per female (74 ± 8). Individuals in the low, medium, and high MeHg treatments had an average of 49 ± 26 , 69 ± 28 , and 10 ± 5 eggs per female, respectively (Figure 3.2). Although not statistically significant, individuals fed the high MeHg diet had the lowest number of eggs per female.

Controls on maternal transfer

T-Hg concentrations in eggs were significantly dependent on maternal T-Hg body burden and showed a strong positive linear relationship ($p < 0.001$, $R^2 = 0.914$; Figure 3.3). Egg T-Hg concentrations were negatively correlated with the number of eggs in the mother ($p = 0.023$, $r = -0.443$; Figure 3.4). T-Hg concentrations in eggs were significantly different between treatments ($p < 0.001$) and egg Hg content was significantly positively correlated with Hg concentration in the maternal diet ($p = 0.003$, $r = 0.894$).

Ambient vs. isotopic mercury

Overall, both ambient and isotopic Hg increased with dose in fish and eggs (Figure 3.5). Adult fish tissue concentrations of ^{199}Hg significantly differed among treatments ($p = 0.006$; Figure 3.5). Individuals in the low treatment accumulated significantly less ^{199}Hg than individuals in the medium ($p = 0.0134$) and high ($p = 0.0079$) treatments. However, there was no difference in isotopic Hg accumulation between medium and high treatments. The concentration of ^{199}Hg in eggs also significantly differed among treatments ($p = 0.019$; Figure 3.5). Eggs from the low treatment had significantly less of the enriched Hg isotope than eggs from the high treatment ($p = 0.0160$).

Patterns of ambient mercury accumulation and maternal transfer were similar. Fish tissue concentrations of ambient Hg significantly differed among treatments ($p = 0.006$; Figure 3.5). Individuals in the low treatment accumulated significantly less ambient Hg than fish in the medium ($p = 0.0134$) and high ($p = 0.0079$) treatments. The concentration of ambient Hg in eggs was also significantly different among treatments ($p < 0.001$; Figure 3.5). Eggs from the low treatment had significantly lower ambient Hg than those from the medium ($p = 0.0235$) and high ($p = 0.0053$) treatments. Additionally, egg concentrations of ambient Hg in all three treatments were significantly higher than control concentrations, indicating these levels were above background mercury from the flake food diet alone.

The percent of total mercury that was ambient did not significantly differ between fish and eggs, or between treatments (Table 3.2). The percent of mercury transferred from a female to her eggs averaged 0.36 % of ambient Hg and 0.44 % of

¹⁹⁹Hg. There was a significant positive correlation between the percent of ¹⁹⁹Hg and percent of ambient Hg transferred ($p < 0.001$, $r = 0.779$; Figure 3.6), suggesting that both mercury isotopes were transferred proportionally. The percent transferred did not significantly differ among treatments for either isotope (Table 3.3).

Discussion

Egg production

There were no differences in egg production among treatments. However, the high variation in number of eggs per female suggests that a larger number of replicates are needed to detect an effect of MeHg. Note that power analysis revealed that our design provided a statistical power to assign statistical significance of only 0.30 (at $\alpha = 0.05$), most likely due to loss of multiple replicates due to water quality issues. Previous studies have found negative effects of dietary MeHg on egg production in fathead minnows (Hammerschmidt et al. 2002). This is likely a consequence of changes in sex hormone levels and decreased vitellogenin production caused by MeHg exposure (Drevnick & Sandheinrich, 2003; Klaper et al. 2006).

Controls on maternal transfer

Because eggs were stripped directly from females after euthanasia, eggs were not exposed to aqueous MeHg. Therefore we can assume all MeHg found in eggs was maternally transferred. We found an average of 0.4 % of female Hg body burden was transferred to eggs. This percentage is similar to T-Hg transfer measured in five different species: 0.3 % (white bass), 0.4 % (smallmouth bass), 0.6 % (rainbow trout), 1.8 % (white sucker), and 2.3 % (yellow perch) (Niimi 1983). A study by

Hammerschmidt et al. (1999) also found 1.9 % transfer in field-collected yellow perch.

The percentage of MeHg transferred to fish eggs was relatively low, compared to observations of transfer of organic contaminants, such as PCBs, pesticides, and fungicides. For organic compounds, studies have found that 5 – 30 % of the maternal burden is transferred to fish eggs, depending on the specific compound and species (Niimi 1983). MeHg transfer is also lower than that observed for some inorganic contaminants in other organisms. One study found that female frogs transfer approximately 50 % of their total selenium burden and 3 – 8 % of their strontium burden into eggs (Hopkins et al. 2006). The low percentage of MeHg transferred from mother to egg suggests that spawning is not a significant mercury depuration route for sheepshead minnows, although this may be the case for other contaminants.

We found a significant negative correlation between the number of eggs in a female and the Hg concentration in her eggs. Additionally, egg mass did not differ between treatments, nor did the percent of Hg body burden transferred (Table 3.3). This suggests that a specific proportion of Hg is partitioned from the female to developing eggs. If this burden is distributed among a larger number of eggs, a lower mercury concentration would be expected in offspring. Therefore, it appears the clutch size of an organism during a particular reproductive event may have important implications for the amount of MeHg transferred and subsequent effects on offspring. This has also been suggested for maternal transfer of selenium in frogs (Hopkins et al. 2006).

We found that maternal body burden was a strong predictor of MeHg content of eggs. Mercury concentrations in eggs increased linearly with female Hg concentration, as observed elsewhere in both field-collected yellow perch (Hammerschmidt et al. 1999) and laboratory-dosed fathead minnows (Hammerschmidt & Sandheinrich 2005). This linear model could be determined for a specific species and then applied to Hg tissue concentrations of wild fish populations, in order to predict exposure and potential risk to offspring.

Ambient vs. isotopic mercury

The most striking result of this study was the increase of ambient mercury in eggs, with increasing dose (Figure 3.5). A constant percentage of ambient Hg was transferred to eggs in each treatment. In this study, ambient mercury represents historical mercury exposure and the enriched Hg isotope (^{199}Hg) represents recent Hg exposure, including the period of oogenesis. Higher ambient mercury in eggs from higher dietary treatments is evidence that a significant portion of maternally transferred Hg is from the burden stored in female tissues. It is clear from our study that recently ingested MeHg is not the only source of maternally transferred mercury. If this were the case, we would have found almost entirely ^{199}Hg in eggs. Ambient Hg concentrations in eggs from Hg-exposed females were significantly higher than in control eggs. Therefore the presence of ambient Hg in eggs cannot be attributed to background mercury levels in flake food. Lastly, ambient Hg and the enriched Hg isotope were transferred proportionally to eggs, further suggesting that both female tissues and the diet during oogenesis are sources of maternally transferred Hg.

