THE EFFECTS OF CHRONIC HABITAT DEGRADATION ON THE PHYSIOLOGY AND METAL ACCUMULATION OF EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) IN THE HUDSON RARITAN ESTUARY

by

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Abstract

THE EFFECTS OF CHRONIC HABITAT DEGRADATION ON THE PHYSIOLOGY AND METAL ACCUMULATION OF EASTERN OYSTERS (CRASSOSTREA VIRGINICA) IN THE HUDSON RARITAN ESTUARY

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The Hudson Raritan Estuary (HRE) was once home an abundant population of the eastern oyster, *Crassostrea virginica*. Years of habitat degradation, via removal of habitat and shell substrate, overfishing of the population, and inputs of organic and inorganic contaminants, all led to the decline of this previous keystone species. The HRE today is a highly eutrophic environment, with increased sediment inputs, periods of low dissolved oxygen, algal blooms, and hotspots of contaminants throughout. The current study was designed to understand how a chronically degraded habitat, as is present in the HRE now, affects both juvenile and adult oyster physiology. There are three parts to this study: in the first, a large scale field transplant study was deployed to determine how juvenile oyster health and subcellular physiology are altered over a continuum of sites across the HRE, and if subcellular metal accumulation related to alterations in physiology. Using eight sites across the HRE, it was apparent that there are many site-specific factors that affect oyster physiology, and the synergistic effects of these abiotic and biotic factors together influence oyster physiology the most. There was no one factor that could be isolated as a key parameter to determine future oyster restoration. The second part used a field transplant study to

examine the role of a degraded habitat on adult oysters and reproduction. Using Vitellogenin protein and energy expenditures to estimate oyster reproduction, it was seen that adult oysters respond much slower than juveniles and no differences were seen between highly degraded habitats and less degraded habitats. Thirdly, both juveniles and adults were observed to accumulate non-essential metals (Cd and Hg) in the field. In order to determine if metal accumulation is the sole cause of physiological alterations, a laboratory exposure was designed to determine if changes in subcellular physiology could be correlated specifically to subcellular accumulation of Cd or Hg, when no other abiotic factors are able to influence oyster health. It was observed that Cd accumulation lead to physiological changes, but Hg accumulation did not. Using this information about the site-specific nature of oyster physiology and how metal accumulation can alter physiology will allow researchers to choose future restoration sites and set up projects that will allow for maximum growth and survival.

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CHAPTER 1

INTRODUCTION

The Hudson Raritan Estuary

Mid-Atlantic estuaries, including the New York-New Jersey Harbor (also known as the Hudson Raritan Estuary), are flourishing ecosystems where saline ocean water mixes with fresh water draining from rivers and creeks (US EPA, 2007). Many areas in these estuaries are prone to anthropogenic impacts; as the surrounding areas become more urbanized, the water is subjected to both physical and chemical disturbances. Though water quality has improved since the passage of the Clean Water Act (1972) and the formation of the National Estuary Program (1987), many parts of the ecosystem are still impaired (US EPA, 2007).

Flowing through the most urbanized city in the United States, the Hudson Raritan Estuary (HRE) has a drainage basin covering 34,000 km^2 of upstate NY and NJ, flowing out the Hudson, Raritan, Passaic, and Hackensack rivers to the Newark, New York, and Raritan Bays (Levinton and Waldman, 2006; Yozzo et al., 2004; Wakeman and Themelis, 2001). The saline wedge of the HRE extends up the Hudson River to Troy (240 km); salinity at Hastings-on-Hudson (near the Tappan Zee Bridge) is between 5-10 ppt, while salinity in Raritan Bay is between 16-32 ppt (Grizzle et al., 2013; this study). Various types of habitats exist in the HRE; freshwater and salt marshes, mudflats, deep water, submerged aquatic vegetation, and small tributary creeks all are present within the estuary, leading to a variety of different habitats (Levinton and Waldman, 2006). Bacterial assemblages, phytoplankton and zooplankton, infaunal and epifaunal benthos (e.g., amphipods, polychaetes, bivalves), and a wide variety of fishes are all found as well (Levinton and Waldman, 2006).

Oysters in the HRE

One of the most ubiquitous invertebrates in the estuary was the eastern oyster, *Crassostrea virginica*. Oysters were very large in the 1600s (described as "dinner plates" by Franz, 1982), and were of great importance to the native people and new settlers, for both food and commerce (Kurlansky, 2006; Franz, 1982). In the late 1600s, it was estimated that 350 square miles of oyster beds existed between Brooklyn, Queens, Jamaica Bay, the East River, Raritan Bay, the lower Hudson River (to Ossining), the Hackensack and Raritan Rivers, and around Staten Island, City Island, Liberty Island, and Ellis Island (Kurlansky, 2006). New York City (NYC) was the capital of the oyster industry for much of the eighteenth and nineteenth centuries, with over half the world's oysters coming from the HRE (Kurlansky, 2006). Oysters were freely harvested, with beds seemingly inexhaustible (Kurlansky, 2006; Crawford et al., 1994, Franz, 1982). Each area of the HRE had its own special "variety" of oysters, with a different flavor from the local waters; these included the large 'Gowanus' oysters, 'Rockaway' oysters from Brooklyn, and 'Princes Bay' in Staten Island. Jamaica Bay and the Rockaways had a rich population of oysters, Staten Island had large oyster beds off the south shore, and Raritan Bay (Keyport) had the 'Great Beds' which were the largest expanse of oyster beds in the HRE at the time (Kurlansky, 2006; Franz, 1982). Over time, oystermen were able to learn where the most growth would occur (where salinity, temperature, and other factors combined to produce the best oysters), and began to cultivate the beds for the largest oysters. Optimal oyster growth and density occurred naturally in the HRE due to the fact that it is a partially mixed estuary, which prevents freshwater from dominating, and lowering the salinity (Kennedy, 1996).

Overharvesting and pollution led to the decline of these large oyster beds beginning in the 1800's (Kurlansky, 2006; Crawford et al., 1994). Beginning in Staten Island and continuing out throughout the rest of Raritan Bay and New York Harbor, oyster beds began to be smothered with increased sedimentation from rivers. Removal of adult oysters left few oysters of reproducing age, and spat settlement declined. After the natural oyster beds were exhausted, oystermen began to transplant seed oysters from other beds (within the HRE and from Chesapeake Bay); these efforts were successful at first but gradually production on the beds declined and after 1909 no harvests were reported.

