



Examining the relationship between metal exposure (Cd and Hg), subcellular accumulation, and physiology of juvenile *Crassostrea virginica*

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Abstract

To assess the toxicity and accumulation (total and subcellular partitioning) of cadmium (Cd) and mercury (Hg), juvenile eastern oysters, *Crassostrea virginica*, were exposed for 4 weeks to a range of concentrations (Control, Low (1×), and High (4×)). Despite the 4-fold increase in metal concentrations, oysters from the High-Cd treatment (2.4 μM Cd) attained a body burden that was only 2.4-fold greater than that of the Low-Cd treatment (0.6 μM Cd), while oysters from the High-Hg treatment (0.056 μM Hg) accumulated 8.9-fold more Hg than those from the Low-Hg treatment (0.014 μM Hg). This fold difference in total Cd burdens was, in general, mirrored at the subcellular level, though binding to heat-denatured proteins in the High-Cd treatment was depressed (only 1.6-fold higher than the Low-Cd treatment). Mercury did not appear to appreciably partition to the subcellular fractions examined in this study, with the fold difference in accumulation between the Low- and High-Hg treatments ranging from 1.5-fold (heat-stable proteins) to 4.6-fold (organelles). Differences in toxicological impairments (reductions in condition index, protein content, and ETS activity) exhibited by oysters from the High-Cd treatment may be partially due to the nature of how different metals partition to subcellular components in the oysters, though exact mechanisms will require further examination.

Keywords *Crassostrea virginica* · Cadmium · Mercury · Physiology · Subcellular · Accumulation

Introduction

Organisms living in heavily urbanized estuaries, such as the Hudson-Raritan Estuary (HRE), are exposed to a variety of inorganic and organic pollutants including PCBs, PAHs, and trace metals (Levinton and Waldman 2006). These

contaminants may ultimately affect the physiology, reproduction, and survival of estuarine species (Oliver et al. 2003; Lannig et al. 2006). Impacts on suspension feeding bivalves, such as the eastern oyster, *Crassostrea virginica*, are of particular concern, as these molluscs are highly vulnerable due to their benthic, sessile existence and are capable of filtering large volumes of water which exposes them to both particulate and dissolved forms of metals (Chu et al. 2002; Volety 2008). Dense populations of oysters, that have historically occurred in the HRE, have the ability to control phytoplankton assemblages, nutrients, and suspended matter within an estuary through their feeding and biodeposition (feces and pseudofeces production), effectively integrating bioavailable metals in the water column and surface sediments (Ringwood et al. 1999; Guo et al. 2002; Shulkin et al. 2003; zu Ermgassen et al. 2013).

Historically, oysters were a keystone species in the HRE but sewage pollution and habitat destruction (e.g., filling, bulkheading, dredging) led to the loss of this ecological engineer (Franz 1982). Studies of the potential restoration of

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oysters at various sites within the HRE have shown varying degrees of survival and growth, indicating that survivorship is likely site-specific (Levinton et al. 2011; Starke et al. 2011; Zarnoch and Schreiber 2012). Contaminant inputs into the lower HRE, along with habitat loss, may increase the difficulty of reestablishing a self-sustaining population of oysters. Organic and inorganic (i.e., metal) pollutants are known to cause physiological changes at the molecular, cellular, and organismal levels in bivalves, which may inhibit growth and reproduction, both of which are necessary for the survival and reestablishment of a healthy oyster population (Roesijadi 1996; Ringwood et al. 1998; G eret et al. 2002; Cherkasov et al. 2007; Volety 2008).

Cadmium (Cd) and mercury (Hg) are non-essential trace metals present in the HRE and can lead to toxicity within oysters and other bivalves when accumulated (Engel 1999; Gagnaire et al. 2004; Sokolova et al. 2005). Physiological impacts may include reductions in growth, survivorship, and alterations in enzymatic and mitochondrial activities (Engel 1999; Kawaguchi et al. 1999; Ringwood et al. 2004; Sokolova et al. 2005; Sanni et al. 2008; Volety 2008; Macey et al. 2010). Preferential binding of Cd or Hg to subcellular fractions in oysters may lead to alterations in metabolism and manifest as physiological changes (i.e., reduced reproductive output, lowered overall condition, changes in filtration rate; Cherkasov et al. 2009; Cooper et al. 2010; Ivanina et al. 2010; Mass Fitzgerald 2013). The amount of energy reserves within the oyster along with reproductive status and environmental parameters (e.g., food, temperature, and salinity of the water) can affect the expression and quantity of metallothioneins present within the oyster, and thus the amount of metal that can be successfully sequestered (Amiard et al. 2006; Erk et al. 2008).

Oysters have the ability to bioconcentrate metals up to several orders of magnitude higher than ambient concentrations, prior to experiencing any toxicity effects (Mouneyrac et al. 1998; O'Connor 2002). Accumulated metals may be sequestered in a variety of subcellular pools, including insoluble metal-rich granules (INS), heat-stable proteins (HSP) such as metallothioneins, cellular organelles (ORG), and heat-denatured proteins (HDP) which would include enzymes (Wallace et al. 2003). These fractions can combine into subcellular compartments relevant to toxicity (metal-sensitive fraction, MSF: ORG and HDP) or detoxification (biologically detoxified metals, BDM: INS and HSP) (Fig. 1; Wallace et al. 2003). Using this subcellular compartmentalization approach to interpret metal accumulation may provide insight into how subcellular storage influences physiology and how changes in subcellular storage of Cd or Hg may be related to toxicity (Goto and Wallace 2007).