Based on these findings, it appears that historical mercury exposure can be important in the context of maternal transfer. While mercury ingested during oogenesis is also important, prior mercury exposure matters. For example, if a fish has minimal MeHg exposure over its early life, but feeds on a highly contaminated diet during oogenesis, maternally transferred mercury will largely reflect recent dietary exposure, as found by Hammerschmidt & Sandheinrich (2005). However, if a fish accumulates high levels of mercury from exposure early in life, but feeds on a relatively uncontaminated diet during oogenesis, maternal mercury transfer may still be high, due to historic exposure. In this case, the source of maternally transferred Hg would be largely from the burden stored in female tissues.

This has important implications for offspring exposure. Egg Hg content may not be as sensitive to variations in the maternal diet during oogenesis as previously thought. Furthermore, an individual's entire history of mercury exposure can affect egg Hg levels. If maternal body burden is high, but current maternal diet is relatively low, Hg concentrations in developing fish may be higher than expected. This is important to consider in the context of trophic transfer. Species feeding on larval or juvenile fish are affected by maternally transferred Hg, as this constitutes a large portion of Hg contamination in young fish.

Table 3.1. Target MeHg concentrations* and corresponding measured concentrations \pm SE for each treatment. Measured concentrations were averaged over the experiment.

Target MeHg concentration ($\mu\text{g g}^{-1}$ dw)	Actual MeHg concentration ($\mu\text{g g}^{-1}$ dw)
0.06	0.04 ± 0.007
1.00	1.04 ± 0.27
5.00	5.02 ± 0.80
10.00	9.90 ± 2.61

* The target MeHg concentration for control food was not zero because background mercury in flake food was unavoidable (ingredients include shrimp and fish meal, which contain MeHg). The target concentration was based on literature values.

Table 3.2. Mean percent ambient Hg \pm 1 SE in fish and eggs for the low (n=3), medium (n=3), and high (n=2) treatments. Values represent a percentage of the total mercury concentration. All values are statistically similar.

Treatment	Mean % ambient Hg	
	Fish	Eggs
Low	13.4 \pm 0.9	7.9 \pm 2.1
Med	10.3 \pm 2.6	14.8 \pm 2.9
High	10.3 \pm 1.0	8.6 \pm 0.5

Table 3.3. Mean percent ambient Hg and percent ¹⁹⁹Hg transferred from female to eggs for each treatment. All values are statistically similar.

Treatment	% ambient Hg transferred	% ¹⁹⁹ Hg transferred
Low	0.31	0.64
Medium	0.60	0.52
High	0.17	0.17

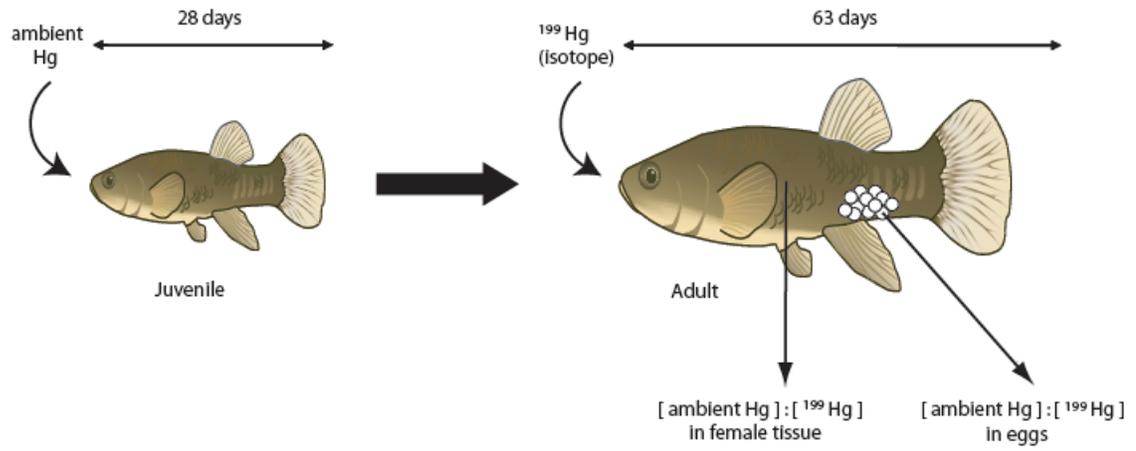


Figure 3.1. Diagram of experimental design and timing of ambient and ^{199}Hg dosing.