Beginning in the mid-1600s, pollution began to increase into the HRE and onto the oyster beds. As the population of New York City and the surrounding areas began to increase, raw sewage and other point-source pollutants were dumped into the Hudson and East Rivers. Historical reports (Earll, 1887, as cited in Crawford et al., 1994) have noted that the decline of the oyster industry in Newark Bay was due to the tainting of the oysters by coal oil from the various refineries in the HRE (Crawford et al., 1994). Industrial discharges into Raritan Bay and the Arthur Kill lead to the demise of the Staten Island beds and the 'Great Beds' area of Keyport Harbor, and the closure of many other beds in the HRE (Gowanus Bay, Jamaica Bay, Newark Bay; Kurlansky, 2006; Crawford et al., 1994). Along with contaminant increases, there were increasing reports linking human diseases (cholera, typhoid fever) with consumption of oysters. As of the 1920s, all oyster beds in the HRE were closed to harvesting.

Discharge of raw sewage into the lower HRE had been going on for many years prior to the installation of a sewer system, and it was not until the mid 1900's that sewage was finally

"treated" before passing into the waterways (Waldman, 1999). Sewage sludge is one of the main causes of oyster (and other benthic invertebrates) decline in the HRE. The thick, black ooze that covered the benthos in the harbor would remove oxygen from the water column, suffocating all biota. This sludge also added chemicals such as carbonic acid and ammonia into the water column, which damage bivalve shells.

Alteration of the natural oyster habitat within the HRE

In addition to pollutant inputs and overharvesting, oyster populations were impacted by habitat alteration and removal. The removal of live oysters by tonging and dredging practices also ruined the accreted reefs below, removing habitat for potential new spat. Additional removal of fringing reefs further altered the habitat, by adding more soft sediment onto hard substrates where oysters would settle. For example, in Jamaica Bay over 50% of salt marshes have been lost since the early 1900s (National Parks Service, 2007); in Newark Bay, over 75% of the original marsh has been destroyed by dredging and fill activities; dams and dykes have altered water flow, and dredging as altered the bottom topography and water currents in the HRE (Crawford et al., 1994). Also important was marsh infilling; sediment settles down on top of municipal wastes in the area and buries them (Crawford et al., 1994). Toxins buried in sediments can still be bioavailable to marine organisms.

Sedimentary movement by dredging also lead to increased sedimentation over oyster reefs and shallow bays (Yozzo et al., 2004). The Port of NY/NJ maintains an active commercial and industrial shipping business, with over 200 miles of navigational channels within the HRE; approximately 6.5 million m^3 of sediment are dredged per year (Wakeman and Themelis, 2001).

Sedimentation rates within the HRE vary from 0.3-5 cm yr^{-1} (depending on the location within the lower HRE), and up to 14 cm yr^{-1} in dredged canals (Bokuniewicz, 2006). Sand and coarse grain materials are transported along the bottom in waves, while fine-grained sediments and mud are transported as suspended load within the currents and deposited onto wetlands and the estuary floor, filling in dredge channels (Bokuniewicz, 2006). This dredged material has been dumped offshore, used to fill in salt-marsh and other coastal habitat (for development), and, most recently, used for restoration of destroyed habitats such as oyster reefs and mudflats (Yozzo et al., 2004).

Pollution inputs to the HRE

The HRE has been characterized by extensive pollution since the 1800's, as industry and population in the area increased (Levinton and Waldman, 2006; Yozzo et al., 2004; Wakeman and Themelis, 2001; Feng et al., 1998). Contaminants from wastewater treatment plants, run-off, combined sewer overflows (CSOs), emissions, industries, chemical effluents, and atmospheric deposition have lead to elevated levels of many organic and inorganic pollutants in the HRE (Wirgin et al., 2006).

The HRE is second only to San Francisco Bay Estuary in terms of metal contaminant levels within an estuary (Levinton and Waldman, 2006). Both essential and non-essential metal ions (Class A and Class B; Hodson, 2004; Duffus, 2002) can affect organisms living in the estuary. Metals that are found within the HRE include mercury (Hg), nickel (Ni), cobalt (Co), chromium (Cr), aluminum (Al), magnesium (Mg), Cadmium (Cd), copper (Cu), mercury (Hg), and zinc (Zn) (Feng et al., 1998; Klerks and Levinton, 1989). Sediment loads of these metals (e.g., Cd,

Cu, Zn, and Hg) are all known to have exceeded the NOAA's effects- range low (ERL) and effects- range median (ERM) levels (Long and Morgan, 1991) at various times since the mid-1800s (Wirgin et al., 2006; Crawford et al., 1994). Cadmium is a non-essential metal and is toxic to an organism that cannot sequester and eliminate the excess metal (Newman and Unger, 2003). The best documented input of Cd to the HRE is that of the NiCad (Nickel- cadmium) battery plant (Marathon Battery) in Foundry Cove, NY (Klerks and Levinton, 1989). This was the site of a Superfund clean-up in 1995, one of the first in the HRE (Levinton and Waldman, 2006; Wirgin et al., 2006; Feng et al., 1998). Sewage sludge, plastic stabilizers and pigments all produce Cd waste that can contaminate the HRE (Newman and Unger, 2003). High concentrations of Cd have been found in sediments from Newark Bay and the Hudson River (Levinton and Waldman, 2006; Feng et al., 1998; Crawford et al., 1994). Upriver inputs of Cd are an important source of this contaminant to the lower HRE (Feng et al., 1998).

In addition to high concentrations of metals within the HRE, many organic toxins are also found to be in excess of NOAA's ERL and ERM guidelines (Wirgin et al., 2006; Crawford et al., 1994). Polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), furans and dioxins were all found above ERL and ERM levels in Newark Bay and the lower HRE (Crawford et al., 1994). PCB levels remain elevated due to the release of large quantities of PCBs from the General Electric plant (in the upper Hudson River) during the 1940s-1970s (Levinton and Waldman, 2006; Dimou et al., 2006; Feng et al., 1998). Dioxins have been found in elevated levels in the Raritan River and the Passaic River (Levinton and Waldman, 2006). Accidental spills of crude oil and other hazardous chemicals has been an ongoing problem in Newark Bay and New York Harbor since the 1800s; oil is often seen floating on top of the water

column in the HRE (Crawford et al., 1994; Brown et al., 1992). In 1990 alone, over 5.5 million liters of fuel oil was spilled into the Arthur Kill and Kill van Kull (Brown et al., 1992). All of these contaminants have been shown to cause mortality, reproductive failure, decreased growth rates, shell deformities, and other pathological lesions in bivalves; additionally, contact with the oil and other petroleum extracts can impair the filter feeding apparatus in bivalves (Roesijadi, 1996; Capuzzo, 1996; Crawford et al., 1994; Brown et al., 1992).

