INDUCTION OF METALLOTHIONEIN IN GRASS SHRIMP (*PALAEMONETES PUGIO*) EXPOSED TO NATURALLY OCCURRING METALS

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

In marine invertebrates, reliance upon metallothionein (MT) for toxic metal detoxification may lead to enhanced trophic transfer of metals. MT concentrations were determined in grass shrimp (*Palaemonetes pugio*) collected from metal contaminated field sites along an established metal impact gradient in Staten Island, NY, (field exposure), as well as in naïve *P. pugio* from a relatively pristine site in Tuckerton, NJ, that were fed prey from the same set of contaminated Staten Island sites via a feeding experiment (dietary exposure). MT concentrations in *P. pugio* collected from the Staten Island sites were elevated in comparison to naïve *P. pugio* from a pristine site in Tuckerton, NJ; however, the concentrations did not vary among shrimp from the impacted sites, possibly due to confounding effects of exposure to an array of contaminants, greater MT turnover or decreasing reliance on MT with increasing metal exposure. MT concentrations in naïve *P. pugio* fed prey from the same sites did not present increases in MT concentration over control following 3 weeks of feeding. For three of four sites, dietary exposure to metals may in large part account for MT induction in grass shrimp, as MT concentrations in field-collected at experimentally fed shrimp for these sites were similar. For one site in the former Fresh Kills landfill, MT concentration in field-collected shrimp was significantly higher, indicating that chronic environmental exposure at this site may supersede dietary exposure in the induction of MT in *P. pugio.* In order to parse the impact of metal exposure on MT concentration, analyses of whole body metals burden and trophically available metals, such as those bound to MT, organelles and heat-sensitive proteins, in the field-collected and dietarily exposed *P. pugio* is required.

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INTRODUCTION

The Hudson River Estuary has been subject to persistent ecosystem degradation from anthropogenic activities such as industrial discharge, combined sewer outflows, stormwater runoff, atmospheric deposition, chemical spills, and leachate from landfills (USACE et al. 2009). Within the estuary, the Arthur Kill/Kill Van Kull wetlands complex is located in one of the most intensively industrialized and urbanized corridors in the northeastern United States (Penhollow et al. 2006). Metal contaminants can persist in the harbor sediments long past mitigation of external sources of contamination. This is due to the interaction of freshwater and seawater, strong bottom currents and the frequent resuspension and upstream transport of contaminant-bound sediment, increasing contaminant accessibility to benthic and pelagic organisms (Lodge et al. 2015). Metal contaminants are of particular concern due to the risk associated with the consumption of contaminated seafood, which has led to several seafood consumption advisories (McGeer et al. 2003; Copat et al. 2013). Additionally, impacts of metal exposure can lead to health and ecological impacts resulting from toxicity, local extinction of metal-sensitive species and shifts in community structure towards more metal-tolerant species (Cain et al. 2004; Perez and Wallace 2004).

Induction of metal-binding proteins (metallothioneins - MT) is one mechanism by which metals detoxification occurs in an array of marine invertebrates (Amiard et al. 2006; Wallace et al. 2003) and is a valuable biomarker for metal pollution in a variety of freshwater and estuarine species (Cajaraville et al. 2000; Cheung et al. 2004; Linde et al. 2001). MT is a ubiquitous, highly conserved, low-molecular weight, cysteine-rich, heat stable protein that maintains cellular homeostasis of essential metals (Zn, Cu) and can

bind and sequester non-essential metals such as Cd and Hg, preventing DNA strand breaks and other sensitive intracellular damage (Vincent-Hubert et al. 2014). MT-metal binding is governed by its thiol group chemistry, and metals such as Cd, Hg, and Ag, which share the stoichiometric characteristics of Cu and Zn, also bind to MT (Templeton and Cherian 1991); however, binding of metal to proteins including MT may increase its bioavailability to predators. In prey organisms exposed to metal contaminants, metals bound to MT, organelles and heat-sensitive proteins have shown to have a greater bioavailability to predators and have been classified as part of a "trophically available metal" or TAM subcellular compartment. Conversely, metals detoxified through formation of metal-rich granules or associated with cellular debris are not as labile are not easily available to predators (Wallace and Luoma 2003). Thus, organisms using MT as a means of metal detoxification could represent an important vector of metal transfer up the food chain (Seebaugh et al. 2005). Greater reliance on MT induction to handle an influx of toxic metals may lead to 'bioenhancement' of metal trophic transfer, since MT-bound metal contained within the TAM compartment would be bioavailable to the organism's predators. This enhancement differs from biomagnification, which is a result of lipophilic metals accumulating in lipid reserves (Seebaugh et al. 2005).

Many studies have demonstrated that pollutant stressed ecosystems can develop communities dominated by pollutant tolerant species (Cain et al. 2004; Goto and Wallace 2010a). Reliance on MT for detoxification in a metal-tolerant community may increase the risk of metal exposure to predators of benthic organisms (Goto and Wallace 2009b). Metal bioenhancement has been demonstrated to occur in laboratory conditions: in response to an environmentally realistic Cd exposure via solution, an increase in TAM-

[Cd] resulting from greater metal binding to heat sensitive proteins resulted in the bioenhancement of Cd transfer to *P. pugio* and mummichog (*Fundulus heteroclitus*) (Seebaugh et al. 2005). In a field setting in creeks adjacent to the heavily polluted Arthur Kill, a tidal strait between NJ and NY, high trophic availability of organic Hg (TAM-associated organic Hg) has been shown in sites with the greatest benthic invertebrate biomass, exceeding the expected availability from sediment organic Hg concentrations at these sites by over two-fold (Goto and Wallace 2009b). Additionally, at various Arthur Kill sites, site-specific benthic community structure has been shown to shift toward metal tolerant species (Goto and Wallace 2009a; Goto and Wallace 2010a; Goto and Wallace 2010b). Hence, the linkage between sediment bound metal and metal trophic transfer up food chains may be driven in a large part by site-specific community structure and that community's collective metal handling strategies.

Study Organism

The grass shrimp (*Palaemonetes pugio*) is an important benthic-pelagic coupler (benthic feeding predator that is prey to more pelagic species) in the Hudson River Estuary (Welsh 1975). Grass shrimp play a vital role in estuarine food webs as they link benthic associated carbon (and pollutants) to higher trophic levels since they are important components of the diets of several finfish, including the mummichog and striped bass (Welsh 1975; Davis et al. 2003). Feeding experiments with *P. pugio* using oligochaete prey collected from Cd-contaminated sites have established that grass shrimp can accumulate toxic metals from ingested prey, can induce MT upon metal exposure, and the accumulated metal can be transferred to their predators (Wallace et al. 2000; Seebaugh et al. 2005). The induction of MT at this critical step in the food chain has the

potential to propagate a bioenhancement of trophic transfer of benthic metal (Seebaugh et al. 2005). *P. pugio* is therefore an ideal species to investigate the linkage between sediment metal, metal accumulation in benthic invertebrates, induction of MT, and the translation of these impacts to higher trophic levels.

Study Sites

The Arthur Kill/Kill Van Kull complex of the Hudson-Raritan Estuary has several tributaries and offers shallow refuge habitat for winter flounder, black sea bass, red hake and other important marine and estuarine fish species. Additionally, it contains deepwater habitats which support a variety of migratory and resident fish species (USACE et al. 2009). Estuarine tidal marshes, including three tributaries of the Arthur Kill tidal strait and one creek at an external site, were sampled for benthic invertebrates and grass shrimp, *P. pugio,* to represent an environmental impact gradient of toxic metals. Sampled sites included the highly-impacted Mill Creek at the confluence of the Arthur Kill and the Raritan Bay. Mill Creek has been historically polluted with toxic metals owing to its proximity until 1970 to the former Nassau Smelting and Refining Company facility (Crawford et al. 1995; Carmody et al. 1973). Another site, Main Creek, was chosen due to its location within the former Freshkills landfill. Neck Creek, a medium-impacted site that is part of the Meredith Wood Park and well within the Arthur Kill proper, was damaged severely by an oil spill in 2001 (Packer 2001) and is in close proximity to several abandoned industrial facilities. Lemon Creek, located on the south shore of Staten Island is a relatively pristine location and represented a low-impacted regional reference site. This environmental impact gradient (Lemon<Neck<Main<Mill) (Figure 1) has been

effectively used in previous studies of field-based toxicological impacts on resident biota (Perez and Wallace 2004; Goto and Wallace 2009a; Seebaugh et al. 2011).