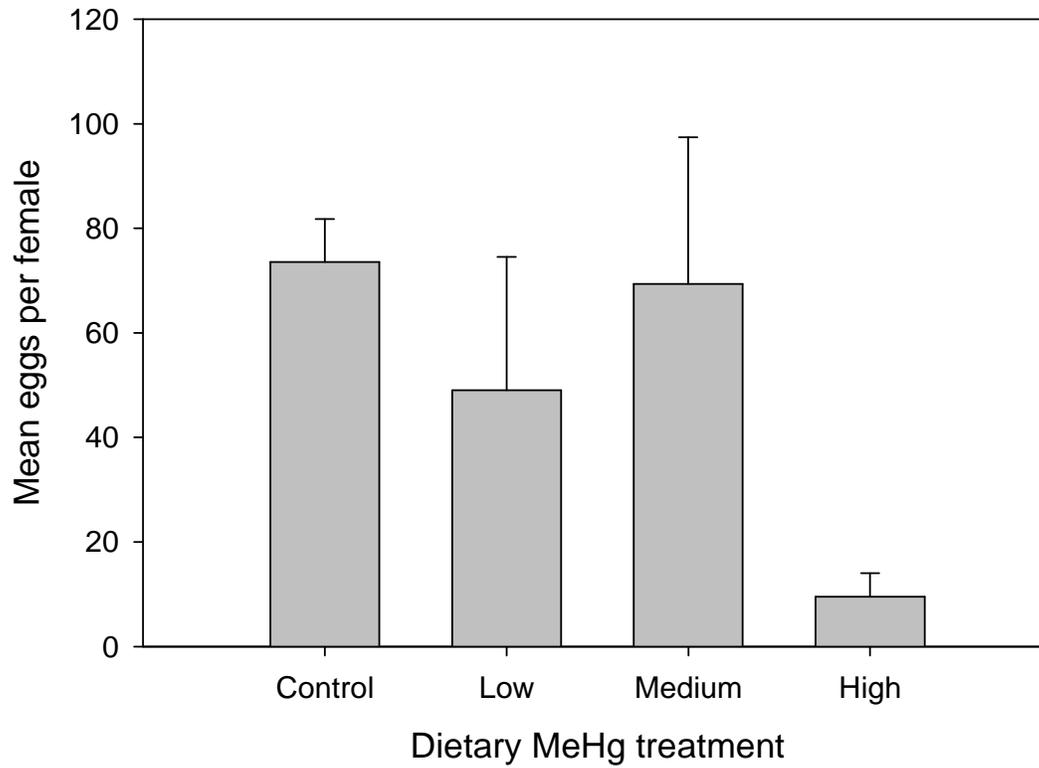


Figure 3.2. Mean number of eggs \pm 1 SE for females from each treatment: control (n=4), low (n=3), medium (n=3), and high (n=2). All values were statistically similar.

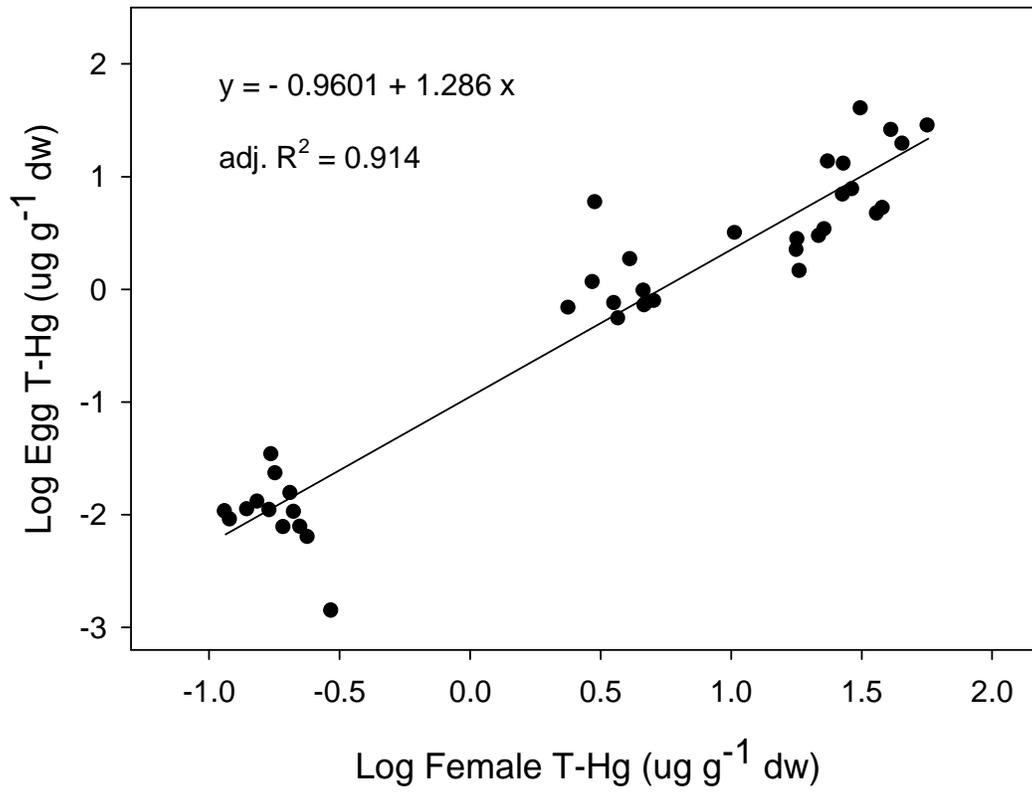


Figure 3.3. Linear regression analysis of mass specific T-Hg concentrations in female bodies versus eggs. Points represent individuals (n=39). All treatments are included.

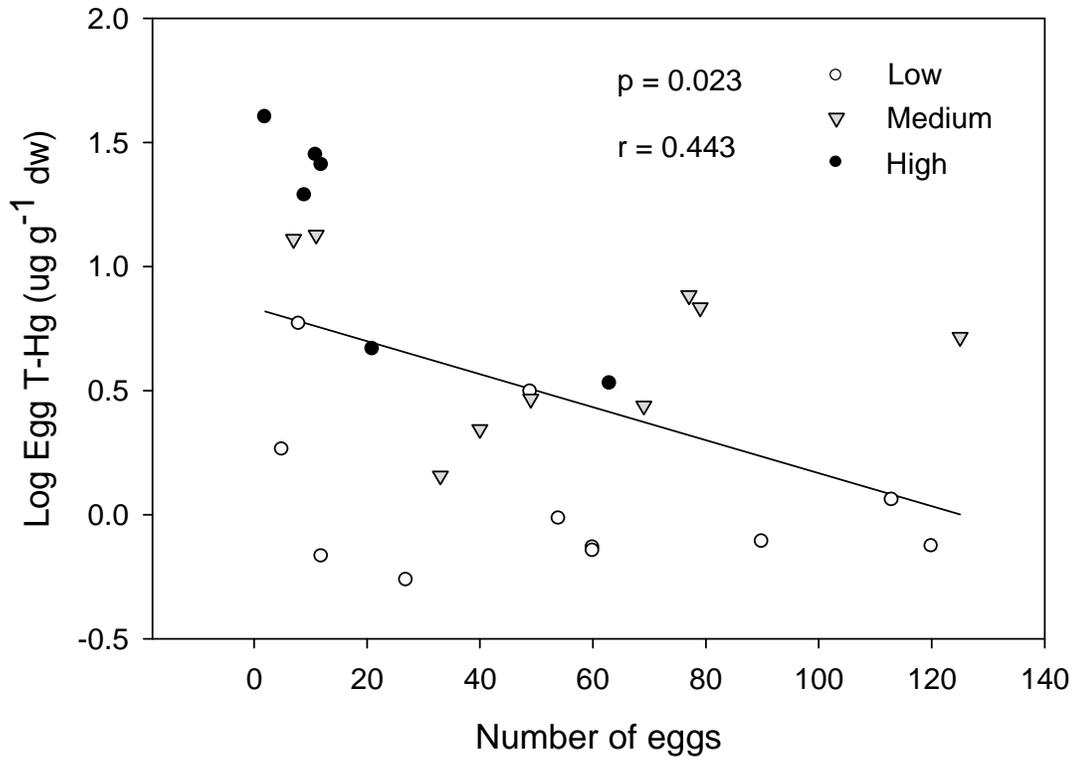


Figure 3.4. Correlation between the number of eggs in a given female and the mass specific T-Hg concentration of her eggs. Points represent individuals (n=26). Controls were excluded due to low concentrations.