Inputs to the HRE can be from either point or non-point sources. In particular, combined sewer overflows (CSOs) pose a particular problem in the lower HRE. CSOs continue to put run-off containing organic and inorganic contaminants into the HRE, despite regulations to control contamination (Dimou et al., 2006; Wakeman and Themelis, 2001; Crawford et al., 1994). CSOs may lead to eutrophication via increased nitrogenous waste inputs, which may lead to lower dissolved oxygen levels (Nezlin et al., 2009). In the early 1900s, oxygen levels in Newark Bay were reported to be less than 0.33 mg/L (dissolved oxygen levels between 0-0.5 mg/L are fatal to most fish and benthos); by the late 1990s, oxygen levels were on the rise; average levels are approximately 3 mg/L in the HRE (Crawford et al., 1994). Depleted oxygen concentrations (hypoxia) and anoxia can have severe effects on the physiology of benthic marine organisms, including oysters. While oysters are adept at handling short-term reductions in dissolved oxygen, oysters may experience reduced growth, cessation of feeding, and ultimately mortality when confronted with hypoxic or anoxic conditions for prolonged periods of time (Baker and Mann, 1992; Widdows et al., 1989). Reefs may see reductions in settlement of new spat, and slower growth of spat and juveniles on the reef (Baker and Mann, 1992). Mortality of larval and juvenile oysters reduces the amount of oysters in a population, which will have effects on the

amount of gametes spawned by adults, thus affecting future recruitment as well as the current population (Baker and Mann, 1992). Hypoxia and anoxia also lead to changes in the oysters' metabolism, shifting to anaerobic metabolism; this type of energy usage requires the changing of pathway enzyme concentrations and enzyme cofactors (Greenway and Storey, 1999).

Current conditions in the HRE show improvement over the previously high pollution levels. Numerous legislative acts were passed in the 1970s-1990s that lead to increased pollution control and waste treatment. Benthic surveys of Newark Bay in the 1980s found increases in species abundance and species richness, where no benthos had been found in previous decades (Crawford et al., 1994). Studies of benthic sediment cores from 1973-1986 found decreases in the surficial sediment concentrations of several metals (Cu, Zn) following legislation (Cochran et al., 2006).

Study organism: The eastern oyster Crassostrea virginica (Gmelin)

Eastern oysters (*Crassostrea virginica*) are a keystone species in intertidal and subtidal estuarine areas along the Atlantic coast (Shumway, 1996). Distributed from northern temperate to subtropical areas along the East coast of North America, *C. virginica* is found in various estuarine salinities and temperatures (Thompson et al., 1996). The bivalve has a flattened right shell and larger, more cupped left shell; this asymmetry is very important to the ecology and behavior of the oyster (Eble and Scro, 1996). Like all other bivalve molluscs, *C. virginica* has a mantle covering the internal organs (visceral mass), which secretes the shell and protects the gills (Eble and Scro, 1996). Adductor muscles help to keep the valves of the shell closed when the oyster is not filtering water, and allow the shells to gape when the oyster is feeding (Eble and

Scro, 1996). Growth is maximized in water with higher food availability and lower thermodynamic stress (Thompson et al., 1996; Shumway, 1996).

Oysters are dioecious, protandric species which spawn gametes into the water column annually, where the fertilized eggs develop into planktotrophic larvae (Eble and Scro, 1996; Newell and Langdon, 1996; Thompson et al., 1996). Spawning is usually coordinated over a reef, as the release of gametes from one oyster stimulates nearby oysters to do the same (Thompson et al., 1996). Environmental conditions, such as temperature, salinity, light, pH, and seston concentrations, can all affect spawning, larval survival, and recruitment (Shumway, 1996). After approximately one year, oysters mature into adults. Male oysters are typically smaller (less than 35 mm) and younger; sex change is influenced by size, environmental conditions and sex ratios within the population (Eble and Scro, 1996; Thompson et al., 1996). Reversal of sex, from female to male, has been documented but is uncommon (Thompson et al., 1996). Sex ratios in an oyster population are important for spawning and fertilization; stress from nutrition, disease, and contaminants have all been showed to skew sex ratios in oyster populations (Thompson et al., 1996).

Reproduction in *C. virginica* occurs in four steps, (1) storage of energy, (2) production and accumulation of gametes, (3) release of gametes, and (4) recovery period (Thompson et al., 1996). The gonads begin as indistinguishable tissue around the digestive tract; as the oyster begins to store glycogen and environmental conditions become favorable, the gonads begin to differentiate into oocytes and spermatocytes (Eble and Scro, 1996; Thompson et al., 1996). Vitellogenesis (formation of an egg yolk for larval nutrition) occurs prior to spawning, and can

be influenced by the health and stress of the adult oyster (Thompson et al., 1996). Vitellin (Vn) and Vitellogenin-like proteins (Vtg) have been found in the gonad tissue of bivalves, and can be altered due to the presence of contaminants (i.e., estradiols; Gagnè et al., 2002). After spawning, any unspent follicles are resorbed by amoebocytes. As water temperatures cool, oysters become dormant for the winter (Eble and Scro, 1996). In the spring, when conditions are again favorable, gonad tissue will redevelop. In the mid-Atlantic, one major spawning period occurs (mid-summer); another smaller spawning period, in the fall, may also occur (Kennedy, 1996; Thompson et al., 1996). It is difficult to accurately measure the amount of gametes spawned per adult per year, since multiple spawning periods may occur, and not all oysters on the reef spawn at exactly the same moment; however, many researchers agree on an estimate of 4-9 million eggs per female per year (Thomspson et al., 1996; Cox and Mann, 1992).

Many internal and external factors control spawning and gametogenesis in *C. virginica*. Environmental conditions (i.e., temperature and salinity) can control the timing of gametogenesis and spawning. A gradual increase in temperature has been shown to initiate gametogenesis, while a sudden increase in temperature induces spawning (Thompson et al., 1996; Shumway, 1996). Pheromones and other chemical cues present in the water column can induce or delay spawning (Thompson et al., 1996; Shumway, 1996). Parasites and disease can reduce fecundity and delay (or terminate) gametogenesis (Thompson et al., 1996). Internal cues, such as stored nutrients and endocrine compounds, can also control the timing of spawning and gametogenesis (Thompson et al., 1996). However, it is the interaction of many of these internal and external cues that is most likely controlling spawning and gametogenesis- no one factor can be singled out for having the greatest impact (Thompson et al., 1996). For example, spawning is often

coordinated with peaks in food availability in the water column, an interaction of temperature, salinity, hydrodynamics of the estuary, nutrient stores of the adult and vitellogenesis of the eggs, and chemical cues from other, nearby oysters that spawn (Thompson et al., 1996).

Post- fertilization, a trochophore larva develops in the water column. Initially, the larva is sustained by egg yolk nutrients; any stress the adult oyster has experienced, both external (i.e., temperature or food availability) and internal (i.e., parasites), can alter the concentrations of lipid and protein in the yolk and lead to reduced growth and survival in larvae (Thompson et al., 1996). Trochophore larvae undergo massive mortality from fluctuating environmental conditions (temperature, salinity, currents), and predation. Oyster larvae have been found in the guts of zooplankton (e.g., ciliates), fish, cnidarians, and even adult oysters (Kennedy, 1996).