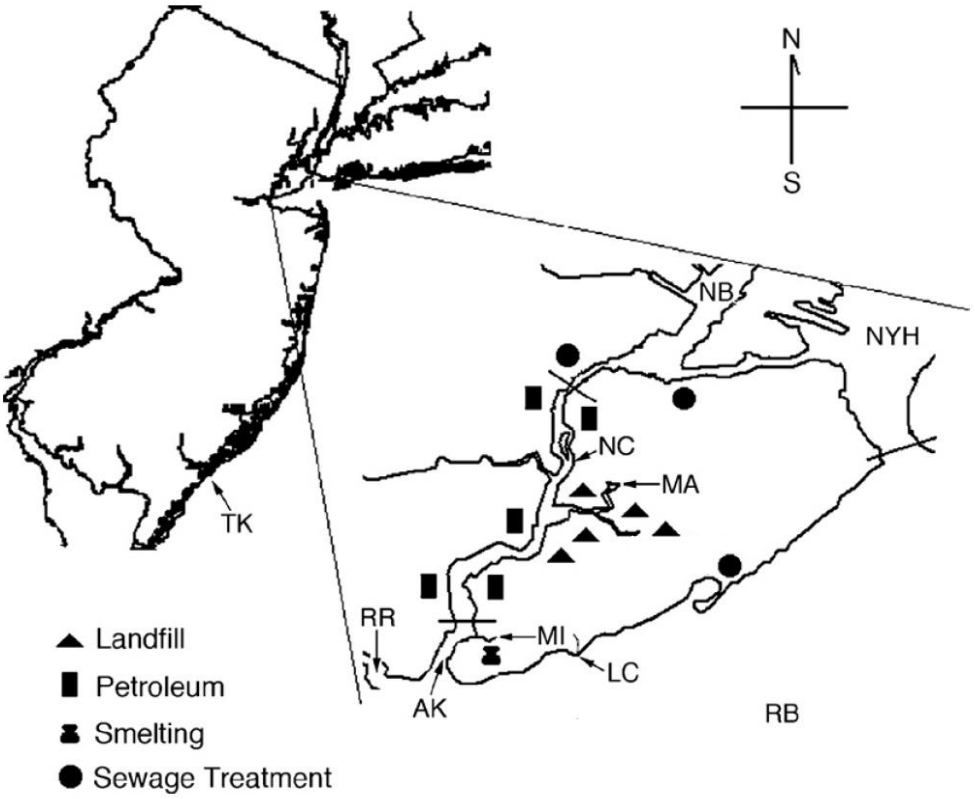

Figure 1: **Map of sampling sites for *P. pugio* and invertebrate prey relative to major waterways. External, relatively pristine site: Tuckerton, NJ – TK. Staten Island Sites: Mill Creek – MI, Main Creek – MA, Neck Creek – NC, Lemon Creek – LC. Also shown: Newark Bay -NB, New York Harbor -NYH, Raritan Bay - RB, Raritan River - RR and Arthur Kill - AK. Adapted from Perez and Wallace (2004); Goto and Wallace (2010a).**

Objectives

Previous studies examining the induction of MT in *Palaemonetes* sp. have often exposed organisms to metals in a laboratory setting with subsequent analysis of MT (Kraus et al. 1988; Howard and Hacker, 1990; Wallace et al. 2000). The impact of dietary metal exposure on MT has been explored by using field-contaminated ragworms (*Nereis diversicolor*) as prey to induce sublethal responses in *Palaemonetes varians* in response to elevated Cu and Zn levels in diet (Rainbow and Smith 2013). A sensitive Cd response has also been noted in *P. argentinus* collected from a metal contaminated site (Boudet et al. 2013). The MT response in rainbow trout (*Oncorhynchus mykiss*) to dietary exposure has been investigated via injection of Cd solution through a catheter (Chowdhury et al. 2005); however, the response of MT to dietary exposure via naturally accumulated metals in invertebrate prey has not been quantified in *P. pugio*. In this study, a 'natural laboratory' approach was employed to quantify MT in *P. pugio* in response to the ingestion of metals associated with a realistic and representative subset of site-specific benthic invertebrates collected along an established metal impact gradient. A feeding experiment was designed to expose naïve *P. pugio* to prey from metal polluted creeks of the Arthur Kill tidal strait in Staten Island, NY, and whole-body MT concentrations were quantified following this dietary exposure. Additionally, *P. pugio* were collected from the same field sites as the prey, and the MT concentration in their tissue was compared to that in experimentally fed *P. pugio* to discern the relative significance of dietary metal exposure from chronic environmental metal exposure via food, water, and sediment in the induction of MT.

It was hypothesized that MT concentrations in *P. pugio* that were fed prey from sites along the metal impact gradient would mimic the trend in contamination. Prey residing in more contaminated sites would have had greater toxic metal availability from site sediment, a portion of which would be compartmentalized into TAM. *P. pugio* feeding on prey with greater TAM metal loads would encounter greater toxic metal availability, resulting in increased MT-bound metal and hence MT concentration. In *P. pugio* collected directly from the contaminated field sites, a similar trend in MT concentrations was hypothesized, mimicking the site impact gradient, but with lower MT concentrations overall, since environmental metal exposure would be limited by the proportional abundance of non-bioavailable metals. In the dietary exposure of naïve *P. pugio*, selective feeding of normalized amounts of prey tissue would allow for concentrated 'dosage' of bioavailable toxic metals present in prey tissue, possibly leading to relatively higher MT concentrations in dietarily exposed *P. pugio.*

METHODS

General Framework

The concentration of metallothionein was determined in (a) *P. pugio* collected from the four metal contaminated field sites in Staten Island previously described (field exposure), and in (b) naïve *P. pugio* from a relatively pristine site in Tuckerton, NJ, that were fed prey from the same set of contaminated Staten Island sites via a feeding experiment (dietary exposure). Following both field collection and dietary exposure, *P. pugio* were depurated, frozen, and analyzed for MT.

P. pugio from Staten Island sites along a metal impact gradient

P. pugio were collected at low tide from four Staten Island sites representing a metal impact gradient (Lemon Creek, Neck Creek, Main Creek, and Mill Creek: Figure 1) by push netting and were transferred to the laboratory in site-specific seawater. *P. pugio* were maintained in site-collected seawater with gentle aeration for ~2 hours at 20^{o}C in 20 L buckets, and were acclimated to laboratory conditions by gradual replacement of site-water with laboratory-prepared 15 ppt seawater (20^{o}C) over a period of 4 hours. Non-gravid adults measuring ~3 cm were removed with a net and placed in labelled, acid-washed aquaria containing 15 ppt seawater at 20^{o}C with gentle aeration (aerated for two days in advance). After 12 hours, they were fed commercial fish flakes. After 24 hours, they were removed, size sorted, and stored at -80^{o}C to await analysis of stable metals (separate project) and MT by the mercury saturation protocol described ahead.