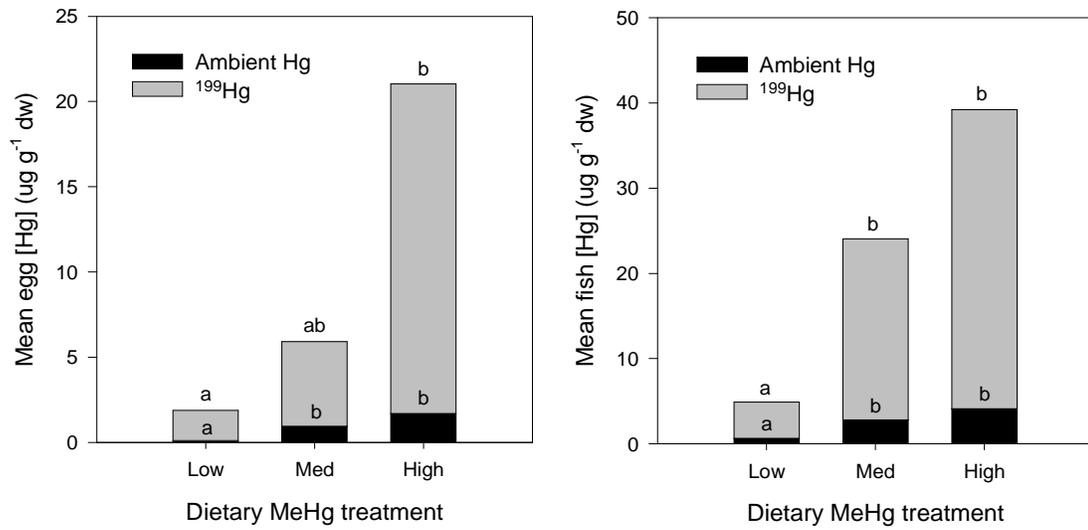


Figure 3.5. Mean concentrations of ambient Hg and ^{199}Hg in eggs (left) and fish carcass (right), for low (n=3), medium (n=3) and high (n=2) treatments. Different letters indicate significant differences among treatments.

Chapter 4: Conclusions, applications, and future research

This research sought to identify important variables affecting accumulation and maternal transfer of methylmercury in fish occupying low trophic levels. Mercury is a contaminant of concern in ecosystems, particularly its methylated form (MeHg) which readily accumulates in fish and other biota. Dynamics of dietary MeHg uptake in relation to fish lifestage, species, and level of exposure are poorly understood in lower trophic levels, particularly estuarine species. Furthermore, little is known about the transfer of this accumulated MeHg from female to offspring. These research questions were addressed in two chapters. Chapter 2 compared dietary MeHg accumulation, as well as growth and survival in two species of estuarine forage fish: *Cyprinodon variegatus* and *Menidia beryllina*. This experiment was conducted over a 70-day dosing period, and included 5 levels of dietary exposure. Chapter 3 examined the source of maternally transferred MeHg in *Cyprinodon variegatus* using a stable mercury isotope approach.

Chapter 2 demonstrated that growth rate and the level of dietary exposure strongly influence tissue concentrations of Hg in *Menidia beryllina* and *Cyprinodon variegatus*. Additionally, results showed that dietary bioaccumulation factors were not consistent across treatments, but rather, decreased with increasing dietary mercury concentration. Controls on bioaccumulation are important to identify in controlled laboratory studies, as they may be unclear or unidentifiable from field data. These findings have important implications for mercury bioaccumulation models. Models in previous studies have used a constant term for Hg assimilation (Trudel & Rasmussen

2001). However, results from Chapter 2 as well as other recent studies (Houck & Cech 2004; Unrine et al. 2004) suggest that the proportion of MeHg accumulated is not constant for a given size and age of fish. In the case of mercury, it appears that this model parameter should vary with dietary exposure.

Concentrations of MeHg in the control and two lowest treatments (0.04, 0.6, and 3 $\mu\text{g g}^{-1}$ respectively) were considered environmentally relevant. These spanned levels of MeHg found in benthic invertebrates (Hall et al. 1998) and crayfish muscle (Allard & Stokes 1989) from relatively uncontaminated systems. When comparing tissue concentrations reached in this study to those measured in young-of-the-year (YOY) white perch and largemouth bass from Maryland reservoirs, the lowest treatment achieved body burdens that were an order of magnitude higher than field-collected YOY fish (Figure 4.1). It is possible that wild fish are feeding on diets with lower MeHg levels than expected. Therefore future studies should use lower dietary MeHg concentrations to reach more realistic tissue concentrations in test species.

Both species tolerated tissue concentrations near 7 $\mu\text{g g}^{-1}$ ww, with little mortality (no mortality in *C. variegatus* and 2.5 % mortality in *M. beryllina*). This is a much higher threshold than expected. It appears that these species are resilient to Hg tissue concentrations greater than those observed in wild fish populations in contaminated areas. For example, catfish (*Ameiurus spp.* and *Ictalurus punctatus*) and largemouth bass (*Micropterus salmoides*) populations in the Savannah River have tissue concentrations that range from 0.3 – 1.0 $\mu\text{g g}^{-1}$ ww (Paller & Littrell 2007). Mercury levels in red drum (*Sciaenops ocellatus*) from Lavaca Bay (TX) have historically ranged from 0.5 – 5.7 $\mu\text{g g}^{-1}$ ww, with the maximum tissue concentration

occurring in 1977, when Hg contamination from a nearby chloralkali plant was high (Sager 1977).