Larvae transition to suspension feeding once the digestive system is developed, using multiple bands of cilia to move through the water and feed on plankton (Newell and Langdon, 1996). The veliger larva (also known as Prodissoconch 1 stage, "D"- veliger, or straight hinge larva) then develop a velum, consisting of two ciliated lobes which help to move the larvae through the water column, feed, and absorb organic matter (Newell and Langdon, 1996; Sellers and Stanley, 1984). Many studies have been performed on trochophore larva, investigating the optimum size range of phytoplankton particles that can be retained by the cilia; Baldwin and Newell (1995) found that a size range between 3-10 μm supported maximum larval growth.

The veliger larvae undergoes metamorphosis, transitioning to a pediveliger larva (also known as Prodissoconch 2 stage; "eyed" larvae), which is ready to settle out of the water column onto the

substrate (Kennedy, 1996; Newell and Langdon, 1996; Sellers and Stanley, 1984). 'Settlement' refers to the metamorphosis of the pediveliger into a benthic, sessile organism which cements down onto the cultch (old oyster shells); the survival of the settled spat over time is referred to as 'recruitment' (Kennedy, 1996; Keogh and Downes, 1982). Recruitment can determine the fate of an oyster reef. A reef must accrete upwards each generation, to keep the oysters from being buried in sediment (Kennedy, 1996; Shumway, 1996). Recruitment is strongly influenced by the spawning capacity of the adult oysters on the reef, environmental conditions in the estuary, and other external cues (e.g., food availability, predators, and disease; Kennedy, 1996). The pediveligers use their muscular foot to crawl around on the benthos, seeking out appropriate substrate. Chemical cues can influence where the oyster settles, preferring to settle down on top of older oyster shells over other substrates (Nesterlode et al., 2007). Pediveligers can reverse swimming directions, and seek out several different substrates before cementing down into a permanent location (Tamburri et al., 1992). Once the pediveliger finds a suitable location, the foot (pedal gland) will secrete a "cement" ($CaCO_3$) substance that binds the left valve of the oyster shell down onto the substrate (Kennedy, 1996). The foot will be resorbed into the body, and the oyster will become sedentary (Kennedy, 1996).

Juvenile and adult *C. virginica* feed using their gills to filter out particles from the water, which are then passed along a basal food tract (at the edge of the gill) on to the gut (Newell and Langdon, 1996). Oysters are omnivores, ingesting a mix of phytoplankton and zooplankton of various sizes, along with bacteria and detritus. Phytoplankton is the principle food source; the size of phytoplankton ingested and retained is dependent on the size of the oyster. Generally, particles between 6-10 μm provide the highest retention efficiencies (Newell and Langdon,

1996). Oysters are able to vary the rate of ingestion by adjusting the degree of particle selection (Beninger et al., 2008). In addition to planktonic food sources, dissolved organic matter can provide nutritional elements to the oyster. Studies with *C. gigas*, the closely related Pacific oyster, have shown that sugars and amino acids are readily ingested by the oyster (Langdon and Newell, 1996), and that seston quality and quantity can alter ingestion rates (Beninger et al., 2008).

Measurements of clearance rates, the volume of water totally cleared of particles per unit of time, can provide evidence as to how much nutritious particles an oyster is ingesting (Newell and Langdon, 1996; Bayne et al., 1985). By monitoring the decrease in the amount of particles in a known volume of water over time, it is possible to ascertain the clearance rate of the oyster (Newell and Langdon, 1996). Clearance rates can be affected by various factors, including shell gape and particle retention efficiency. Shells gape in response to environmental conditions (the mantle has sensory tentacles on the edges; Newell and Langdon, 1996); if the shells do not open or close correctly, it will adjust the clearance rate. Oysters are also known to have multiple "speeds" of clearance rate, switching between "low" and "high" clearance rates depending on environmental conditions and growth rates (Newell and Langdon, 1996; Powell et al., 1992). The flow of water, and associated hydrodynamics of the estuary, have an effect on clearance rates by altering the amount of food and oxygen delivered to the oyster, which will change the ventilation and clearance rates (Newell and Langdon, 1996).

Effects of trace metal pollutants on oysters

Organisms exposed to contaminants may experience toxicity effects at various levels of biological organization. Changes may be seen at the molecular, cellular, tissue, organismal, and population levels of organization within oysters [see Table 1 (reprinted from Capuzzo, 1996)]. The first response of the oyster to contamination is to activate a compensatory mechanism, which can sequester the contaminant (Capuzzo, 1996). Detoxification mechanisms, such as the induction of metallothioneins (MTs) and metallothionein-like proteins (MTLPs), help to sequester and detoxify contaminants in tissues. When the compensatory mechanisms are overloaded, toxicity may occur (Capuzzo, 1996). Toxicity may progress through the levels of biological organization, from the cellular and organismal levels up through populations (Newman and Unger, 2003; Capuzzo, 1996). Chronic exposure to contaminants may lead to altered developmental and reproductive processes in a population by first overloading individuals (Capuzzo, 1996).

Many metals, including Cu, Cd, Hg, and Zn, are known to bioaccumulate in soft tissues of oysters. Bioaccumulation is the net accumulation of a contaminant from all sources in the environment (Newman and Unger, 2003). Bioaccumulation depends on many factors, including the speciation of the metal and the geochemistry of the environment (Newman and Unger, 2003; Roesijadi, 1996). For example, Cd is able to complex with chloride ions in salt water, and the resulting $CdCl_2$ is not bioavailable (oysters are unable to accumulate); Cu is able to form organic complexes with dissolved and particulate organic matter and is also unavailable (Newman and Unger, 2003; Roesijadi, 1996). Season and location have a strong influence on bioaccumulation of metals in oyster tissue. Studies have shown that the temperature of the ambient water is

correlated to the uptake amount of Cd in oysters (Cherkasov et al., 2007). Oyster populations have shown variability in metal accumulation within and between populations, over seasons, and from year to year (Roesijadi, 1996). Seasonal differences of temperature and rainfall can lead to differences in the amount of bioavailability metals due to changing salinity. Many metals, including Cd, behave differently in high versus low salinities, and this may affect the uptake of metals into the oyster (Apeti et al., 2009; Rainbow, 1995). In addition, salinity can change in an estuary due to location- the closer to the mouth of the estuary, the higher the salinity may be (Apeti et al., 2009). Differences in uptake of ambient metals can lead to impacts on physiological function, and may be detrimental to oyster health.