Dietary exposure of naïve *P. pugio*

i) Collection and processing of naïve *P. pugio* for the feeding experiment

Naïve grass shrimp for use in the feeding experiment were collected from a relatively pristine site at the Rutgers University Marine Field Station in Tuckerton, NJ, (TK, Figure 1) at low tide by push netting and were transferred to the laboratory in TK seawater as described above. Non-gravid adults (~3 cm) were first collected and similarly acclimated, and then maintained for ~2 weeks in two large aquaria 15 ppt seawater (20^{o}C), fed commercial fish flakes, and subsequently used in the feeding experiment.

i) Field Sediment Collection

Reference sediment was collected from the relatively unpolluted TK site, sieved through a 300 µm screen and used for use in the purge technique described ahead. To obtain prey for the feeding experiment with naïve *P. pugio*, bulk sediment from the top ~15 cm of the four Staten Island sites was first sampled. Sediment was collected into 20-L buckets from 3-4 locations within each Staten Island creek at low tide and transported to the laboratory with site-specific seawater for processing.

ii) Laboratory Bulk Cultures and Harvesting of Macroinvertebrate prey

'Bulk cultures' were set up from the raw sediment (containing sediment, organisms and debris) from the Staten Island sites. Sediment was stored in covered 200-L storage containers, maintained at 20^{o}C and in 15 ppt seawater and were exposed to artificial light for 12 hours/day. The bulk cultures were fed two teaspoons of commercial powdered rice cereal twice weekly for approximately eight weeks. Their salinity was monitored, and half of the seawater was replaced bi-weekly. To harvest macro-

invertebrates, sediment from each bulk culture was sieved through a 500µm screen and the material retained (debris, large-grain sands, and benthic macroinvertebrates, >500 µm) was spread into large collecting trays with 15 ppt seawater. All live organisms <1 cm in width were collected using forceps and 3 mL graduated plastic pipettes (bivalves and gastropods >1 cm in length were excluded). Immediately after collection, organisms were placed in holding aquaria containing sediment from the external reference site TK (< 300 µm) for 24 hours to exclude or 'purge' any contaminated sediment from the invertebrate's surfaces and intestines. This approach normalized sediment metal loads associated with prey from the different Staten Island sites. The organisms were then sieved out of the sediment using a 500 µm screen, rinsed with clean 15 ppt seawater, gently dabbed dry and stored in acid-washed glass scintillation vials at -80^{o}C.

iii) Preparation of Food Rations

A preliminary experiment was conducted to assess dietary organic carbon (OC) needs for grass shrimp. Nine adult *P. pugio* were individually placed in 250 mL beakers containing 15 ppt seawater. Varying amounts of live sewage worms (*Tubifex tubifex*) were added to the beakers (10 mg, 20 mg and 30 mg wet weight, three replicates each). After 24 hours, the grass shrimp and their fecal matter were removed, and the remaining *T. tubifex* OC was weighed. The experiment validated the 20 mg ideal wet weight of prey needed to feed one grass shrimp/day that was used in a previous study (Wallace et al. 2000). To control for the variability in organic content in the invertebrates collected from different sites, the OC content of 20 mg of brine shrimp (*Artemia sp.*) (prey for Control tanks in the feeding experiment described further) was set as the ideal OC level for each 20 mg 'meal' regardless of site (~8.3 mg OC/shrimp/day).

To prepare food rations, all collected organisms were partially thawed, mixed together and weighed. If prey included gastropods and bivalves, these were lightly crushed in a mortar and added to the mixture. This sample was divided into weekly portions and further into daily portions based on the dietary requirements of *P. pugio* as described above. To make daily portions, the bottom edge of a plastic Ziploc® bag was first marked in 20 sections. The weekly portion was added and pushed to the bottom of the bag using two rulers, forming an even line of prey. The bag was then sealed with a heat sealer and frozen at -80^{o}C. Frozen prey bags were then sliced at the marked intervals, producing 20 meals - 18 "tank meals" for the feeding experiment (three replicate tanks/treatment x six days/week), one random meal for TAM analysis and one random meal for OC analysis. To estimate OC content, one meal was dried at 60^{o}C for 24 hours, then incinerated in a muffle furnace at 500^{o}C for six hours. Following preliminary analysis of OC in site-specific prey, the OC weights for meals from all sites were adjusted ~8.3 mg by trial and error and meals were stored at -80^{o}C in acid washed 24-well trays.

iv) Feeding Experiment

For the feeding experiment, fifteen 9 L tanks were set up with 7 L of aerated 15 ppt seawater at 20^{o}C, carbon filters and plastic grids for habitat enrichment. The tanks were labeled as follows: three replicates (A, B and C) for the four sites (Lemon Creek, Neck Creek, Mill Creek and Main Creek) and for the Control treatment. Initially, 16 naïve *P. pugio* (from TK) were added to each tank, fed commercial fish flakes and allowed to further acclimate for 24 hours. One shrimp from each tank (three per treatment) was then collected, dabbed dry and stored at -80^{o}C to await MT and T_0 metal

analysis. The remaining 15 *P. pugio* were fed the prepared site meals for six days/week, with a planned experimental length of eight weeks, for which ~50 g of tissue was collected and processed from each Staten Island site. The experiment was started in a staggered fashion by initiating three treatments on day One and the two remaining treatments on day Two. At 1:00 pm on each feeding day, debris and waste from the previous day's feeding was siphoned, ½ seawater was renewed with fresh 15 ppt seawater maintained at 20^{o}C and a new 'tank meal' was added. Consumption of crushed gastropod and bivalve tissue was confirmed. Live *P. pugio* were counted twice a week and were removed in the event of mortality. A minimum threshold of six shrimp/tank was set. In response to a high level of conspecific feeding in the shrimp being fed Lemon Creek and Mill Creek prey, this threshold was reached for some tanks in three weeks and the experiment was concluded at the three-week mark. The staggered start allowed for the experiment to end over a course of two days, with three site treatments ending on day One, and the remaining two ending on day Two, each followed by a day of depuration with commercial fish flakes as prey. Upon removal from tanks, *P. pugio* were washed in fresh 15 ppt seawater, weighed and frozen at -80^{o}C until analysis.

Determination of metallothionein concentration

For *P. pugio* collected from the Staten Island sites, metallothionein (MT) concentration in six replicate samples with three shrimp each were analyzed per site, yielding 24 samples (six Lemon Creek + six Neck Creek + six Main Creek + six Mill Creek = 24 samples). The T_0 subset of *P. pugio* (naïve TK shrimp, n=15) yielded five samples of three shrimp/each. For the feeding experiment, two replicate samples with 3 shrimp/each were analyzed from each tank, yielding six replicate samples per treatment

and 30 samples in total (six Control + six Lemon Creek + six Neck Creek + six Main Creek + six Mill Creek = 30 samples).

The concentration of MT within grass shrimp tissue was analyzed using the radioactive mercury saturation technique (Dutton et al. 1993; Klaverkamp et al. 2000; Cooper and Fortin 2012). This approach relies on the high binding affinity of mercury (greater than that of other metals Zn, Cd, Cu) to metal-binding sites within MT (Roesijadi 1992; Dutton et al. 1993). *P. pugio* from each tank were thawed on ice, weighed, and placed in a 30-mL centrifuge tube. TRIS buffer (25mM Omnipur, maintained at 4ºC and adjusted to pH 7.2) at a tissue:buffer ratio of 1:19 was added. The mean wet weight for all sets of three was 0.78115 ± 0.01 g. The tissue was subsequently homogenized using a Polytron® tissue homogenizer. The homogenate was then vortexed and two subsamples were immediately taken from each homogenate: a 5 mL subsample for use in the MT analysis protocol described below, and a 2 mL subsample that was dried in a glass scintillation vial at 65ºC for 48 hours to determine the dry/wet weight ratio. The dried 2 mL subsample and the remaining homogenate were processed for analysis of total *P. pugio* metal burden and TAM/non-TAM metal analysis respectively (separate project).