Mercury concentrations in the diet of Lavaca Bay fish (Sager 1977) were similar to those in this study. T-Hg concentrations in estuarine algae, detritus, bivalves, polychaetes, and crustaceans, ranged from $0.5 \mu\text{g g}^{-1}$ ww to a maximum of $19 \mu\text{g g}^{-1}$ ww in some polychaetes and detritus (Locarnini & Presley 1996). However, consumers in this system (such as red drum described above) had lower mercury accumulation than fish in our study. This is likely due to differences in mercury speciation in the diet. Although Hg species were not reported in the study, Lavaca Bay benthic organisms likely contained both inorganic and organic mercury. A high proportion of inorganic Hg is typical in organisms occupying lower trophic levels (Francesconi & Lenanton 1992; Mason et al. 2000; Kehrig et al. 2001). Small forage fish, such as those in our study, feed on algae, zooplankton, and benthic invertebrates, which usually contain between 5 and 50 % MeHg (Francesconi & Lenanton 1992; Morel et al. 1998). The proportion of MeHg increases over trophic levels, reaching 95-99 % in higher level consumers (Morel et al. 1998; Mason et al. 2000; Kehrig et al. 2001). Fish consuming prey with high inorganic Hg content would likely accumulate less mercury than the individuals in our study, which consumed entirely MeHg. This is due to the higher assimilation efficiency of MeHg compared to Hg^{II} (Wong & Wang 2003).

It is important to expose organisms to realistic ratios of inorganic and organic mercury compounds in laboratory dosing studies. For high level consumers, such as piscivorous fish, a diet that contains a large proportion of MeHg is realistic. However,

for species occupying lower trophic levels, exposure to both inorganic and organic mercury is significant. Therefore, future studies should incorporate realistic proportions of mercury compounds in the diet. This is important not only for realistic accumulation, but also for realistic toxicological responses, as inorganic Hg and MeHg may have different mechanisms of toxicity.

Although our assessment of resiliency is only based on survival, future studies should focus on more sensitive sub-lethal endpoints, such as reproduction. It is likely that the tissue burdens observed in this study have adverse effects on reproduction, based on previous studies of a freshwater minnows with similar Hg burdens (Hammerschmidt et al. 2002). Sub-lethal effects of MeHg are also likely to vary between species. Chapter 2 demonstrated species-specific differences in mortality related to dietary MeHg exposure. There is a lack of species comparisons in mercury literature, therefore studies that compare the Hg-sensitivity of different species continue to be important.

Chapter 3 demonstrated the unique information that can be obtained from mercury stable isotope studies. In addition to tracing maternal transfer, the use of an enriched stable isotope allows us to calculate depuration of ambient mercury over time (Figure 4.2). This is another parameter in bioaccumulation models that is important, but poorly understood. Mercury mass balance models have used an elimination rate (ng / day) that is constant for a given fish size and water temperature (Trudel and Rasmussen 2001). However, it appears that fish mercury burden is also a controlling factor. In this study, *Cyprinodon variegatus* were capable of excreting a consistent percentage of their Hg body burden over time (Figure 4.2), causing

individuals in high Hg treatments to depurate a larger mass of Hg per day. A recent study also suggests that the exposure pathway affects elimination rates of metals. Depuration of cobalt appears to be dependent on both the route and duration of exposure (Mansouri et al. 2011).

Factors controlling Hg depuration are an important area of future research. The use of stable isotopes as tracers allows both accumulation and elimination to be measured simultaneously (Evans et al. 2002). In the future, stable isotopes could be used to label different exposure routes or compare accumulation and depuration at different time points, via multiple enriched isotopes. This type of technique is also useful for other metals, which could have varying dynamics of uptake and elimination.

Accumulation and depuration are important processes not only in the context of Hg exposure, but ultimately affect the amount of Hg transferred to offspring. Results from Chapter 3 indicate that a significant portion of maternally transferred Hg is from the burden stored in female tissues. This burden is directly related to the rate at which Hg is accumulated and eliminated, as well as the dietary history of an individual. It is clear from this study that recently ingested MeHg is not the only source of maternally transferred mercury and that historical Hg exposure is important when assessing potential exposure of offspring.

Although both species in this study appeared very resilient to dietary MeHg exposure, it is still unclear if MeHg can have significant effects on the reproductive fitness of fish in contaminated ecosystems. There is limited research on the effects of maternally transferred MeHg on offspring health, particularly in fish. Even though

maternal contribution is small, exposure to MeHg during sensitive early life stages could have effects on an individual's reproductive success later in life. Thus far, no studies have investigated reproduction in the *second generation* after MeHg exposure. Cross-generational effects of maternally transferred MeHg are important areas of future study, in order to better understand possible population-level responses.