The objective of this study was to determine the effects of metal exposure (either Cd or Hg) on various physiological endpoints of juvenile *C. virginica*, within a controlled

laboratory setting. While field surveys are important for determining the survival of oysters for restoration, it is difficult to tease apart how varying environmental parameters impact physiology. It was hypothesized that oysters exposed to elevated concentrations of Cd or Hg would exhibit impaired health as determined by lowered overall condition indices and protein storage, and increases in metabolic activity. The results from this study, along with related field work (Mass Fitzgerald 2013), could aid in site selection for future *C. virginica* restoration efforts within the urbanized HRE.

Methods

Laboratory exposures

Juvenile (2–3-month post-settlement) eastern oysters, *C. virginica*, were obtained from Aeros Cultured Oyster Company in Southold, NY. This location was chosen as it is outside the urbanized HRE watershed. Oysters used for Cd exposures were obtained in June 2011 (22.9 ± 3 mm shell length; 0.045 ± 0.015 g dry weight); a second group used for Hg exposures was obtained in July 2011 (40.6 ± 7.5 mm shell length; 0.189 ± 0.108 g dry weight). Oysters were transported on ice back to the laboratory, placed into aquaria with a salinity of 25 ppt and temperature of 25 °C (to approximate ambient conditions from Southold, NY). Seawater was prepared with artificial sea salts (Reef Crystals®) and deionized water. Oysters were acclimated to this temperature and salinity for 72 h prior to being transferred into treatment aquaria.

For each metal (Cd and Hg), three treatment levels were used (Control, Low (1×), and High (4×)) in triplicate using 19-L aquaria. The Low exposures were designed to be more realistic field concentrations, and the High exposures were set at an elevated, yet sublethal, dose that was above the EPA maximum. Fifty oysters were placed in each aquarium and supplied with gentle aeration. The nominal concentrations of Cd used were 0.6 μM (Low-Cd treatment) and 2.4 μM (High-Cd treatment). Nominal Hg concentrations used were 0.014 μM (Low-Hg treatment) and 0.056 μM (High-Hg treatment). Stock solutions of Cd (CdCl₂) and Hg (HgCl₂) were used to prepare the exposure media. The stock solutions were acidified and 0.5 N NaOH was used to adjust the solution to a neutral pH. Recent background concentrations of Cd in the HRE were reported to be 0.0044 μM and were found to be as high as 0.044 μM (Bopp et al. 2006). Background concentrations of Hg in the HRE were reported to be 0.0009 μM and were found to be as high as 0.024 μM (Bopp et al. 2006). Both the Low-Cd and the Low-Hg treatments were above the EPA guideline for maximum concentration Cd (0.36 μM) and Hg (0.009 μM) in marine systems (US EPA 1999,

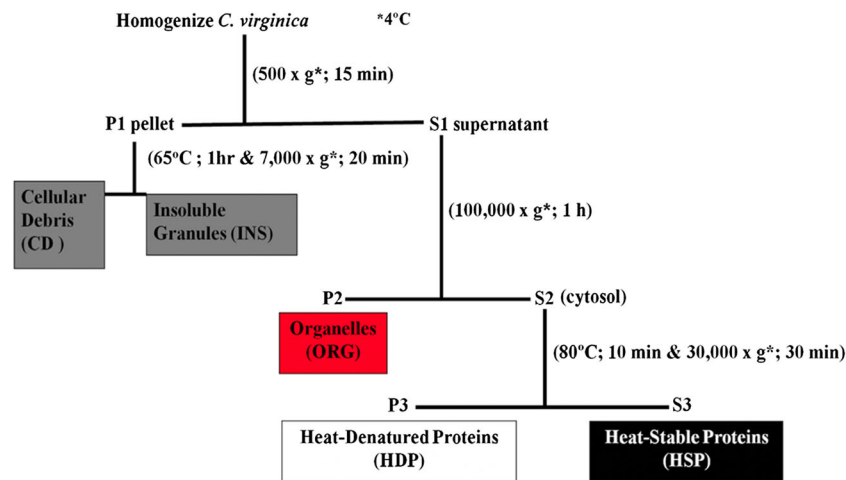


Fig. 1 Subcellular fractionation methodology, based on procedures developed in Wallace et al. 2003. During the process, sequential centrifugation and heat steps divide the tissue into five fractions: INS (insoluble metal-rich granules), HSP (heat-stable proteins; including metallothioneins), ORG (organelles), and HDP (heat-denatured proteins; including enzymes). These fractions can combine into subcellular

compartments relevant to toxicity (metal-sensitive fraction, MSF: ORG and HDP) or detoxification (biologically detoxified metals, BDM: INS and HSP). A fifth fraction, CD (cell debris) is produced during centrifugation steps; this fraction was not included in analysis due to lingering questions about the relevance of this fraction in toxicity and detoxification processes (Wallace et al. 2003; Cain et al. 2004)

2001). These concentrations, however, are below historical heavy metal loadings found in the HRE which may still be present in some locations (NYSDEC 2003). Preliminary experiments revealed that the chosen concentrations used were sublethal and did not result in significant mortality during the 3-week preliminary exposure period. Control treatments (Con-Cd and Con-Hg) contained no added metal. Metal was added in the appropriate concentrations to 16 L of artificial seawater in each treatment aquaria. Every 48 h, aquaria were drained of exposure media (via slow controlled pumping), refilled with fresh artificial seawater, and respiked with the appropriate concentrations of metal. Oysters were fed daily with Instant Algae® commercial algal blend (2 mL), added after the water was changed, metals added, and thoroughly mixed.