Subsamples of 5 mL were placed in 15 mL centrifuge tubes and heat treated at 95 ºC for five minutes to denature non-MT (heat-sensitive) proteins. MT was isolated from the homogenate by centrifugation at 30,000 x g for 30 minutes at 4ºC and collecting the supernatant (pellet discarded). Four replicate 1.5 mL Eppendorf tubes were set to receive the homogenate subsamples, with 200 µL of supernatant added to each tube. To initiate mercury saturation of MT, 200 µL of Hg working solution containing ^{203}Hg-labelled $HgCl_2$ (300,000 cpm ^{203}Hg and 10 µg $HgCl_2$ in 10% trichloroacetic acid) were

added to each Eppendorf tube. The tubes were then incubated on ice for 15 minutes. To remove excess mercury, 400 µL of 50% (w/w) egg white solution in 0.9% NaCl were added to each tube, followed by centrifugation at 16,800 g at 4°C for 20 min. As a measure of quality control, replicate tubes for total cpm added, blank cpm and an MT standard (prepared from rabbit liver MT-2, Enzo) were processed similarly and subjected to MT saturation. Following centrifugation, supernatants and pellets were separated and placed in a gamma counter (Perkin Elmer-Wallac Wizard 1480) and analyzed for 60 seconds each. To correct for propagated counting error in the case of low sample activity, the counting time was appropriately increased to yield the cpm value. The percent recovery for the MT standard will be quantified in the future using ICP-MS.

Metallothionein was quantified within each homogenate using the Hg sequestration capacity of the tissue, using the following equation:

$$nmol\ metal\ binding\ sites \cdot g^{-1}\ tissue\ (wet\ weight) = [(replicate\ cpm - average\ blank\ cpm) / average\ total\ cpm] \times D \times C$$

Where *D* is the dilution factor (20) and *C* is the nominal concentration of stable Hg in a dose of 200 µL Hg working solution (249.265 $nmol \cdot mL^{-1}$) (Cooper and Fortin 2012; Klaverkampa et al. 2000).

Statistical Analysis

All data are expressed as mean ± standard error. The assumptions of parametric tests (single-sample ANOVA and Student's t-test) were checked using Shapiro-Wilk's *W* test of normality and Leven's test for equality of variances. When these assumptions were not met, non-parametric alternatives were used, including the Wilcoxon signed-rank test

and Kruskal-Wallis one-way ANOVA. In the case of a rejection of a Kruskal-Wallis null hypothesis, the Conover-Iman test for stochastic dominance was used as a post hoc test. In order to control for the false discovery rate, the obtained *p*-values were adjusted using Benjamini-Hochberg method. The Conover-Iman test has shown to be more powerful than Dunn's test for non-parametric post hoc analysis (Conover and Iman 1979; Conover 1999).

Graphing and analyses were conducted using RStudio (Version 1.0.143) and Microsoft Excel (Version 1704). In RStudio, the ggplot2 and plyr packages were used to create plots with summary statistics; the Rcmdr, FSA and conover.test packages were used to carry out statistical analyses. The level of statistical significance was set as alpha = 0.05.

RESULTS

Metallothionein in *P. pugio* from Staten Island sites along a metal impact gradient

Whole-body MT concentrations of field-collected *P. pugio* are presented in Figure 2. All concentrations are expressed in terms of nmol binding sites of MT/wet weight of shrimp tissue (g). At T_0, the MT concentration in naïve grass shrimp from TK was measured to be 12.11 ± 0.78 nmol binding sites/g tissue. MT concentrations in the tissue of grass shrimp collected from Lemon Creek, Neck Creek, Main Creek, and Mill Creek were: 18.80 ± 1.9; 17.64 ± 1.2; 34.43 ± 6.95; and 19.42 ± 1.82 $nmol \cdot g^{-1}$ respectively.

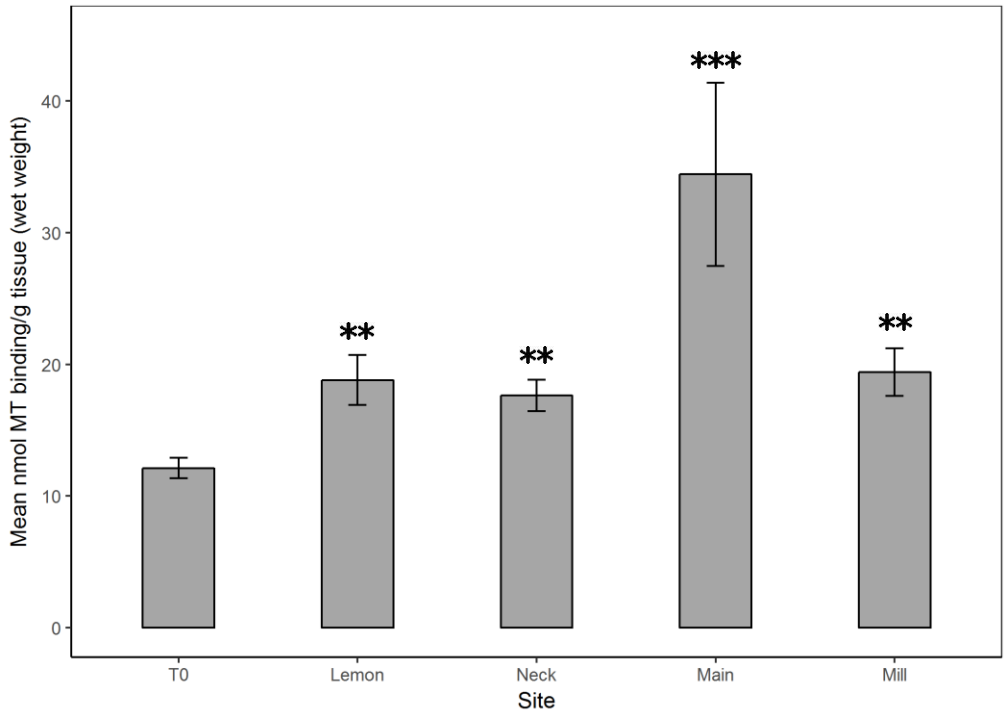

Figure 2: **Metallothionein binding site concentrations in *P. pugio* ± S.E. at T_0 (naïve TK *P. pugio*), in *P. pugio* collected from the sites (Lemon Creek, Neck Creek, Main Creek, Mill Creek). Asterisks indicate significant differences in MT concentrations compared to MT in T_0 grass shrimp (** $p<0.01$, *** $p<0001$).**

A Kruskal-Wallis one-way ANOVA revealed significant differences in MT concentrations among grass shrimp collected from different sites (*chi-squared* = 13.35, $p < 0.01$; including T_0). Pairwise comparisons from a post hoc analysis of MT concentrations in field-collected *P. pugio* using the Conover-Iman test are presented in Table 1. MT concentrations in field-collected *P. pugio* was significantly higher in Main Creek shrimp ($p < 0.001$) and significantly higher in Lemon Creek, Neck Creek and Mill Creek shrimp ($p < 0.01$), than in T_0 shrimp (naïve, from TK).

Table 1: Conover-Iman t-test statistics and significance from multiple pairwise comparisons of MT concentrations (nmol binding sites/g wet weight tissue) in field-collected *P. pugio* (Benjamini-Hochberg corrected p-values)

	Lemon Creek	Neck Creek	Main Creek	Mill Creek
TK (naïve)	3.09**	2.92**	-4.53***	3.38**
Lemon Creek	-	0.17	-1.52	-0.30
Neck Creek	-	-	-1.69	-0.48
Main Creek	-	-	-	-1.21

** $p < 0.01$.
*** $p < 0.001$.

Dietary exposure of naïve *P. pugio*

Standardization of meal organic carbon content

The mean wet, dry and OC weights of randomly sampled "tank meals" from each treatment of the feeding experiment are shown in Figure 3. While the wet weights and dry weights for randomly sampled tank meals varied among treatments (wet: 0.15 – 0.20 g; dry: 0.03 – 0.12 g), the OC content for the meals were close to the OC target (OC of control meals of 8.3 ± 0.36 mg). T-tests comparing the OC content of various sites with that of the target (Control meal OC) showed no significant differences for meals of different sites (Table 2).