The effects of multiple contaminants, as is present in the HRE, may cause high rates of toxicity and possibly mortality in oysters (Roesijadi, 1996). Suites of contaminants, (i.e., multiple metals, or metals and organic contaminants together) may lead to high toxicity within the oyster (Roesijadi, 1996). MacInnes (1981) found that exposure to a mixture of Cu, Hg, and Zn lead to disruption of embryonic development in oysters. In addition, the effects of environmental parameters (e.g., temperature and salinity) with the effects of metal contaminants, can lead to toxicity and mortality among oysters (Roesijadi, 1996; Shumway, 1996; MacInnes and Calabrese, 1979)

Oysters are able to concentrate metals in the soft tissues to very high concentrations (Roesijadi, 1996). The use of metallothioneins (MT) and metallothionein-like proteins (MTLPs) and metal-rich granules (MRG) aid in detoxification by sequestering and binding certain metals (i.e., Cd, Hg, Cu, Zn). Once these metal sequestering vesicles become overwhelmed, then toxicity will

occur (Roesijadi, 1996). Cysteine molecules allow the MTs to bind large quantities of metal atoms to each MT, making them extremely effective in sequestering metals (Amiard et al., 2006; Newman and Unger, 2003). MTs are inducible; meaning that exposure to one of the metals will lead to the formation of more MTs (Amiard et al., 2006; Newman and Unger, 2003; Roesijadi, 1996). MTs are important in maintaining the balance of trace metals within the cell, and sequestering excess metals (Newman and Unger, 2003). They function to both (1) keep the concentration of essential metals in balance within a cell (i.e., Cu and Zn), and (2) detoxify any non-essential metals when concentrations increase (i.e., Cd, Hg; Amaird et al., 2006). A number of factors, aside from the presence of metals, can induce MTs (Erk et al., 2008; Amiard et al., 2006). Any stress an oyster undergoes, including experimental handling, starvation, oxygen deprivation (anoxia or hypoxia), freezing, and any contaminants in the environment (metals, antibiotics, herbicides) can induce MTs (Erk et al., 2008; Amiard et al., 2006). Additionally, food availability and quality, reproductive cycle in females, temperature, salinity, seasonal and spatial scales all play a part in the amount of MTs found in an organism (Erk et al., 2008; Amaird et al., 2006; Ivanković et al., 2005).

Granular hemocytes (also termed metal-rich granules) concentrate metals (i.e., Cu, Zn) to high levels (Rainbow and Smith, 2010; Roesijadi, 1996). Eastern oysters contain granular hemocytes that sequester both Cu and Zn. In *C. gigas*, over 90% of the total body burden of Cu and Zn was found to be in granular hemocytes (Thompson et al., 1985). Granular hemocytes have been found in the interstitial spaces in oyster tissue, especially around the gills, mantle, and digestive gland (Roesijadi, 1996), indicating that these tissues are very important in the defense of oysters against contamination. Granular hemocytes have also been shown to initiate an inflammatory

response in some bivalves, and were found at the site of tissue damage (Roesijadi, 1996). Elevated levels of Cd were shown to reduce the amounts of granular hemocytes found in oysters, and to inhibit the phagocytic activities of the hemocytes (Cheng, 1996; Cheng 1988; George et al., 1983).

Reproductive cycles of oysters lead to variations in the amount of metal that is found in the soft tissues. Reproduction changes the amount of soft tissue found in the oyster, by increasing the size of the gametes during gametogenesis. Post-spawning, metal reserves within the soft tissues can fluctuate due to a loss of nutrients and tissue in the oyster (Roesijadi, 1996). Páez-Osuna et al. (1995) found seasonal fluctuations in *C. iridescens* with respect to the concentration of several trace metals, including Cd, Cu, and Zn; trace metal concentrations decreased as gonadal tissue increased. In addition, Cd exposure may cause poor spawning effort and abnormal larvae from affected adults. The metal can have a toxic effect on the adult oysters' gonads, which in turn transfers to the larvae (Roesijadi, 1996). Exposure of adult *C. virginica* to "Class B" metals has also been shown to affect the energetics and metabolism of the gametes (Roesijadi, 1996). Oyster larval stages are the most sensitive to bioaccumulation (Roesijadi, 1996). At this stage, cells are rapidly dividing and DNA/RNA is constantly being replicated. Cu and Cd inhibit the activity of RNA polymerase, which is necessary in cell differentiation and the formation of new cells (Roesijadi, 1996). Calabrese et al. (1977) found that Hg, Ag, Cu, and Ni all caused toxicity in *C. virginica* embryos. Larval stages are less sensitive than embryos; however, there is still rapid differentiation at these stages, and the larvae are still more susceptible to toxicity than adults (Roesijadi, 1996). Metal binding to intracellular ligands also differs between larval phases. Studies have found that *C. virginica* embryos exposed to elevated contaminant levels

bind more Cu and Zn to MTs than in other intracellular pools (Ringwood and Brouwer, 1995). Additionally, metamorphosis from a larva into a sessile adult can be disrupted by exposure to metals (i.e., Cu; Roesijadi, 1996).

Using MTs and metal-rich granules to bind metals, oysters can sequester contaminants and avoid toxicity. However, once a threshold concentration is reached, either due to long-term exposure or high quantities of contaminants, the oyster will experience effects of toxicity (Roesijadi, 1996). Long-term studies have shown that toxicity effects such as reduced shell growth and emaciation of soft tissues can occur with exposure to elevated Cd levels (Roesijadi and Klerks, 1989). Changes in shell thickness have been reported with exposure to elevated Cd, Cu, and Zn levels; this is due to inhibition of enzymes that form the shell matrix (Hinkle et al., 1987). Increases in metal exposure can affect detoxification, such as induction of more MTs and granular hemocytes. The "spill-over" hypothesis (Campbell et al., 2005; Brown and Parsons, 1978) states that a reduction of MT concentrations at high exposures is due to the fact that the excess toxins are preventing the MTs from being fully functional, and therefore less metal is sequestered (Amiard et al., 2006). Also, the MTs that are already present do not perform well, as studies have shown failure of MTs to suppress reactive oxygen species at high Cd concentrations (Amiard et al., 2006).