Each laboratory exposure was continued for 4 weeks. Prior to the start of the experiment, an initial subsample of oysters was taken to evaluate physiological condition. Upon completion of the exposure period, all oysters were removed from each aquarium and enumerated to determine mortality. Mortality was minimal for all treatments (< 10%). Subsamples of oysters were removed and rinsed two times (approximately 30 min) in clean artificial seawater (25 ppt) to allow oysters to clear their mantle cavity. The oysters were then flash-frozen on dry ice and stored for future analysis (−80 °C). Per replicate aquaria, a random 34 oysters were chosen for sampling: 20 oysters were subsampled to analyze condition index and tissue protein content, ten oysters were subsampled for ETS activity, and four oysters were subsampled for metal analysis

Physiological endpoints

Condition index ($n = 20$ per replicate) was determined using the methods of Crosby and Gale (1990), using the whole oyster, dry shell, and dry tissue weights (g). The stoichiometric ratio of protein in dried tissues (pooled tissue of 20 oysters, $n = 3$ per treatment) was measured by determining nitrogen content on a Perkin Elmer CHN elemental analyzer and then multiplying by 5.8 to obtain protein content (Gnaiger and Bitterlich 1984). To estimate energy usage, analysis of the electron transport system (ETS) was completed using the modified methods of Madon et al. (1998). Briefly, oysters ($n = 10$ per replicate) were homogenized in ETS-B buffer (pH 8.5) at a 1:4 ratio of wet weight:buffer volume (mL). A subsample (2 mL) of homogenate was centrifuged at 10,000g for 20 min at 4 °C. Afterwards, the reaction was completed by adding the final electron acceptor, INT-formazan, to solution with substrate (β -NADH) and homogenate. The reaction was stopped after 20 min by adding 1:1 phosphoric acid/formalin quench solution and read at 490 nm on a UV spectrophotometer. ETS activity was then calculated from the following equation: $\text{ETS } \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1} = \{ (E_{\text{corr}} \times V_{\text{hom}} \times 60/t \times 1 \times V_{\text{rxn}}) / (V_{\text{inc}} \times DW \times 31.8) \}$ where E_{corr} is the corrected sample absorbance (cm^{-1}), V_{hom} is the total homogenate volume (mL), t is the incubation time (min), 60 is the constant used to express activity per hour, V_{rxn} is the final reaction volume (mL), V_{inc} is the homogenate volume used during the reaction (mL), and DW is the dry weight of oyster tissue placed in the initial homogenate (g) (Garcia-Esquivel et al. 2001; Zarnoch and Sclafani 2010).

Subcellular fractionation and metal accumulation

Oyster tissue was separated into operationally defined subcellular fractions based on the methods of Wallace et al. (2003). Briefly, oysters ($n = 4$ per replicate) were shucked, weighed for wet weight (g), and homogenized with 6 mL TRIS buffer (pH 7.6). A subsample (1/6th the total volume, 1.0 mL) was then removed to determine total body burden (TOT). Sequential centrifugation and heat treatment steps were used to separate the oyster tissue into the following relevant operationally defined fractions: INS, HSP, ORG, and HDP (Wallace et al. 2003). The subcellular fractionation process also produces an additional fraction (“cell debris”), which would contain metals that were solubilized from tissue fragments of the oyster. This fraction was not included in metal analysis due to questions about the relevance of metal in this fraction to toxicity or detoxification processes (Wallace et al. 2003; Cain et al. 2004).

To analyze for Cd, subcellular fractions (as well as TOT) were placed in a drying oven (60 °C) for a minimum of 3 days, digested for approximately 2 days with trace-metal grade nitric acid, resuspended in 2% nitric acid, filtered through a 0.45- μm Millipore filter, and subjected to graphite furnace atomic absorption spectrophotometry (Brown and Luoma 1995). To determine Hg concentrations, fractions were dried at 60 °C for a minimum of 3 days and then digested with 1:4 nitric acid:sulfuric acid in a 60 °C hot water bath. Afterwards, the addition of potassium permanganate to the sample oxidized all mercury present. Samples were then analyzed by cold vapor atomic absorption spectrophotometry (Perkin Elmer FIMS-100 Hg analyzer) using standard techniques (SnCl_2 was added to digested tissue prior to analysis; Hatch and Ott 1968; Klajović-Gašpić et al. 2006).

Data treatment and statistical analysis

Data were analyzed using Nested ANOVA to determine the effect of metal exposure on differential subcellular binding and physiological endpoints (overall condition, energy storage, and ETS activity). Where appropriate, data was transformed (either log-10 or arcsine transformations; Zar 1999) for meeting assumptions of Nested ANOVA. Bonferroni's post hoc tests were used to identify differences between treatments. The Kruskal-Wallis ANOVA with Dunn's post hoc test was used when data could not be transformed to meet ANOVA assumptions. When metal burdens were analyzed, Student's t tests were used to determine the differences between Low and High treatments, as none of the Control treatments (Cd or Hg) was found to have any detectable metal. All analyses were completed using Statistica 7.1 (StatSoft Inc.®) and Sigma Plot 10 (Systat Software Inc.®).