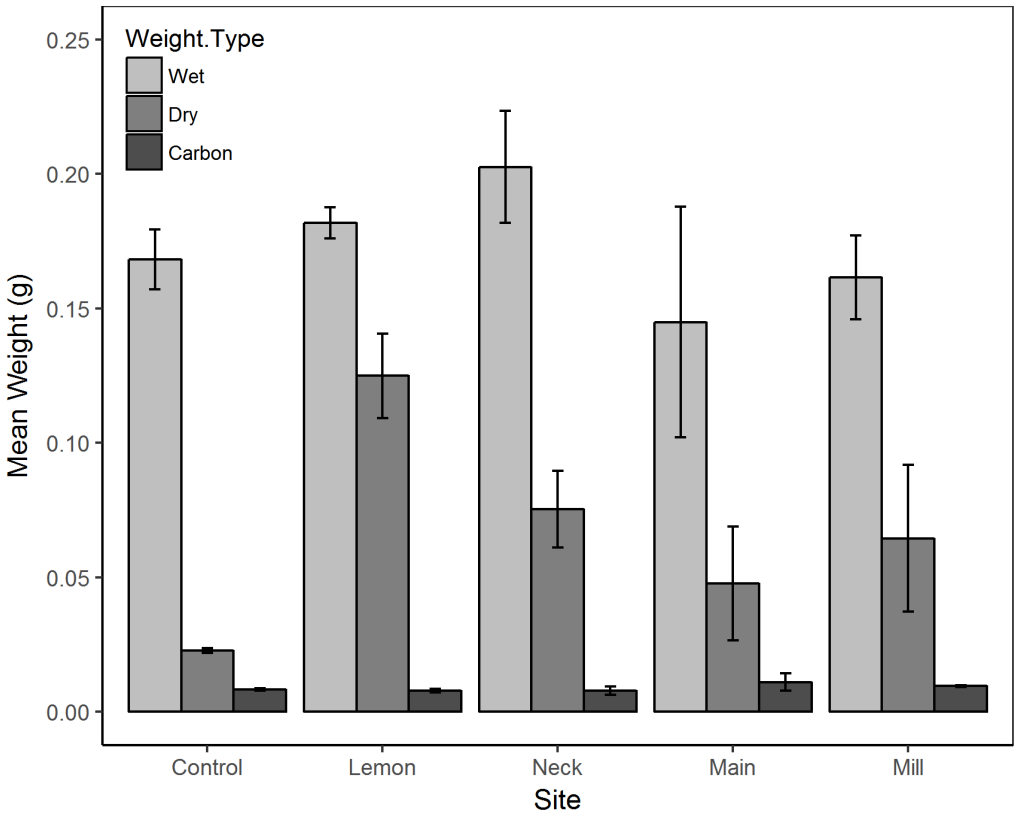

Figure 3: **Mean wet (light gray), dry (medium gray) and carbon (dark gray) weights ± S.E. for randomly selected meals (1/week of the feeding experiment). Mean Carbon weight of the Control meal (8.3 ± 0.36 mg) was the organic carbon target for site meals.**

Table 2: **T-test statistics for Organic Carbon Weight Comparisons of meals from field sites to Control meals (all non-significant)**

	Lemon	Neck	Main	Mill
Control	0.54	0.33	-0.83	-2.66

Feeding Experiment

Bi-weekly counts of surviving *P. pugio* during the feeding experiment revealed similar final survivorship in the Control, Main Creek, and Neck Creek treatments (80%, 77.8%, and 71.1% respectively), and much lower survivorship in the Mill Creek and Lemon Creek treatments (48.9% and 42.2% respectively) (Figure 4). Coincidentally, the greatest degree of cannibalistic behavior was observed in the Lemon Creek and Mill Creek tanks; two of the three Lemon Creek tanks reached the minimum threshold of six remaining *P. pugio* at the end of week Three, leading to the termination of the experiment.

The availability of OC per shrimp over the course of the feeding experiment is shown in Figure 5. In agreement with the steepest decline in survivorship, the maximum fold increase in OC availability/shrimp occurred in Lemon Creek treatments (×1.96 increase). For Mill Creek, Neck Creek, Control, and Main Creek, the OC availability increased by ×1.61, ×1.25, ×1.18, and × 1.15 respectively. The average weight of *P. pugio* at T_0 was 0.27±0.01 g. After the feeding experiment, the average weight of *P. pugio* were as follows: Control - 0.25±0.02 g; Lemon Creek - 0.24±0.02 g; Neck Creek - 0.27±0.01 g; Main Creek - 0.27±0.02 g; and Mill Creek - 0.24±0.03 g. A comparison of *P. pugio* weights from T_0 and following the feeding experiment did not reveal any significant differences (Kruskal Wallis *chi-squared* = 8.803, *p value* = 0.117) (Figure 6).

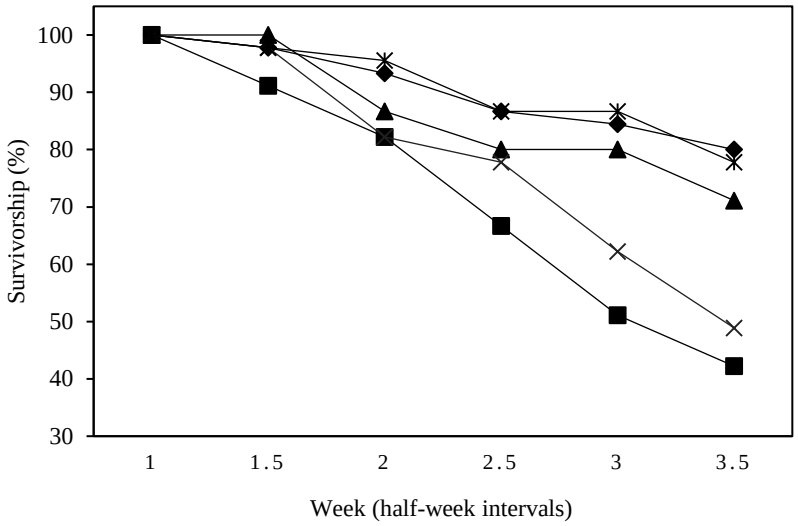

Figure 4: **Percent survivorship of *P. pugio* over the course of the feeding experiment (♦ – Control, ■ – Lemon Creek, ▲ – Neck Creek, ∗ – Main Creek, × – Mill Creek)**

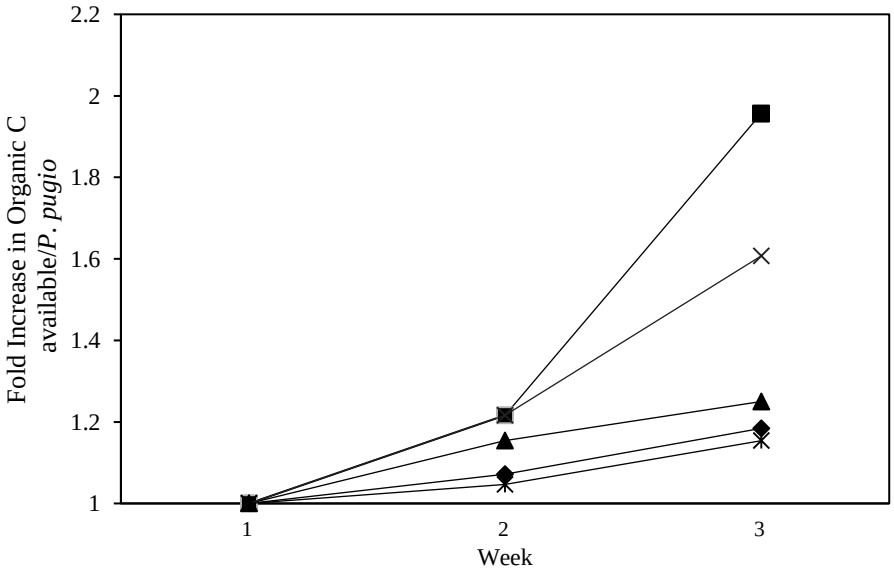

Figure 5: **Fold increase in Carbon weight available to each *P. pugio* over the course of the feeding experiment (♦ – Control, ■ – Lemon Creek, ▲ – Neck Creek, ∗ – Main Creek, × – Mill Creek)**