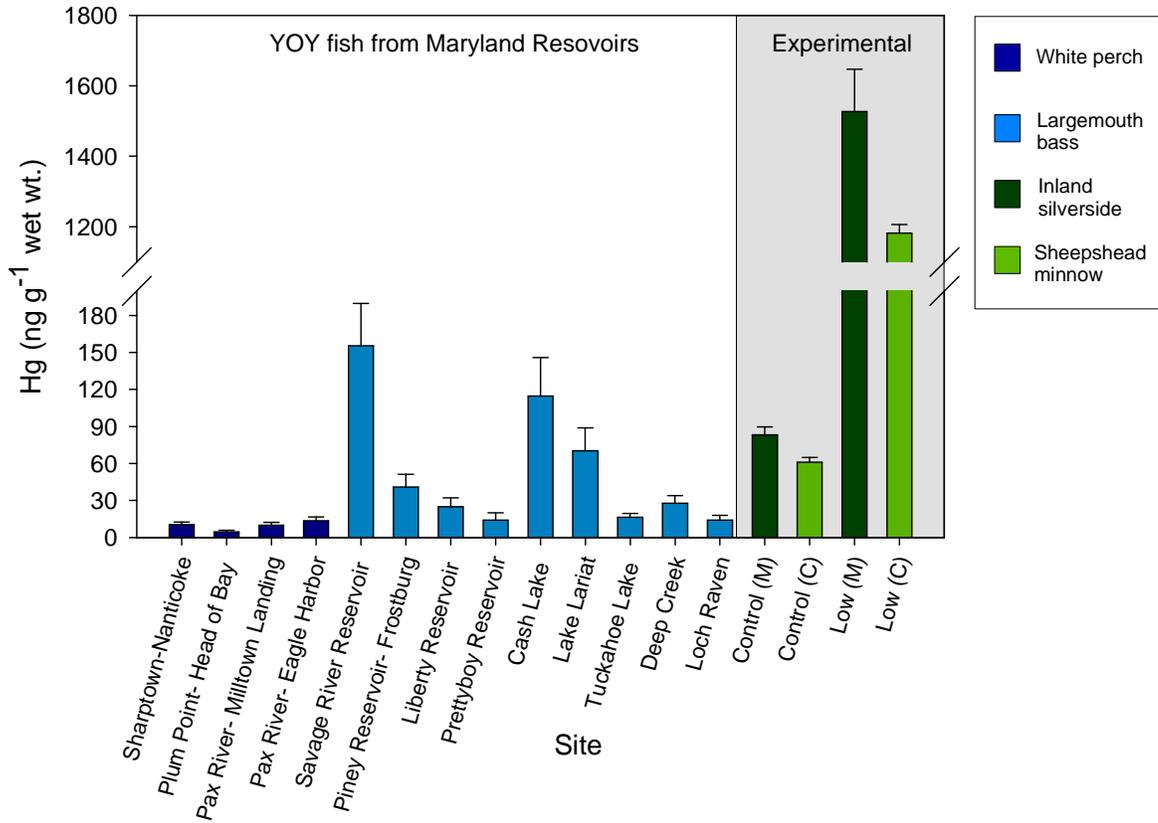


Figure 4.1. Mercury concentrations in young of the year fish in Maryland reservoirs from 2008 – 2010. Experimental concentrations in *M. beryllina* and *C. variegatus* in the control and lowest treatment are included for comparison.

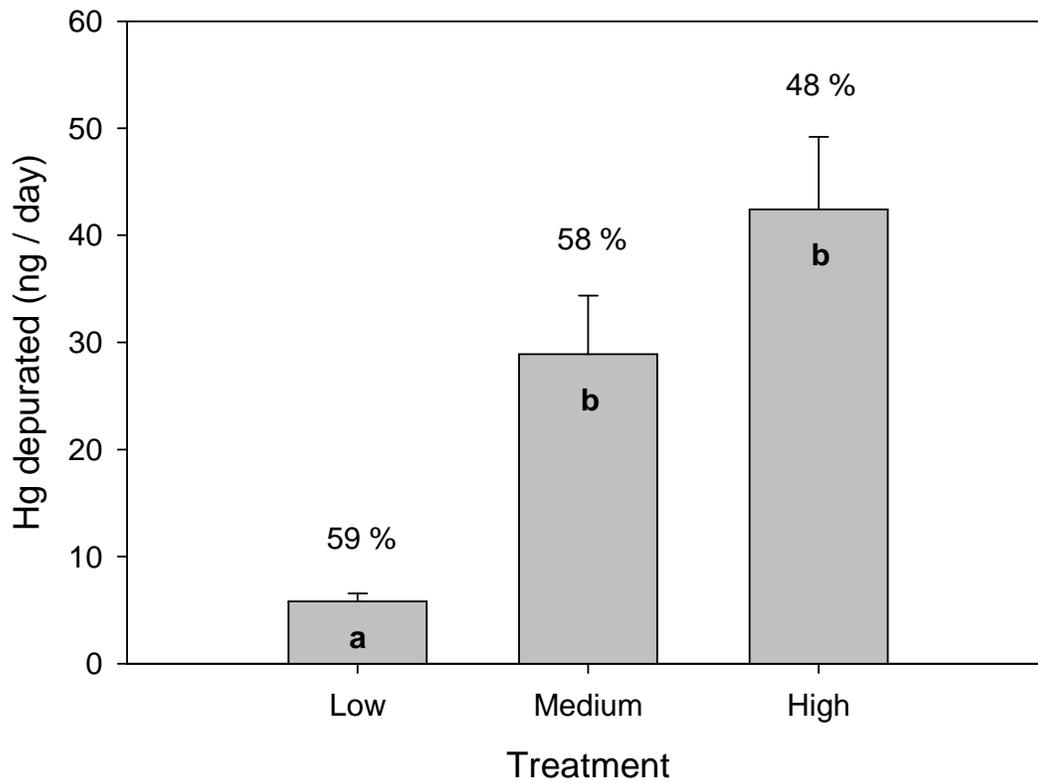


Figure 4.2. Estimated depuration of ambient mercury (ng/day) in each treatment, over the 63 days of ^{199}Hg dosing. Percentages of total burden depurated are also included. Different letters indicate significant differences in depuration (ng/day). Note that percentages did not differ among treatments.

Appendix: Mercury partitioning in water

Background

Many toxicological studies that focus on dietary exposure fail to measure aqueous levels of contaminants. Although diet is thought to be the major source of Hg in fish (Phillips & Buhler 1978; Hall et al. 1997), it is important to characterize all exposure routes in laboratory dosing experiments. In this study dosing was conducted in closed systems, which had the potential to accumulate Hg over time. Aqueous mercury likely included Hg partitioned from un-eaten food and feces, as well as Hg excreted by fish. Over time, demethylation of aqueous MeHg was expected; therefore it was important to measure both inorganic and organic Hg concentrations. We also determined the concentration of dissolved MeHg, as chemical form affects bioavailability.

Methods

Water samples were collected from each tank every two weeks, filtered through muffled 0.7 µm glass microfiber filters (GF/F, Whatman), preserved with 0.5 % HCl, and refrigerated for later analysis. Additionally, a sub-set of water samples were filtered to 3kDa via centrifuge ultrafiltration, in order to measure dissolved Hg. Water samples were analyzed for T-Hg and MeHg to track partitioning of Hg within aquaria over time.

Analysis of total mercury (T-Hg) was conducted on a Tekran Model 2600 Mercury Analyzer with a Model 2620 Autosampler (Tekran Instruments, Canada). The instrument measures T-Hg via cold vapor atomic fluorescence spectrometry

(CVAFS). Briefly, all mercury species within the sample were reduced to elemental mercury (Hg^0) by stannous chloride (SnCl_2). Hg^0 was then concentrated on a gold trap, thermally desorbed, analyzed by CVAFS, and quantified according to EPA method 1631 (US EPA, 1996).