Results

Cadmium exposures

Physiological endpoints

Condition index in oysters decreased over the gradient of Cd exposure (ANOVA, $p < 0.05$) (Fig. 2). After 4 weeks, condition index was found to be the highest (6.17 ± 0.10) in Con-Cd oysters and the lowest (4.99 ± 0.30) in High-Cd oysters; Low-Cd oysters had an intermediate condition index of 5.83 ± 0.17 .

Protein content ($\mu\text{g mg DW}^{-1}$) in oysters was found to decrease upon 4 weeks of exposure to Cd (Kruskal-Wallis, $p < 0.01$). Con-Cd oysters were found to have a protein content of $511.92 \pm 4.05 \mu\text{g mg DW}^{-1}$, compared with $493.59 \pm 7.42 \mu\text{g mg DW}^{-1}$ and $461.15 \pm 11.06 \mu\text{g mg DW}^{-1}$ for Low-Cd and High-Cd, respectively (Fig. 3).

Aerobic potential (estimated by the ETS assay) was also influenced by Cd exposure (Kruskal-Wallis, $p < 0.05$), with oysters from the High-Cd treatment exhibiting a 50% reduction in aerobic potential as compared with Con-Cd oysters (103.11 ± 16.74 vs $201.16 \pm 7.49 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$, respectively). There was no significant difference between the Low-Cd ($202.24 \pm 54.84 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$) and Con-Cd oysters (Fig. 4).

Metal accumulation and subcellular fractionation

Following the 4 weeks of exposure, oysters from the Low-Cd ($0.6 \mu\text{M Cd}$) treatment attained a total Cd body burden of $29.54 \pm 5.19 \mu\text{g Cd g DW}^{-1}$, while those from the High-Cd ($2.4 \mu\text{M Cd}$, a 4-fold higher exposure) treatment accumulated a body burden ($70.89 \pm 11.12 \mu\text{g Cd g DW}^{-1}$) that was 2.4-fold higher (Student's t test, $p < 0.05$) (Table 1). As compared

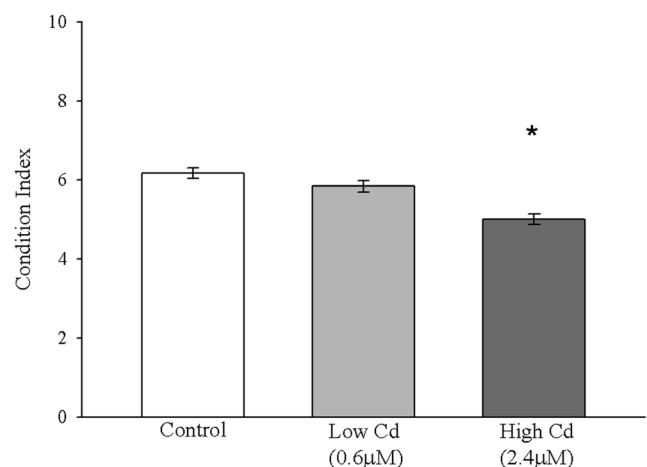


Fig. 2 Condition index of oysters exposed to Low-Cd ($0.6 \mu\text{M}$) or High-Cd ($2.4 \mu\text{M}$) treatment for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ($n = 60$) ± 1 SE. Asterisk indicates a significant difference between the High-Cd treatment and Con-Cd and Low-Cd treatments (Kruskal-Wallis, $p < 0.0001$)

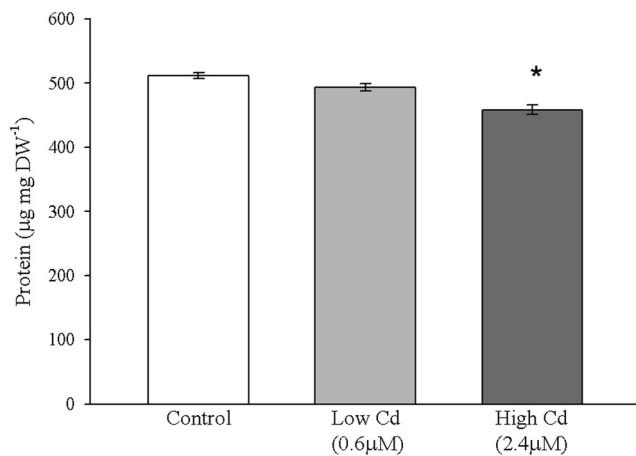


Fig. 3 Protein concentration of oysters exposed to Low-Cd (0.6 µM) or High-Cd (2.4 µM) treatment for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ($n = 20$) \pm 1 SE. Asterisk indicates a significant difference between the High-Cd treatment and Con-Cd and Low-Cd treatments (Kruskal-Wallis, $p < 0.01$)

with those from the Low-Cd treatment, all subcellular fractions of oysters from the High-Cd treatment attained a higher Cd burden (Table 1; Student's t test; $p < 0.05$) with fold differences, in general, mirroring that of total Cd burdens (~2.4-fold higher); binding to heat-denatured proteins, however, was depressed (only 1.6-fold higher) in oysters from the High-Cd treatment (Low-Cd 3.25 ± 0.59 µg Cd g DW⁻¹; High-Cd 5.30 ± 0.55 µg Cd g DW⁻¹) (Table 1). In contrast, Cd binding in the ORG fraction was elevated (2.8-fold higher) in oysters from the High-Cd treatment (Low-Cd 2.91 ± 0.52 µg Cd g DW⁻¹; High-Cd 8.12 ± 1.85 µg Cd g DW⁻¹) (Table 1). Cd burdens (total and in subcellular fractions) of oysters from the Con-Cd treatment were below the detection, and as there were no differences between treatment aquaria, oysters from each treatment were pooled ($n = 24$). Similar to the individual subcellular fractions, both subcellular compartments (MSF and