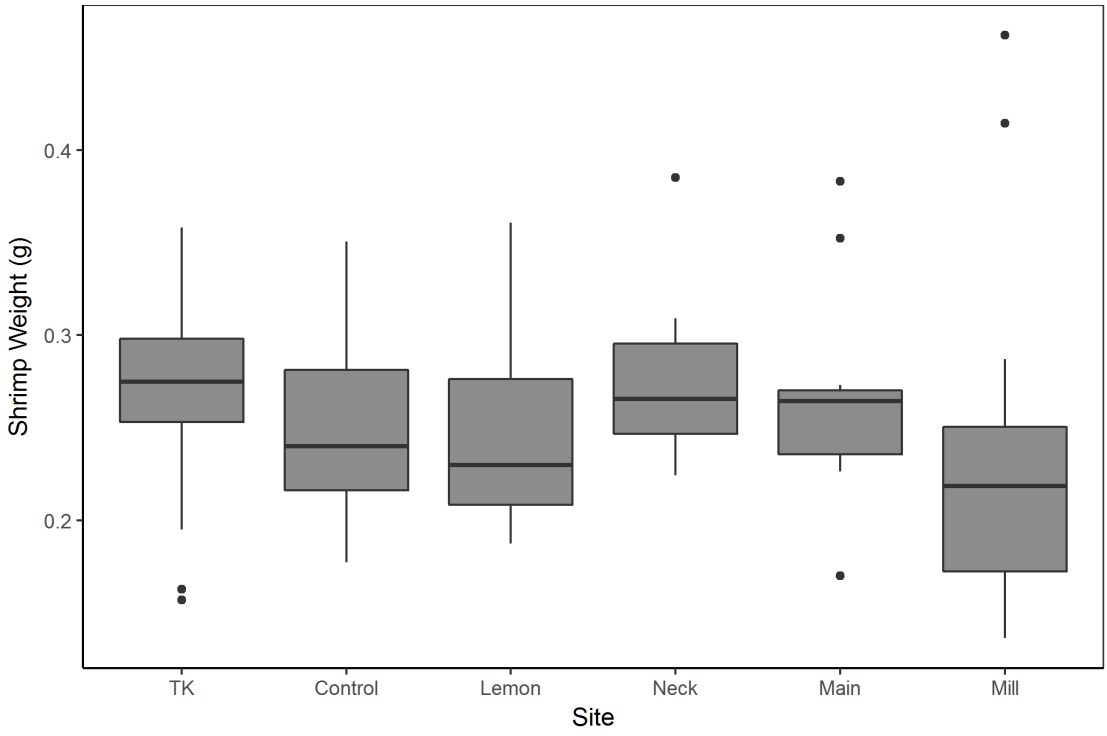

Figure 6: **Weight of naïve *P. pugio* from Tuckerton, NJ (TK, n= 25) and after the feeding experiments (n=12/site treatment). No significant differences.**

Metallothionein in *P. pugio* after the feeding experiment

MT concentrations in the whole-body tissues of naïve *P. pugio* fed prey from contaminated sites (and *Artemia* for Control) are presented in Figure 5. All concentrations are expressed in terms of nmol binding sites of MT/wet weight of shrimp tissue (g). The MT concentrations in the tissue of grass shrimp that were experimentally fed prey in the Control, Lemon Creek, Neck Creek, Main Creek, and Mill Creek treatments were: 13.97 ± 2.05; 13.21 ± 2.53; 13.27 ± 2.42; and 20.71 ± 3.25 $nmol \cdot g^{-1}$ respectively. A Kruskal-Wallis one-way ANOVA did not reveal any significant differences in MT concentrations among the experimentally fed *P. pugio* (*chi-squared* = 8.94, $p = 0.06$; including T_0).

Differences in MT concentrations in field-collected and experimentally fed *P. pugio*

Comparing MT concentrations between field-collected and experimentally fed *P. pugio* (as per site) revealed a significant difference for the Main Creek site (t = 2.88, p<0.05) and no significant differences for the Lemon Creek, Neck Creek and Mill Creek sites (Figure 7). MT concentrations were also similar in the T_0 and experimental Control shrimp. Kruskal-Wallis one-way ANOVAs to analyze possible tank-related effects on MT induction during the feeding experiment did not detect any significant differences in MT concentrations among shrimp from different tanks within each treatment.

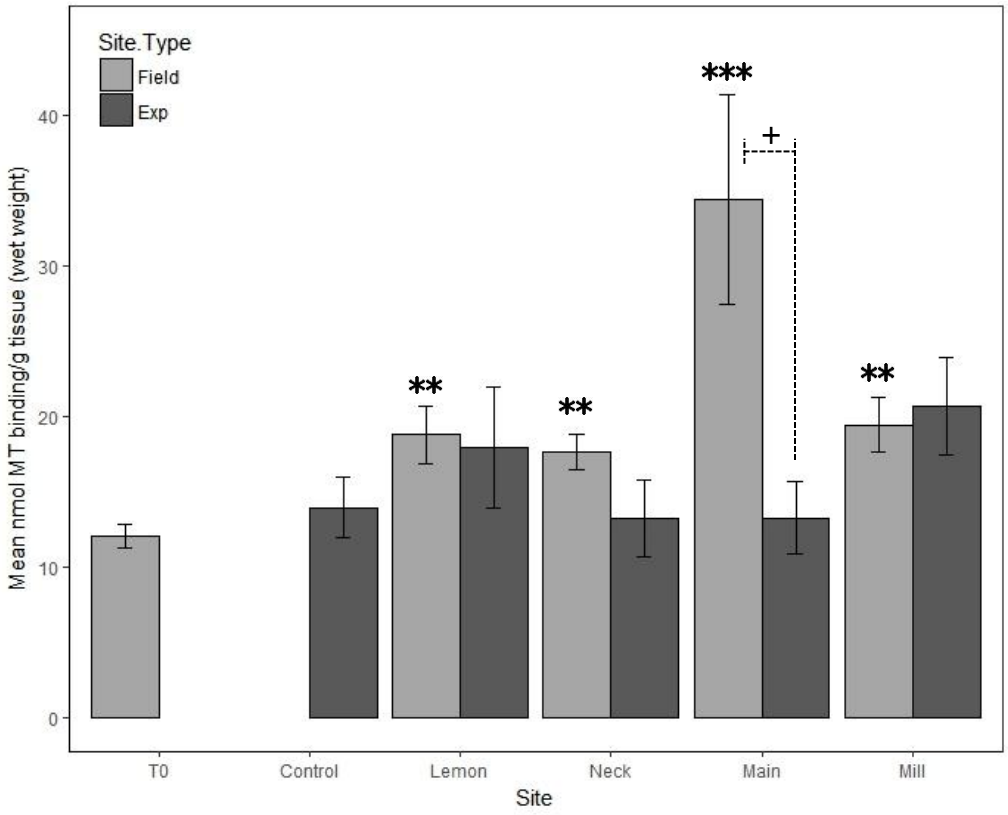

Figure 7: **Metallothionein binding site concentrations in *P. pugio* ± S.E. at T_0 (Initial), in *P. pugio* collected from the sites (Lemon Creek, Neck Creek, Main Creek, Mill Creek; light gray) and in *P. pugio* after the feeding experiment (Control, Lemon Creek, Neck Creek, Main Creek, Mill Creek; dark gray). Asterisks indicate significant differences in field-collected grass shrimp against T_0 grass shrimp (** $p<0.01$, *** $p<0001$). + indicates significant difference between field and experimentally fed grass shrimp for the same site.**