Examining metal accumulation in oysters: physiological endpoints

Energy reserves: carbohydrate, lipid, and proteins

Oysters are able to obtain nutrition during times of high food availability (summer, fall) and store it for use in the winter, when food availability is lowest (Thompson et al., 1996). In the HRE,

the phytoplankton bloom is the winter-spring bloom. These energy reserves are used for basal metabolism ("maintenance" metabolism) and also for gametogenesis (Thompson et al., 1996). Carbohydrates, specifically glycogen, are the most important molecule stored in *C. virginica*. This biomolecule provides energy storage, and is the main component fueling gametogenesis and vitellogenesis (Thompson et al., 1996). Glycogen is stored in the interstitial spaces between the gonad tissue and digestive gland, in specialized Leydig cells. These cells are used during gametogenesis to produce gametes for spawning (Eble and Scro, 1996; Thompson et al., 1996). Immediately after spawning, glycogen stores are at the lowest levels in oyster tissue (Shumway, 1996; Thompson et al., 1996). Lipid levels vary seasonally, and are heavily related to reproduction and gonadal tissue concentrations (Abad et al., 1995). Other than during vitellogenesis, lipid stores are maintained at approximately the same levels throughout the year. During vitellogenesis excess lipids are stored in oocytes, and decrease once the eggs are spawned (Thompson et al., 1996). Protein levels remain relatively constant in oyster tissue throughout the year. However, long term deviations of protein levels may be due to seasonality (temperature and salinity changes). Protein levels may be elevated in response to exposure to contaminants, due to the increase of defensive mechanisms (such as metallothioneins; Smolders et al., 2003). Glycogen stores and lipid stores were shown to decrease in marine bivalves in response to effluent exposure, and protein elevation could be due to a trade-off between declining glycogen levels and increasing protein pools (Smolders et al., 2003).

Eastern oysters ingest a variety of plankton and seston through their filtration activities, leading to fluctuations in nutritional intake per day (Langdon and Newell, 1996). These fluctuations can affect the growth rate of the oyster by altering the energy reserves within the oyster's tissues.

Low protein amounts in the phytoplankton can lead to better growth rates, by providing a higher C:N ratio. Higher carbohydrate amounts can lead to better growth rates (again by altering the C:N ratio; Langdon and Newell, 1996). Amino acid amounts can change the lipid stores in an oyster, and ingestion of certain amino acids (i.e., 20:5ω3 and 22:6ω3, two fatty acids found in certain algal species) can lead to higher growth rates (Langdon and Newell, 1996). Food quality can alter clearance rates in larvae (Baldwin and Newell, 1995) and adult (Beninger et al., 2008) oysters.

Energy usage: the electron transport system (ETS) assay

Determination of subcellular energy usage, using the ETS assay, has been used in several species to estimate metabolic rates *in situ*. Measurement of respiration rates *in situ* are important, since stress from sampling and laboratory conditions can lead to altered physiological rates in oysters, and inaccurate respiration rate readings may occur (Fanslow et al., 2001). ETS studies were first performed with zooplankton, but methods have been adapted for use on larger organisms, including bivalves (Erk et al., 2008; Olsen et al., 2007; Smolders et al., 2004; Smolders et al., 2003; Fanslow et al., 2001). The ETS assay measures activity of the enzymes involved in the electron transport chain, which consists of a chain of cytochromes, flavoproteins, metallic ions, and other enzymes located in the mitochondria (Fanslow et al., 2001; Cammen et al., 1990). The assay is performed by measuring the activity of the final electron acceptor for oxygen, INT-tetrazolium. INT-tetrazolium is reduced to INT-formazan when O_2 is substituted for the terminal electron acceptor; INT-formazan is optically reactive and is able to be easily measured on a spectrophotometer (at 490 nm; Fanslow et al., 2001; Madon et al., 1998). Then, a stoichiometric

relationship is used to convert the formazan reading to amount of oxygen consumed (De Coen and Janssen, 1997).

Madon et al (1998) analyzed zebra mussel (*Dreissena polymorpha*) respiration rates and observed that ETS rates had a strong correlation with respiration rates, and with wet weight of the organism. The correlation of respiration and ETS rates supports the conclusion that the ETS assay is an acceptable metric for estimating the energy consumption in bivalves. A low R/ETS ratio has been found for sessile organisms (Fanslow et al., 2001; Madon et al, 1998), due to the fact that ETS only measures a small percentage (<10%) of cellular respiration; active organisms require more enzyme activity and consume more energy, so their R/ETS ratio is high (the respiration value increases drastically, while the ETS value still stays low). Oysters are sessile bivalves, so the ETS assay is an acceptable metric for determining energy consumption in juvenile and adult oysters. Energy usage in oysters is also dependent on size. As adult oysters grow larger, less energy is partitioned for growth and more is spent on reproduction; larger oysters can produce more eggs than smaller oysters (Thompson et al., 1996). R/ETS ratios show that there are differences in usage with males versus females, and also with the size of organisms (Fanslow et al., 2001; De Coen and Janssen, 1997; Cammen et al., 1990). ETS activity also shows strong seasonal variation, related to the temperature of the environment and the food quality in the area (Fanslow et al., 2001; Cammen et al., 1990). Studies with zebra mussels in Lake Huron found that ETS activity increased as the temperature of the bay increased, and that this correlation can be attributed to differences in food quality over the seasons (Fanslow et al., 2001). Additionally, the quality of the food particles being filtered by the bivalve has an effect on ETS rates, by altering the amount of filtering that must be done and the amount of nutrition

that is ingested (versus the amount of pseudofaeces produced; Fanslow et al., 2001). Poor food quality can lead to a reduced respiration rate, and therefore a reduced R/ETS ratio (Fanslow et al., 2001).

Advantages of the ETS assay are that (1) the organism does not become acclimated during the collection period, due to the fact that ETS responds slower to environmental change and stress than physiological respiration rate, (2) ETS has an allometric relationship with body mass, and scales at the same coefficient as respiration rate (meaning both have the same correlation with body size, and can be scaled similarly), and (3) ETS assay is able to be performed at a later date (after being flash frozen and stored at -80^{o}C), which allows for more samples to be run simultaneously and a more efficient use of time in the laboratory and field collection (Madon et al., 1998). ETS is able to determine energy consumption on a finer scale than physiological respiration studies; ETS is more sensitive, and responds more slowly (to avoid affects of sampling stress on the oysters; Madon et al., 1998). ETS activity tends to require a time period of days to weeks to adjust to changing environmental conditions; reflects the conditions the organism is in at that moment in time, not a condition being brought on by sampling stress or starvation in a laboratory tank (Fanslow et al., 2001; Cammen et al., 1990).

Study sites in the HRE

The lower HRE (Figure 1) is a geographically small area with a wide range of environmental parameters. Temperature, salinity, and dissolved oxygen can vary on microscales, spatially and temporally. Several sites were chosen to study oyster physiology, in order to give an overview of the HRE on both large and small spatial scales. Sites chosen had a link to oyster restoration in

the HRE (either current or future research). All sites had historically been home to populations of native *C. virginica* in the estuary (Kurlansky, 1996; Franz, 1982), though at the present time only one (Soundview Park) was observed to have a small population of wild oysters surviving (Medley, 2010).