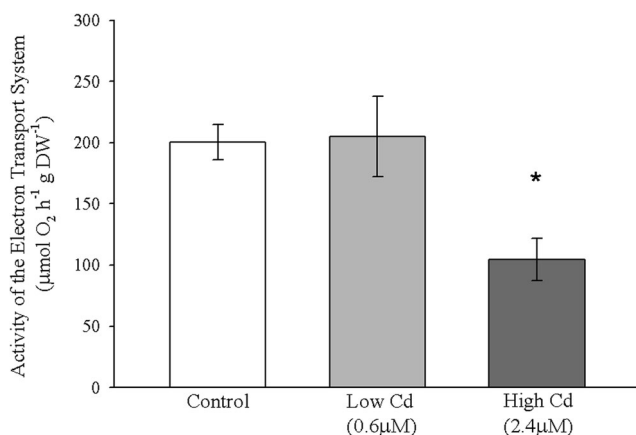


Fig. 4 Activity of the electron transport system of oysters exposed to Low-Cd (0.6 µM) or High-Cd (2.4 µM) treatment for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ($n = 30$) \pm 1 SE. Asterisk indicates a significant difference between the High-Cd treatment and Con-Cd and Low-Cd treatments (Kruskal-Wallis, $p < 0.05$)

BDM) accumulated more metal when exposed to the increased Cd exposure (Low-Cd vs High-Cd (Table 1; Student's t test, $p > 0.05$). Cadmium in both subcellular compartments reflected the fold differences of the TOT burdens (2.3-fold higher for BDM and 2.2-fold higher for MSF; Table 1).

Mercury exposures

Physiological endpoints

After 4 weeks, there were no differences, among treatments, in oyster physiology (ANOVA, $p > 0.05$). The average condition index for oysters ranged between 5.02 and 5.14; average protein content was between 476.98 and 484.72 µg mg DW⁻¹; and average aerobic potential (via ETS assay) ranged from 85.60 to 106.59 µmol O₂ h⁻¹ g DW⁻¹. Specific values for each treatment can be found in Table 2. Though there were no significant differences, a trend of decreasing overall condition and protein content was observed; however, aerobic potential showed the opposite trend, with an increase in values with increasing Hg exposure (Table 2).

Metal accumulation and subcellular fractionation

Oysters from the High-Hg (0.056 µM Hg) treatment accumulated significantly more Hg (131.75 ± 27.94 µg Hg g DW⁻¹) than those from the Low-Hg (0.014 µM Hg) treatment (14.75 ± 4.61 µg Hg g DW⁻¹) (Table 3; Student's t test, $p < 0.01$). This accumulation of Hg in oysters from the High-Hg treatment, in contrast to the Low-Hg treatment, was greatly enhanced over that of the exposure gradient (a 4-fold difference in exposure vs a 8.9-fold difference in attained body burden) (Table 3). Although most subcellular fractions of oysters from the High-Hg treatment had significantly more Hg than those from the Low-Hg treatment (Student's t test; $p < 0.0001$) (i.e., INS, HDP, and ORG), fold differences in subcellular partitioning between the Low-Hg and High-Hg treatments were greatly reduced as compared with the fold difference in total body burdens (i.e., 3.5-fold for INS, 3.7-fold for HDP, and 4.6-fold for ORG). There was no difference in the accumulation of Hg in the HSP between these treatments (~0.40 µg Hg g DW⁻¹) (Table 3). The subcellular compartments (MSF and BDM) of High-Hg exposures had significantly more metal accumulated in the (Student's t test, $p > 0.05$), but the fold differences were not as high as the TOT burdens (2.6-fold higher for BDM and 4.2-fold higher for MSF in High-Hg treatments; Table 3). Mercury burdens (total and in subcellular fractions) of the oysters from the Con-Hg treatment were below detection, and as there were no differences between treatment aquaria, oysters from each treatment were pooled ($n = 24$).

Table 1 Metal accumulation in oysters exposed to Low (0.6 μM) or High (2.4 μM) doses of Cd for 4 weeks. All values are reported as mean μg metal g DW⁻¹ (n = 3) ± 1 SE. The “fold difference” represents the difference in metal accumulation between the Low-Cd and High-Cd treatments. The level of metal in the Control-Cd treatments was non-detectable. As per Wallace et al. (2003): *TOT*, total body burdens; *INS*, insoluble

granules (i.e., metal-rich granules); *ORG*, organelles; *HDP*, heat-denatured proteins (i.e., enzymes); *HSP*, heat-stable proteins (i.e., metallothionein-like proteins); *BDM*, biologically detoxified metal (INS + HSP fractions); *MSF*, metal-sensitive fraction (ORG + HDP fractions). ^ASignificant difference between the Low-Cd and High-Cd treatments (Student’s *t* test, *p* < 0.05)