DISCUSSION

Chronic metal exposure can increase the metal tolerance of entire communities of marine invertebrates residing in metal-impacted ecosystems (Cain et al. 2004; Goto and Wallace 2010a). Detoxification of metals within the organisms can lead to pollutant-induced community tolerance, wherein the species adapt to withstand contaminant stress by development of relevant physiological mechanisms (Blanck 2002). The cysteine-rich and highly conserved protein metallothionein (MT), which is integral to the homeostasis of essential metals such as Cu and Zn, can be induced further in several marine invertebrates upon toxic metal exposure, often in a dose-dependent manner (Amiard et al. 2006). Since MT-bound metals may be bioavailable to predators, quantifying the MT response in *Palaemonetes pugio* - a key benthic-pelagic coupler and prey species in the Hudson-Raritan Estuary – may help to elucidate the trophic transfer of toxic metals in impacted systems. This study investigated the concentration of MT in *P. pugio* collected from metal contaminated sites in Staten Island, as well as in naïve *P. pugio* from an external reference site that were fed prey from the same Staten Island sites in an effort to discern the impact of dietary metal exposure from environmental metal exposure (in the form of water, sediment, and prey) on the whole-body MT response.

The concentration of MT in *P. pugio* collected from sites representing an established metal impact gradient in the Arthur Kill (Mill Creek<Main Creek<Neck Creek), as well as from a local reference site (Lemon Creek), was significantly elevated in comparison to that in grass shrimp from a pristine site in Tuckerton, NJ (TK). Surprisingly, despite the MT concentration in Main Creek grass shrimp being slightly (but not statistically) higher, MT concentration in the tissue of *P. pugio* from the Mill

Creek, Neck Creek, and Lemon Creek sites was fairly uniform. MT concentrations in *P. pugio* that were experimentally fed prey from the same set of field sites were not elevated, with concentrations similar to T_0 observed in the control and the site treatments. The similar MT concentrations in *P. pugio* at T_0 (field) and in *P. pugio* following a Control exposure of the feeding experiment, indicated that laboratory conditions had no impact on the MT response.

MT concentration in field-collected *P. pugio*

The elevated MT in *P. pugio* from the Staten Island sites in the current study accompanied by the uniformity of MT concentration among sites, where sediment metal concentrations have shown to differ greatly (Goto 2009), is not without precedent. In shore crabs collected along a Cu gradient in the Fal Estuary, UK, midgut gland Cu concentrations reflected the field gradient, but gland MT concentrations did not, possibly due to maintenance of constant MT levels due to increased turnover of MT-bound Cu through the cystolic compartment for storage in MRG (Pederson and Lundebye 1996). Along a pan-metal impact gradient in an estuary in the Netherlands, MT concentrations in the periwinkle, *Littorina littorea*, while elevated in comparison to a distant pristine site, did not increase in response to a steep field Cd gradient or increasing tissue metal burdens, despite the potential of Cd in inducing MT in this species (Van den Broeck et al. 2010).

P. pugio in the field are chronically exposed to an array of contaminants, many of which (herbicides, antibiotics, hormones, oxidants, etc.) can induce MT synthesis, albeit not as sharply as metals (Amiard et al. 2006). Estrogenic contaminants can modulate MT levels in lake trout through a decrease in liver- and increase in kidney-MT (Werner et al.

2003) On the other hand, polycyclic aromatic hydrocarbons have been repeatedly shown to inhibit MT induction (Maria and Bebbiano 2011; Faverney et al. 2000). Furthermore, *P. pugio* can become acclimated to metals in sediment, but when exposed to sediment containing a variety of pollutants including metals and PAHs, competing responses to different contaminants may decrease resistance to certain pollutants, while also impacting metal detoxification mechanisms (Klerks 1999). Hence, while the overall MT concentration in field-collected *P. pugio* was elevated, the elevation could have arisen from a combination of toxic effects and detoxification mechanisms, which may have confounded the relationship between metal exposure and MT concentration.

Toxic metal stress can induce MT in malacostracan crustaceans in a matter of 1-5 days, but may lead to increased MT turnover as well as decreases in MT levels in response to the general toxic effects of long-term or high-dose metal exposure (Amiard et al. 2006; Couillard et al. 1995). MT turnover, encompassing the induction, metal chelation and lysosomal degradation of MT in the cell, represents a cellular response to changes in the cellular environment, such as toxic metal or ionizing radiation stress, and as a general antioxidant defense (Isani et al. 2000; Amiard et al. 2006). The half-lives of MT-bound metals and MT molecules often differ greatly within marine invertebrates. Nassiri et al. (2000) demonstrated that in the amphipod *Orchestia gammarellus* experimentally exposed to Cu, Cd, and Zn, despite similar uptake rates for all 3 metals, Cd remained attached to successive MT molecules in the cytoplasm and did not appear in the lysosomes while degradation of MT in the presence of Cu and Zn in lysosomes was confirmed. Increased MT turnover has also been reported in the green mussels *Perna viridis* exposed to Cd (Ng et al. 2007). As metals in the lysosome, along with several

other organelles, can be part of the TAM compartment (Wallace and Luoma 2003), the forthcoming quantification of TAM-associated metals in *P. pugio* tissue from study sites may help ascertain whether elevated MT concentrations, followed by increased MT turnover, was indeed responsible for the lack of variation in MT concentrations in site-collected *P. pugio.* A high turnover may be hypothesized in the case of elevations of TAM-associated metal levels in field-collected *P. pugio* without concurrent increases in MT concentrations. Future studies should consider MT-mRNA expression and steady state MT concentration in concert to more accurately quantify MT response and turnover in response to metal exposure in *P. pugio*.

The uniformity of MT concentrations in field-collected *P. pugio* despite chronic metal exposure across a metal impact gradient, may indicate a plateauing of MT concentration, possibly arising from a proportional decrease in reliance on MT for metal binding with increasing metal exposure or increasing metal burdens in the TAM compartment. A correlational increase in TAM-metal with increases in whole-body metal for Cd, Pb, Cu, and Zn has been demonstrated in *P. pugio* from the same sites sampled in this study; however, no proportional increase in TAM partitioning has been observed in concert. On the contrary, in the case of Pb, the increase in whole-body metal burden was accompanied by a proportional decrease in partitioning to TAM (Goto and Wallace, 2009a). If the imminent analysis of whole-body and TAM metal burdens in the field-collected *P. pugio* from this study emulate the correlations described in Goto and Wallace (2009a), consistent MT concentrations in *P. pugio* from these sites may indicate an inverse relationship between TAM-associated metals and MT concentrations, and no correlation with whole-body metal burdens.