Soundview Park (SVP) is a large (205 acres) park located in the south Bronx, where the Bronx River and East River flow together (NYC Parks, 2013). The park was originally comprised of mostly marshland, which has since been filled in with sediment, boulders, and rip-rap. In the early 2000s, wild oysters were noticed growing along the rocks and assorted trash (including submerged tires and discarded auto parts) along the shoreline, and in 2004 the NYC Department of Parks and Recreation (Natural Resources Group- NRG) began surveying the wild population and initiating restoration activities. A large salt-marsh restoration project has also been on-going in the park since 2008 (NRG). This site was incorporated into a larger, estuary wide program (Oyster Restoration and Recovery Partnership, ORRP) in 2009.

Jamaica Bay was home to a large population of oysters in the past, with heavy concentrations in the small creeks that ring the bay (Franz, 1982). The majority of Jamaica Bay is protected by the National Parks System (Gateway National Recreation Area), and restoration activities within this area are limited. The NY Department of Environmental Protection and the NYC Department of Environmental Conservation have begun restoration projects at some sites in the bay (i.e., Dubos Pt), and feasibility studies have shown that oysters grow well in the eutrophic creeks (Zarnoch and Schriebman, 2012). The creeks chosen for this study (from east to west: Spring Creek, Fresh Creek, and Paedergat Basin) have all been discussed as possible restoration projects in the

bay though no current work is underway. Within Spring Creek, a study across a small spatial scale was performed by using two sites in the same creek; one was close to the mouth of the creek, where water is readily exchanged with the rest of the bay (Spring Creek South), while the other is located at the back of the creek, where flushing is limited, and next to a wastewater treatment plant (Spring Creek North). These creeks have been known to have poor water quality, including low dissolved oxygen and high levels of coliform bacteria (Isleib et al., 2005). The areas chosen at Spring Creek (South) and Paedergat Basin were within small parks, and along salt marsh areas. Fresh Creek and Spring Creek (North) were along streets and highly subjected to run-off and other urban inputs (i.e., solid waste and other assorted trash items). During times of heavy rain, run-off and CSO input is high in all creeks.

Raritan Bay was previously known for the largest oyster reefs in the HRE (Kurlansky, 1996). The "Great Beds" were extensive oyster reefs covering much of the bottom of Raritan Bay, along the NJ coastline. Keyport, NJ, was also known to have an active oyster industry. Heavy silt input from the Raritan River (Western Raritan Bay) and overharvesting likely lead to the decimation of the "Great Beds" and collapse of the oyster fishery (MacKenzie, Jr., 1990). A history of pollution and diseases linked to oysters (i.e., typhoid fever; Kurlansky, 1996; MacKenzie, Jr., 1990) permanently closed the fishery in the early 1900s. Raritan Bay had seen a resurgence of oysters in the early 2000s, with the implementation of the NY/NJ Baykeeper's "oyster gardening" program and implemented reefs in the Navesink River and Keyport. Water quality has improved in the bay, but many pollutants are still present in the sediments, including trace metals and organic PAHs/PCBs (Hudson River Foundation, 2004). As of 2010, the NJ-

DEP has banned all oyster gardening, research, and restoration activities in Raritan Bay and the rest of the NJ part of the HRE (Ravit et al., 2012).

Research questions

Oysters and other bivalves are often used as "biosentinels"- organisms that are used to monitor the health of an environment (Kim et al., 2008). They are ideal organisms because (1) they are sessile, and therefore cannot move if conditions become unfavorable, (2) they filter water and sediment through feeding activities, and thus are exposed to both particulate and dissolved forms of contaminants, (3) they are easy to collect and analyze, as they occur in intertidal to shallow subtidal areas along shorelines, and (4) they have short generation times, allowing for multiple life stages to be observed. Additionally, bivalves concentrate metals and organic contaminants in tissues at levels above ambient conditions (Kim et al., 2008).

Restoration of previous oyster habitat and populations within the HRE has been the subject of discussion for the past decade. Though several small restoration studies have taken place (i.e., NY/NJ Baykeeper, NYC Parks) at sites in the HRE, no study has attempted to understand the relationship between the impacted estuary and oyster physiology. The HRE has changed drastically since the time when oysters flourished. Inputs of toxic chemicals, including organic and inorganic contaminants, have been buried in sediments and are released through dredging and tidal activities. Removal of habitat via dredging, bulkheading, and marsh infilling have lead to increases in sedimentation and decreases in hard substrate. There are no more oyster reefs in the HRE, and very few wild oysters to reproduce. A recent survey of the HRE found only seven sites (out of 16 surveyed) with small populations of oysters (Medley, 2010). The HRE is both

spat and substrate limited; meaning that in order to rebuild a population an addition of both live oysters and hard substrate would be required. This requires an understanding of how the transplanted oysters would react to the impacted HRE and how physiology may impact future growth and reproduction.

The current study is designed to understand how a chronically degraded habitat, characterized by lowered dissolved oxygen concentrations, eutrophication, inputs of trace metal contaminants, and loss of habitat, affects both juvenile and adult oyster physiology. There are three parts to this study, (1) how is juvenile oyster health and subcellular physiology is altered over a continuum of sites across the HRE, and is subcellular metal accumulation related to alterations in physiology?, (2) how is oyster reproduction (estimated by changes in Vitellogenin protein and energy expenditures) affected by changes in environmental parameters (both natural and anthropogenic)?, and (3) can changes in subcellular physiology be correlated specifically to subcellular accumulation of Cd or Hg, when no other abiotic factors are able to influence oyster health?

The first part, "Changes in physiology and metal accumulation of juvenile *Crassostrea virginica* at various sites within the Hudson-Raritan Estuary" (Chapter 2) examines the impacts of a degraded habitat on subcellular physiology (energy reserves and energy usage) and subcellular metal accumulation (Cd or Hg). Relationships between metal accumulation and environmental parameters (i.e., seston characteristics), and physiological endpoints are investigated in order to determine if the factors causing physiological change can be determined. The second part, "The effects of an impacted estuary on adult *Crassostrea virginica:* Are changes in subcellular

physiology linked to metal accumulation or environmental influences?" (Chapter 3) examines how adult physiology may be altered when broodstock (reproducing adult oysters) are transplanted to sites within the HRE. If future restoration projects hope to rebuild a self-sustaining population within the HRE (Bain et al., 2007), then reproduction is a key factor that must be examined and understood. Thirdly, "Examining the relationship between metal exposure (Cd and Hg), subcellular accumulation, and physiology of juvenile *Crassostrea virginica*" (Chapter 4) examines how exposure of juvenile oysters to elevated concentrations of trace metals will alter physiology. Oysters are exposed to sublethal concentrations of Cd or Hg during a controlled laboratory experiment, and relationships between subcellular physiology and metal accumulation were determined. Following the three parts of this study is a review of oyster restoration activities in the HRE and recommendations for future studies based on the integrated results of the three research questions (Chapter 5).