	Treatment		Fold difference 4×
	Low-Cd (0.6 μM)	High-Cd (2.4 μM)	
Total metal (μg g DW ⁻¹) (TOT)	29.54 ± 5.19	70.89 ± 11.12 ^A	2.4×
Subcellular fraction (μg g DW ⁻¹)			
INS	0.82 ± 0.20	1.77 ± 0.22 ^A	2.2×
HSP	5.88 ± 1.54	13.22 ± 1.92 ^A	2.2×
HDP	3.25 ± 0.59	5.30 ± 0.55 ^A	1.6×
ORG	2.91 ± 0.52	8.19 ± 1.85 ^A	2.8×
Subcellular compartment (μg g DW ⁻¹)			
BDM (INS + HSP)	6.56 ± 1.66	14.83 ± 1.93 ^A	2.3×
MSF (ORG + HDP)	5.89 ± 0.96	13.04 ± 1.68 ^A	2.2×

Discussion

Changes in oyster physiology with Cd accumulation

Exposure of juvenile *C. virginica* to elevated, but sublethal, concentrations of Cd (2.4 μM) led to changes in physiology at different levels of biological organization, including whole body and subcellular levels. Condition index significantly decreased in both treatments after 4 weeks of exposure (Fig. 2). Oysters in the High-Cd treatment had significantly lower protein content (Fig. 3) and ETS activity (Fig. 4) than those in the Control and the Low-Cd treatments, suggesting that sublethal exposures of Cd impair oyster health.

Cadmium exposure has been linked to oxidative stress in a variety of bivalves. By producing damaging reactive oxygen species (ROS), the antioxidant functions of cells are reduced and the whole oyster will be put into a state of physiological stress (Macías-Mayorga et al. 2015). In the present study, an approximate 50% reduction in aerobic potential with an increase in Cd concentration was observed, strengthening the conclusion that subcellular physiology is affected by exposure to elevated Cd.

Similar results were observed when Gêret et al. (2002) exposed *Crassostrea gigas* to 1.8 μM for 21 days and found significant accumulation within gills and digestive glands while exhibiting lowered protein content. Reductions in mitochondrial activity (increases in mitochondrial uncoupling and proton leak, inhibition of oxidative respiration) and low levels of ATP (used in response to oxidative stressors) were also found when exposing oysters to low levels of Cd (Sokolova 2004; Ivanina et al. 2010).

Cadmium accumulation in juvenile oysters

Although there was a 4-fold difference in exposure concentrations between the Low-Cd (0.6 μM Cd) and High-Cd (2.4 μM Cd) treatments, there was only a 2.4-fold difference in accumulated total body burdens of Cd (Table 1). Previous field and laboratory studies with bivalves have indicated a linear relationship between exposure regimes and Cd accumulation (Serra et al. 1995; Markich et al. 2001; Perceval et al. 2006). The suppression of total body Cd accumulation during the present study could be due to saturation effects, as

Table 2 Condition index, protein content (μg mg DW⁻¹), and aerobic potential (as estimated by the ETS assay; μmol O₂ h⁻¹ g DW⁻¹) of oysters exposed to varying concentrations of Hg for 4 weeks (± 1 SE). Control =

no metal, Low-Hg = 0.014 μM, High-Hg = 0.056 μM. There were no differences found among treatments

Parameter	Control-Hg	Low-Hg	High-Hg
Condition index	5.14 ± 0.66	5.02 ± 0.65	5.06 ± 0.65
Protein (μg mg DW ⁻¹)	484.02 ± 5.38	484.72 ± 4.69	476.98 ± 9.93
Aerobic potential (ETS) (μmol O ₂ h ⁻¹ g DW ⁻¹)	85.60 ± 39.50	92.57 ± 18.30	106.59 ± 24.70

Table 3 Metal accumulation in oysters exposed to Low (0.014 μM) or High (0.056 μM) doses of Hg for 4 weeks. All values are reported as mean μg metal g DW^{-1} ($n = 3$) \pm 1 SE. The “fold difference” represents the difference in metal accumulation between the Low-Hg and High-Hg treatments. The level of metal in the Control-Hg treatments was non-detectable. As per Wallace et al. (2003): *TOT*, total body burdens; *INS*,

insoluble granules (i.e., metal-rich granules); *ORG*, organelles; *HDP*, heat-denatured proteins (i.e., enzymes); *HSP*, heat-stable proteins (i.e., metallothionein-like proteins); *BDM*, biologically detoxified metal (INS + HSP fractions); *MSF*, metal-sensitive fraction (ORG + HDP fractions). ^ASignificant difference between the Low-Cd and High-Cd treatments (Student’s *t* test, $p < 0.05$)

	Treatment		Fold difference 4 \times
	Low-Hg (0.014 μM)	High-Hg (0.056 μM)	
Total metal ($\mu\text{g g DW}^{-1}$) (<i>TOT</i>)	14.75 \pm 4.61	131.75 \pm 27.94 ^A	8.9 \times
Subcellular fraction ($\mu\text{g g DW}^{-1}$)			
<i>INS</i>	0.60 \pm 0.08	2.07 \pm 0.22 ^A	3.5 \times
<i>HSP</i>	0.32 \pm 0.06	0.48 \pm 0.07	1 \times
<i>HDP</i>	1.01 \pm 0.07	2.67 \pm 0.28 ^A	2.6 \times
<i>ORG</i>	2.19 \pm 0.36	8.02 \pm 1.08 ^A	3.7 \times
Subcellular compartment ($\mu\text{g g DW}^{-1}$)			
<i>BDM</i> (<i>INS</i> + <i>HSP</i>)	2.71 \pm 0.28	12.42 \pm 1.96 ^A	4.6 \times
<i>MSF</i> (<i>ORG</i> + <i>HDP</i>)	4.91 \pm 0.60	20.43 \pm 2.59 ^A	4.2 \times