MT concentration in naïve *P. pugio* fed site-collected prey

A sharp decline in *P. pugio* survivorship due to the consumption of conspecifics was observed in the Lemon Creek and Mill Creek treatments of the feeding experiment. This behavior is common among crustaceans (Dick 1995; Dutil et al. 1997; Marshall et al. 2005), especially in captivity (Sarda and Valladares 1990). A study of grass shrimp feeding preferences with *Palaemonetes antennarius* adults (Costantini et al. 2001) demonstrated that the shrimp had a strong preference for the isopod *Proasellus coxalis*, both dead and live, while shelled organisms were only consumed in their larval phases; crushed snails (such as those used in the feeding experiment) were consumed, but not by preference, and grass shrimp also consumed conspecifics at their vulnerable post-molt stage. The Lemon Creek and Mill Creek prey in the current study also had the greatest proportion of shelled organisms (~43% and ~70%, respectively) such as gastropods and bivalve, compared to prey for the Main Creek and Neck Creek treatments, which comprised of ~100% soft-bodied organisms such as small amphipods and isopods (< 1 cm) and polychaete worms; this may partly explain the higher degree of conspecific feeding in experiments for these sites. Refuge density has been associated with lower rates of cannibalism in some shrimp and crab species. While habitat enrichment was provided in the tanks of the feeding experiment (plastic grids), introducing enclosed refugia to combat consumption of vulnerable post-molt shrimp would have risked potential monopoly of tank meals by individual *P. pugio*, possibly limiting OC availability or causing unequal dietary contaminant exposure among tank shrimp.

The impact of prey-associated metal on feeding behavior and energetic requirements of *P. pugio* cannot be discounted for the Lemon Creek and Mill Creek site

treatments. It is difficult to say whether observed instances of cannibalism were true cannibalism or scavenging of dead conspecifics. In case of the latter, given the nearly twofold lower survivorship in Lemon Creek and Mill Creek treatments compared to the Control, dietary exposure to metal may have played a role in *P. pugio* mortality for these treatments. Despite the close to two-fold increase in OC availability to grass shrimp in the Lemon Creek and Mill Creek treatments from prey (Figure 4), and additional OC and bioavailable contaminant exposure from consumption of conspecifics, MT concentrations in the surviving *P. pugio* from these treatments were not significantly greater than control values, and were similar to values for the Main Creek and Neck Creek treatments. While there are no known studies of the impact of cannibalism on metal bioaccumulation in crustaceans, in the case of polychlorinated biphenyls (PCBs), the self-biomagnification effect of cannibalism and conspecific scavenging has been shown to be small and unlikely to exceed 5% over average (Fraser et al. 2005). The lack of an MT "spike" in the *P. pugio* from these treatments may either be a result of a similar insignificant impact of self-biomagnification, or may indicate a plateau in MT concentration for the treatments.

The lack of significant increase in MT concentrations among *P. pugio* fed prey from the sites in the metal impact gradient compared to control was surprising. Data on the whole-body and subcellular partitioning of metal in the meals for *P. pugio* (which is underway) will lend itself to a better understanding of the processes governing the uniform MT concentrations in all feeding treatments. Goto and Wallace (2009a) have shown that in the polychaete, *Nereis acuminata,* collected from the Lemon Creek, Neck Creek and Mill Creek, despite positively correlated increases in TAM-metal and whole-body metal burdens, no proportional increase in TAM partitioning was observed for Cd,

Pb, Cu, and Zn; however, TAM-associated Cd, Pb, and Cu were disproportionately low in *N. acuminata* from Main Creek in comparison to the other sites. This may partly explain the greater MT concentration in *P. pugio* collected from Main Creek compared to that in naïve *P. pugio* fed prey from the Main Creek site in this study. The lack of such differences in MT concentrations between the field-collected and experimentally fed *P. pugio* for the Lemon Creek, Neck Creek, and Mill Creek sites indicated that dietary exposure to metals may in large part account for MT induction in grass shrimp in the Hudson-Raritan Estuary.

The Main Creek site is directly connected to the Arthur Kill and is surrounded by the largest landfill in the US, the Fresh Kills landfill. Sediment core analysis from this site has revealed a high level of metal contamination throughout the core depth, with 2x and 3x greater mean enrichment factor for Cd compared to the Neck Creek and Lemon Creek sites, respectively (Nichols 2012*)*. The higher MT concentration in field-collected *P. pugio* from Main Creek as compared to the experimental Main Creek treatment, may also be associated with the response of MT in the presence of other contaminants at this site, while the feeding experiment only exposed *P. pugio* to the contaminants that were bioaccumulated by the prey from each site. Additional variability in contaminant exposure may have been introduced by the diversity of organisms within and among the prey communities of different sites, with different bioaccumulation and subcellular partitioning for metals. In the absence of competing environmental exposure to toxicants, *P. pugio* in the feeding experiment may have effectively depurated metals by excretion of metal-rich granules and through ecdysis. The imminent measurements of whole body

metal burdens of the experimental *P. pugio* will shed further light upon the efficiency of metal depuration.

Considerations in the measurement of MT

The quantification of whole-body MT in *P. pugio* in this study may obscure organ- or body section-level fluxes of MT in response to dietary toxic metal stress. In *Palaemonetes argentinus* exposed to high Zn concentrations, significant increases in cephalothorax MT levels have been observed, with no concurrent increase in the abdomen, even at maximum exposure concentrations, possibly due to the location of hepatopancreas within the cephalthorax (first body section) (Bertrand et al. 2015). An opposite response in MT concentrations has been demonstrated in *P. argentinus* exposed to the broad-spectrum organophosphorus pesticide chlorpyrifos, with significant decreases in cephalothorax and increases in abdominal MT concentrations (Bertrand et al. 2016). Chlorpyrifos is the most widely used conventional pesticide by weight in the United States (Solomon et al. 2014). Its possible presence at the field sites - along with that of a range of other organic contaminants - may play an essential role in the modulation of MT concentration in the field-collected *P. pugio*. Competition between mechanisms of metal sequestration such as non-MT cystolic ligands, MRG and tertiary lysosomes may interfere with MT response to metal exposure (George and Olsson 1994). Additionally, in the case of Cu contamination and a possible increase in TAM-Cu, the presence of the Cu-binding pigment haemocyanin in malacostracan crustaceans such as *P. pugio* can complicate these relationships (Rainbow 1993). The molt cycle of decapods – a factor that could not be controlled for within the feeding experiment in this study - is

also associated with pronounced cellular and tissue effects on haemocyanin and copper metabolism, introducing further variability in MT (Engel and Brouwer 1993).

Imminent work

In order to parse the impact of metal exposure on MT concentration, the following will be evaluated using organisms from the current study:

(1) The relationships between *P. pugio* MT and TAM-metal in prey from the feeding experiment. Especially in the case of Cd, prey TAM has been successfully used to estimate metal transfer to predators (Seebaugh and Wallace 2009; Wallace and Luoma 2003).

(2) The relationships between MT concentrations, TAM-metal burdens, and whole-body metal burdens in *P. pugio* from the feeding experiment. Correlations between these will shed light on the proportional reliance of MT for metal detoxification, and whether there is indication of elevated MT turnover.

(3) The relationships between MT concentrations, TAM-metal burdens and whole-body metal burdens in field-collected *P. pugio* and comparisons of these with (2). With similar MT concentrations measured in field-collected and experimental fed *P. pugio* for all but one site, significant differences in metal partitioning to TAM may indicate differential reliance on MT in response to chronic field and short-term dietary metal exposures.

(4) Correlation of sediment metal loads at the sites with whole-body and TAM-metal burdens in field-collected *P. pugio* and site-specific prey. Subcellular metal partitioning may be better explained in response to sediment loads of specific metals.

Establishing linkages between metal loads in sediments and impacts on marine biota is essential in environmental risk assessment**.** Chronic metal exposure can increase the metal tolerance of an entire community. This process, referred to as pollutant-induced community tolerance, can be due to detoxification of metals within organisms (Blanck 2002); however, the content that is detoxified by binding to MT may increase the bioavailability of these metals to predators, and a greater reliance on MT could lead to a greater-than linear trophic transfer of metal in stressed ecosystems, causing alterations in nutrient cycles, species-species interactions, and transfer of energy (Seebaugh et al. 2005). These effects could be compounded at each subsequent trophic level due to the reliance on MT for detoxification at the previous level.

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