Prior to analysis, excess oxidant was neutralized in samples with 10 ul of hydroxylamine hydrochloride. T-Hg standards were prepared from a NIST stock solution in concentrations of 5, 10, 25, 50, 75, 100, 150, and 200 ng L^{-1} . A matrix blank was prepared, according to the type of samples being analyzed. When analyzing fish samples, the matrix blank consisted of 0.2 % digest acid and 0.08 % BrCl and when analyzing water samples, the matrix blank consisted of 0.5 % BrCl. Quality control included calibration blanks, replicate standards and samples, duplicate dilutions, and duplicate SRMs (DORM-2, National Research Council Canada).

Water samples were acidified with 0.5 % sulfuric acid one day prior to analysis and KOH was used to adjust pH between 3 and 9 the following morning. Aliquots of sample were added to bubblers containing a citrate buffer (pH = 4.8). The solution was ethylated with sodium tetraethylborate, converting MeHg to gaseous methylethylmercury (Bloom 1989). This was purged from solution and concentrated on Tenax traps. Methylethylmercury was then thermally desorbed from traps, separated by gas chromatography, and detected by CVAFS (Tekran Model 2500). Each run included a set of standards (25-500 pg in volume), as well as blanks and sample replicates.

Summary of aqueous mercury

T-Hg concentrations were dose dependent and seemed to stabilize after approximately 2 weeks of dosing (Figure A.1). Aqueous mercury was slightly higher in *Cyprinodon variegatus* tanks, possibly due to higher waste loads. An average of 46 % of aqueous Hg was methylated on Day 15 and an average of 23 % was methylated on Day 70 (Figure A.2). Percentages of MeHg were consistent across treatments, with a lower proportion of MeHg at the end of the dosing period. This suggests that Hg was demethylated throughout the experiment. Furthermore, the half-life of inorganic Hg is much lower than that of MeHg. In a 3 g fish, the half-life of inorganic Hg is approximately 10 days, while the half-life of MeHg is over 250 days (Trudel and Rassmussen 1997). Therefore, it is likely that depuration contributed to the inorganic Hg load, in addition to demethylation. Approximately 50% of MeHg was in the dissolved phase (Figure A.3). Most aquaria had dissolved MeHg concentrations of 1-5 ng/L, which are concentrations that occur in natural waters.

Overall, waterborne mercury likely had little influence on fish Hg accumulation in this study. Previous studies have shown that the diet is the primary Hg exposure pathway in fish (Phillips & Buhler 1978; Hall et al. 1997), and that less than 0.1 % of accumulation is the result of direct uptake from water (Trudel et al. 2000). Additionally, there were low concentrations of dissolved MeHg, which is the form of Hg that would most readily accumulate in fish (Trudel et al. 2000).

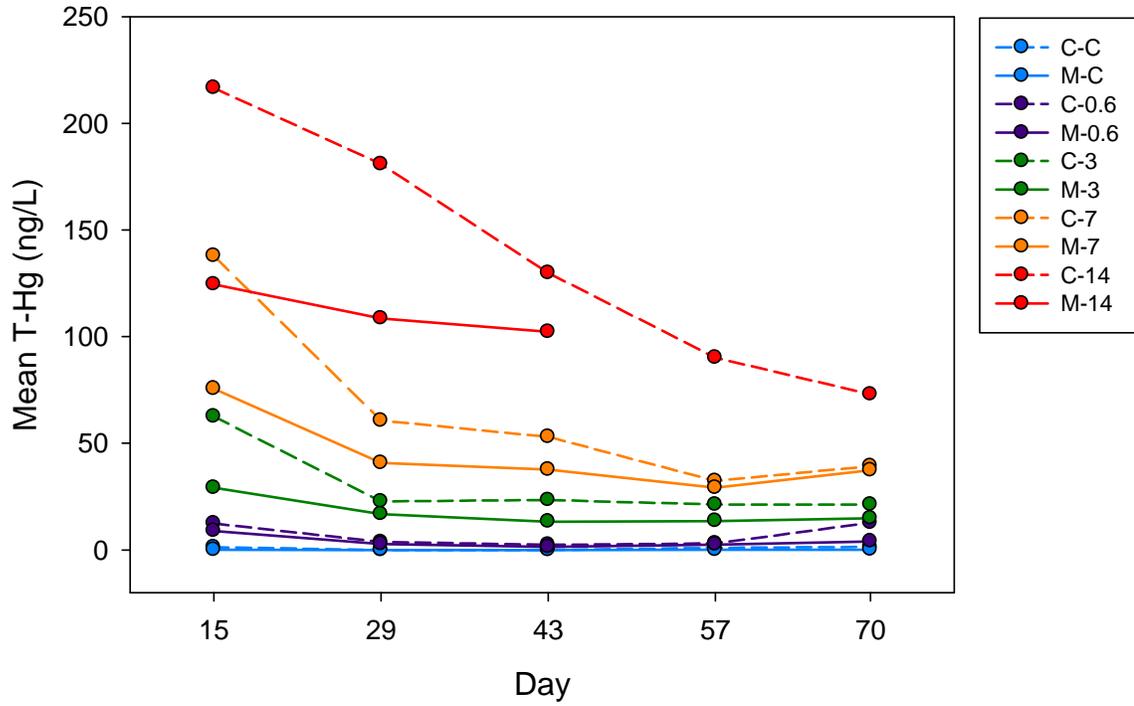


Figure A.1. Mean T-Hg concentrations in water over time. Each point represents a treatment average (n=3).