subcellular compartments become filled with Cd. The saturation of certain subcellular compartments and subcellular binding mechanisms (i.e., metallothionein-like proteins) with Cd can lead to a decline in the rate of uptake (Serra et al. 1995). While the concentrations used in the present study did not result in significant mortality, the sublethal concentration may have been high enough to elicit responses at the subcellular level, resulting in a slower uptake rate of Cd accumulation. Declines in the amount of protein, and the observed energy expended by the oysters (ETS assay) indicate that the oysters may have been performing physiological functions (i.e., filtering water) below the optimal rate when exposed to 2.4 μM Cd for 4 weeks (Figs. 2 and 3; Bartlett et al. 2018). Slower filtration could lead to reduced exposure to dissolved Cd, and thus a decline in accumulation as dissolved metal was likely the dominant exposure pathway. Future studies examining the filtration rate of juvenile oysters exposed to sublethal levels of Cd will help to elucidate the relationship between Cd exposure and filtration rates.

To our knowledge, this is the first study of juvenile *C. virginica* and subcellular fractionation of non-essential trace metals. The subcellular partitioning of Cd within bivalves may be species-specific and related to age or size. Previous studies have examined different bivalves (or *C. virginica* adults) and shown similar trends and results to what was found here. Sokolova et al. (2005) examined adult *C. virginica* and exposed the oysters to sublethal cadmium concentrations (0.22 μM). Subcellular fractionation and mitochondrial efficiency tests revealed that increases in the amount of Cd bound to the “metal-sensitive” organelle fraction resulted in lower mitochondrial and lysosome function in the digestive gland tissues (Sokolova et al. 2005). These results also suggest that Cd accumulation will strongly affect energy budgets (storage and

usage). With respect to the age/size class of the oysters, Ng et al. (2010) collected juvenile *C. gigas* (similar to the size used in the present study) from an exposure gradient in an impacted estuary. The tissues were examined for subcellular distribution of Cd as per the same methods used here (Wallace et al. 2003), and no significant differences between accumulation in the *BDM* and *MSF* compartments were seen (Ng et al. 2010). In that study, accumulation of Cd in the *HSP* fraction was more responsive to elevated exposures, which was similar to what was found in this study. It is possible that changes in subcellular physiology in juvenile oysters that results from exposure to Cd in the environment may affect key enzymatic pathways for survival and detoxification.

Increased binding of Cd in the *BDM* compartment could be indicative of the induction of MTLPs (Wallace et al. 2003). During the present study, Cd did bind to the *HSP* fraction (2.2-fold difference between the Low-Cd and High-Cd treatments; Table 1). Short, low-level exposures may not induce metallothioneins, but longer exposures, or higher doses of metal, can lead to increases in the intracellular concentrations of metallothionein-like proteins (MTLPs) (Roesijadi and Robinson 1994; Cooper et al. 2010). The 4-week exposure used in the current study was likely long enough to induce metallothionein production, as there was a higher concentration of the total body burden in the *HSP* fraction than any other subcellular fraction (for both Low-Cd and High-Cd exposures; Table 1). While the concentration of MTLPs, and Cd bound to them, was not specifically analyzed during this study, there is ample evidence that exposure to this level of Cd would cause the increased production of these proteins (Roesijadi and Klerks 1989; Roesijadi 1996; Geffard et al. 2002; Cain et al. 2004; Macias-Mayorga et al. 2015). Similarly, juvenile *C. gigas* accumulated more Cd in the

MTLP fraction and had higher metallothionein concentrations, at concentrations similar to the Low-Cd treatments used here (field concentrations between 0.36 and 0.78 μM ; Ng et al. 2010). Therefore, it is very likely that MTLTPs were induced and accumulated Cd in the present study, and may have even reached saturation upon exposure during the High-Cd treatments (Lim et al. 1998; Cain et al. 2004; Ivanina et al. 2009; Macías-Mayorga et al. 2015).

Physiological effects of Hg accumulation

Even though Hg exposure regimes were up to eight times higher than those in the EPA guidelines (0.009 μM ; US EPA 1999, 2001), oysters from both treatments did not exhibit significant changes in condition indices, protein content, or ETS activity (Table 2). A preliminary experiment to determine the exposure regimes used 0.056 μM as the highest concentration; when no mortality was found during the preliminary 3-week exposure, this was set as the High-Hg exposure. While a higher concentration could have been used and may have led to observable impacts on physiology, it would have been environmentally unrealistic. It is important to note that the oysters used in this aspect of the study were significantly larger than the oysters used in the Cd exposures and there may be size-specific differences in metal accumulation and associated physiological responses (Mubiana et al. 2006; Zhong et al. 2013). Furthermore, the organic form of Hg (methyl-Hg, MeHg) may be present in the HRE along with inorganic Hg. Though the toxicity of MeHg may be higher, most Hg in the estuary is stored in sediments (Balcom et al. 2008; Goto and Wallace 2009). Future studies should examine the role of MeHg in the toxicity of juvenile *C. virginica*.

Mercury accumulation in subcellular fractions

Studies have shown that upon exposure to environmental pollutants, bivalves such as oysters shunt energy from normal metabolic activities (such as shell growth) to help alleviate the impacts of exposures (Engel 1999; Kawaguchi et al. 1999; Mouneyrac et al. 1998; Ringwood et al. 2004; Sokolova et al. 2005; Sanni et al. 2008; Volety 2008; Macey et al. 2010). While the High-Hg exposure was 4-fold higher than the Low-Hg exposure, the total body burden of oysters from High-Hg treatment was approximately 9-fold higher than those from the Low-Hg treatment (Table 3). To our knowledge, this is the first study examining subcellular Hg accumulation of juvenile *C. virginica*; however, previous studies using other marine bivalves have shown increased accumulation of Hg in tissues. *Perna perna* mussels exposed to 0.25 μM Hg for 24 days were found to accumulate 87 μg Hg g DW⁻¹ in soft tissues, which was a 348-fold increase over the exposure concentration (again, illustrating tissue concentrations accumulating at a faster than linear rate) (Anandraj

et al. 2002; Gregory et al. 2002). Accumulation in the *Perna perna* mussels led to reduced filtration rates and damaged gill filaments; however, that study used Hg levels 4.5 times higher than the High-Hg exposure in the current study, which is unrealistic in terms of environmental Hg levels in the HRE (Anandraj et al. 2002; Gregory et al. 2002). Exposure of the Japanese scallop *Chlamys farreri* to very low concentrations of Hg (2.4×10^{-4} μM) did not elicit significant whole-body physiological changes, but did cause declines in subcellular function (Zhang et al. 2010). Activity of a key enzyme in metabolic activity, GPx, was inhibited with mercury exposure, which may lead to oxidative stress and alterations of protein activity (Zhang et al. 2010). It is possible that the exposure regime used here caused impaired enzymatic pathways that were not examined during the current study. Cunningham (1976) examined the effects of sublethal Hg exposure on juvenile oysters, though only shell growth was measured and not tissue physiology and subcellular accumulation of Hg. During the experiment, juvenile oysters were exposed to very low concentrations of Hg (100 ppb; roughly 100 times lower than the High-Hg treatment), which resulted in significantly reduced shell growth after a 7-week exposure. Oysters were then left to depurate and it was found that the growth rates rebounded within 4 weeks, indicating that the physiological mechanisms controlling shell growth were not permanently impacted (Cunningham 1976). Oysters were likely allocating energy to alleviate toxicity, rather than using the energy for shell growth.

Conclusion

Exposure of juvenile *C. virginica* to elevated, yet sublethal, concentrations of Cd (2.4 μM) for 4 weeks resulted in significant changes in physiology (i.e., lowered physiological condition, decreases in protein content, and changes in aerobic potential). Cadmium was found to accumulate at a higher concentration (μg g DW⁻¹) in the subcellular fractions HSP, ORG, and HDP compared with that in INS. The accumulation of Cd onto the HDP fraction likely impacted enzymatic activity involved in oyster physiology, resulting in lowered activity of the electron transport chain, lowered protein synthesis/storage, and lowered overall condition. Accumulation in the ORG fraction could also decrease activity in the electron transport chain, as this is part of mitochondrial function in *C. virginica* (Sokolova et al. 2005). Accumulation in the HSP fraction could lead to alterations in protein storage as metallothioneins accumulate Cd; alterations in key enzymatic pathways meant to protect the oysters have been induced at similar exposure regimes in other studies (Macías-Mayorga et al. 2015; Meng et al. 2017). In this study, Cd accumulated at a less than linear rate in the HSP but greater than linear rate in ORG which may have allowed for preservation of key enzymatic pathways

necessary for detoxification. Further research is required to understand the mechanisms of subcellular binding of Cd and how it relates to the enzymatic pathways involved in detoxification in juvenile oysters.

In contrast, none of the physiological endpoints examined illustrated discernible changes in physiology when juvenile *C. virginica* were exposed to elevated, yet sublethal, concentrations of Hg (0.056 μM) for 4 weeks. Mercury accumulated most heavily in the ORG and HDP fractions, both fractions of the MSF compartment (Wallace et al. 2003). While no physiological endpoints were altered during exposures, it is possible that subcellular changes may have occurred that were not examined during this study. It is important to note that the total burden was 8.9-fold higher for High-Hg oysters versus Low-Hg oysters, yet the individual fractions were only 1.5- to 4.6-fold higher in High-Hg treatments. Studies have shown that Hg preferentially binds to membranes (Pan and Wang 2011; Velez et al. 2016), and it is likely that the majority of Hg that the oysters accumulated was indeed associated with membranes (which would be a part of the cell found in the “cell debris” fraction that was not analyzed during this study). Future studies should examine the role of the “cell debris” fraction in Hg accumulation and overall physiological effects of oysters exposed to environmentally realistic Hg concentrations.

The results of this study suggest that exposures of juvenile *C. virginica* to environmentally relevant concentrations of Cd (0.6 μM) or Hg (0.014 μM) are not likely the primary drivers of physiological stress within an urbanized estuary, such as the HRE, and would not limit restoration efforts. However, the synergistic effects of metals along with other stressors found in an urbanized ecosystem (e.g., eutrophication) may impair the overall health of *C. virginica* by altering accumulation rates, subcellular partitioning, and associated physiological impacts (Cherkasov et al. 2007; Ivanina et al. 2010, 2011). Field exposures, measuring metal accumulation, and subcellular partitioning along with physiological endpoints and environmental parameters (i.e., dissolved oxygen, total particulate matter) will provide further guidance as to future restoration projects within the HRE.

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