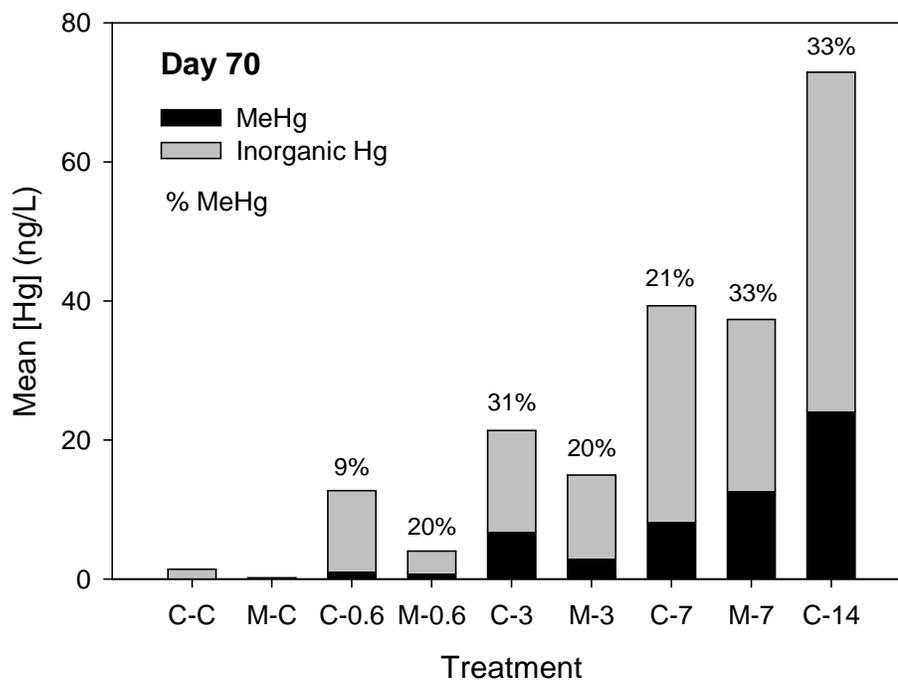
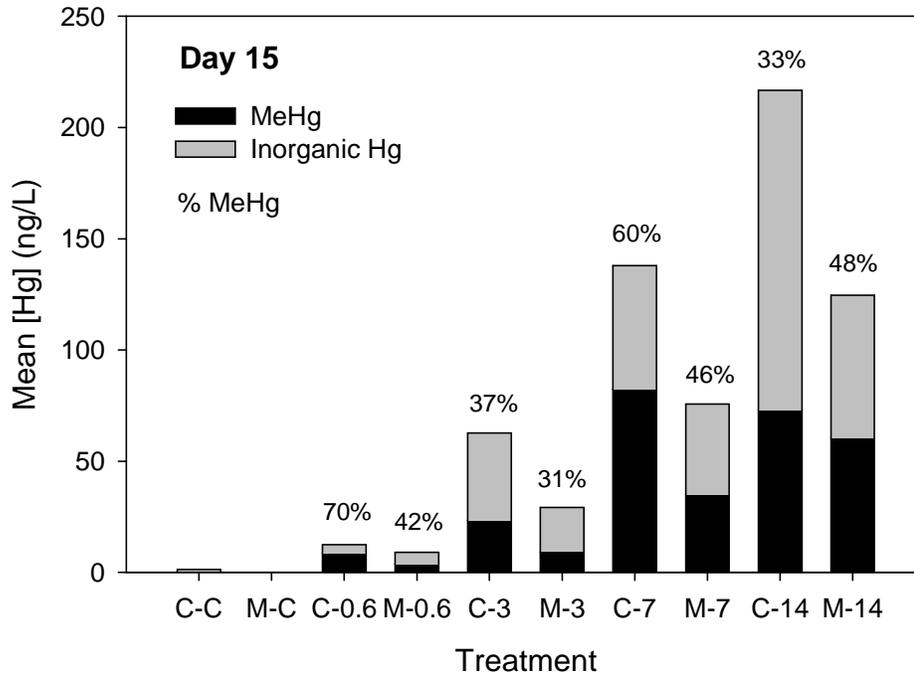


Figure A.2. Mean MeHg and inorganic Hg concentrations in each treatment for the first sampling point (Day 15- above) and the last sampling point (Day 70- below). Percentages represent the portion of T-Hg that was MeHg.

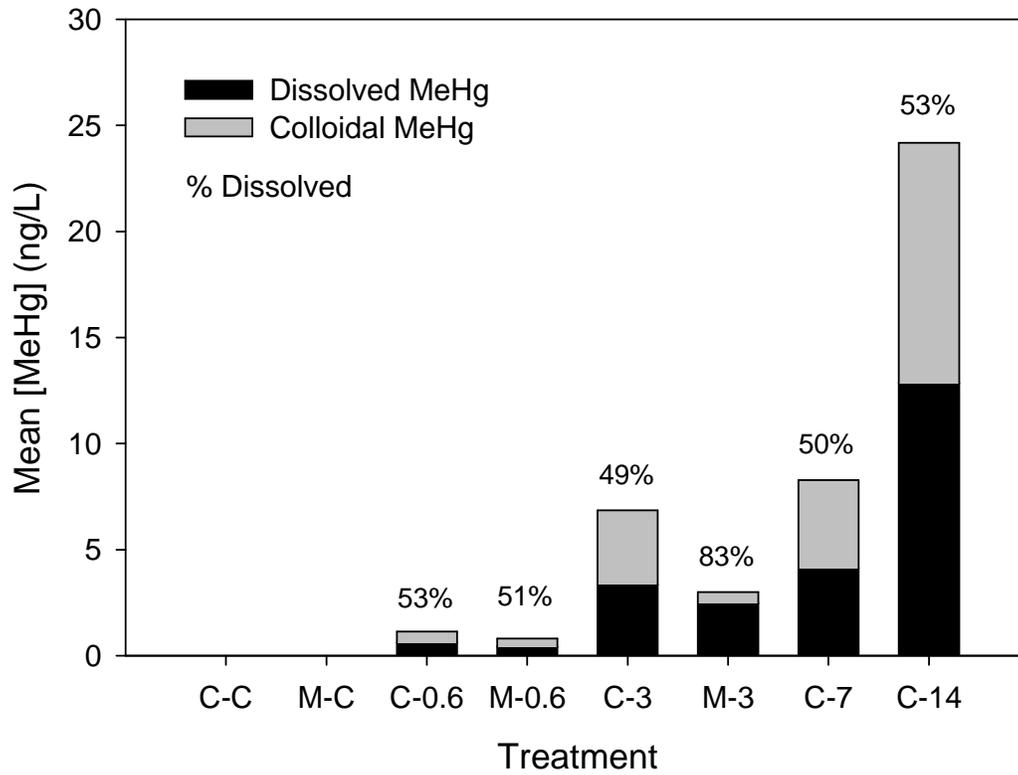


Figure A.3. Mean dissolved and colloidal MeHg concentrations for each treatment on Day 70. Percentages represent the portion of MeHg that was in a dissolved form.

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