Level	Types of Responses	Effects at Next Level
Biochemical- Cellular-Tissue	Toxication	Toxic metabolites
	Metabolic impairment	Disruption in energetics and cellular processes
	Cellular damage	
	Detoxication	Adaptation
Organismal	Physiological changes	Reduction in population performance
	Behavioral changes	
	Susceptibility to disease	
	Reproductive effort	
	Larval viability	
	Adjustment in rate functions	Regulation and adaptation of populations
	Immune responses	
Population	Age/Size structure	Effects on species productivity and coexisting species and community
	Recruitment	
	Mortality	
	Biomass	
	Adjustment of reproductive output and other demographic characteristics	Adaptation of population
Community	Species abundance	Replacement by more adaptive competitors
	Species distribution	
	Biomass	Reduced secondary production
	Trophic interactions	
	Ecosystem adaptation	No change in community

Table 1: Responses of marine organisms to chemical contaminants at different levels of biological hierarchy (Capuzzo, 1996).

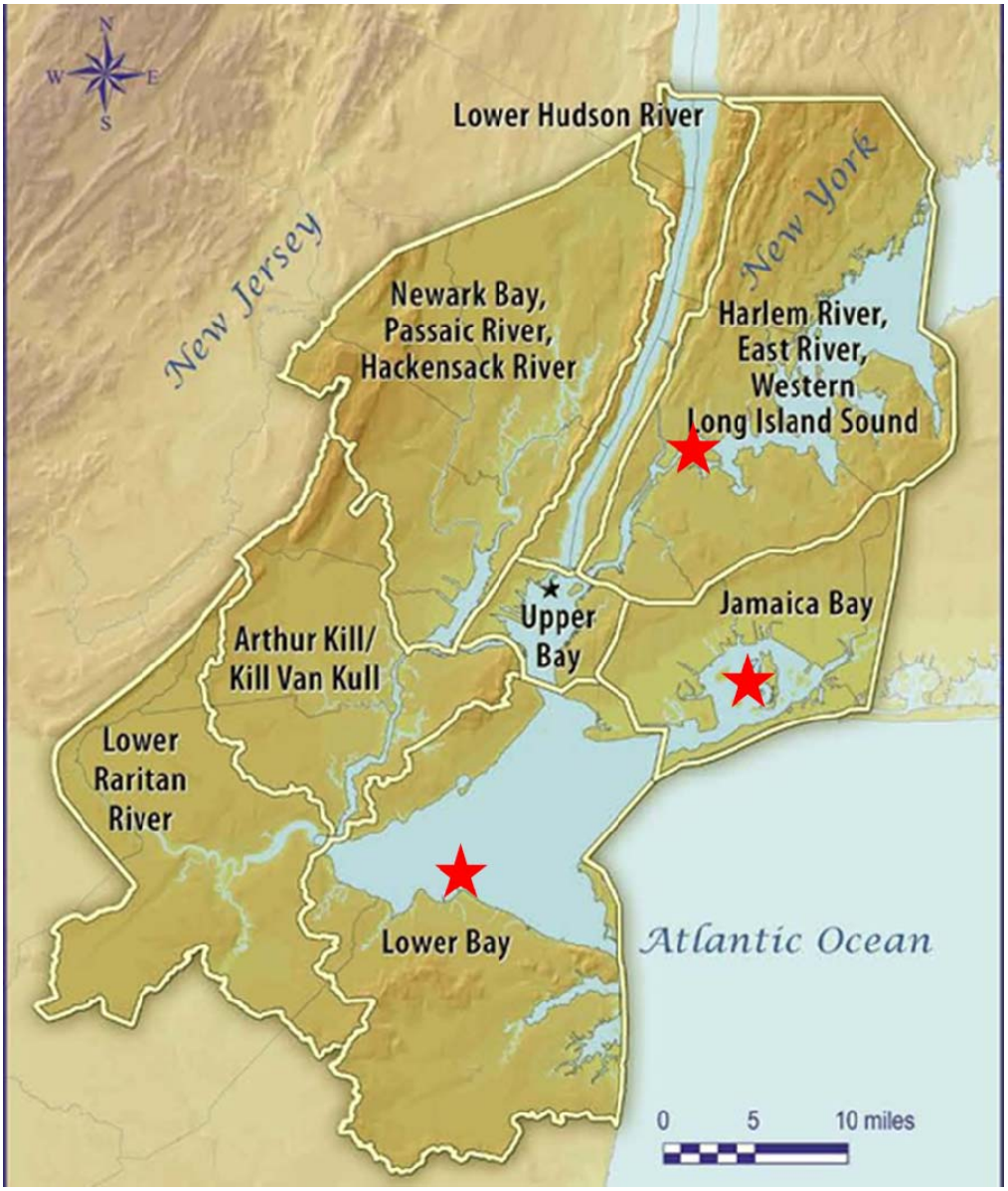

Figure 1: Map of the Hudson Raritan Estuary (reprinted from Hudson Raritan Estuary Comprehensive Restoration Plan, 2009). Clockwise from the top, stars represent sites used during this study: Soundview Park, Bronx River/ East River; Jamaica Bay; Raritan Bay.

CHAPTER 2

CHANGES IN PHYSIOLOGY AND METAL ACCUMULATION OF JUVENILE *CRASSOSTREA VIRGINICA* AT VARIOUS SITES WITHIN THE HUDSON-RARITAN ESTUARY

INTRODUCTION

The Hudson-Raritan Estuary (HRE) is a small (13,000 mi^2) partially-mixed estuary which surrounds the most populous city in the United States, New York City (Levinton and Waldman, 2006). This highly urbanized estuary has been impacted by a long history of pollution and eutrophication, and therefore has experienced alterations in key environmental parameters (i.e., dissolved oxygen) which have affected biota residing in the estuary. Within the HRE, benthic filter-feeding bivalves are valued for their ability to filter the water column and control sediment dynamics. Organisms such as epifaunal and infaunal bivalves are known as 'benthic-pelagic couplers' and are able to remove organic material and suspended particulates from the water column and transfer the material (as feces and pseudofeces) onto the benthos (Newell, 2004; Brumbaugh et al., 2000; Gerritsen et al., 1994). A keystone species in many estuaries, the eastern oyster *Crassostrea virginica* filters the water column, allowing for increasing amounts of light and oxygen to penetrate and become available to other organisms, affecting the entire estuarine community (Nelson et al., 2004; Coen and Lukenbach, 2000). This species has been 'functionally extinct' within the HRE for the previous century (Franz, 1982); prior to the 1900s, oyster reefs in the HRE were some of the most dense on the eastern seaboard, and were able to is able to control phytoplankton assemblages, nutrients, and suspended matter within the estuary (Nelson et al., 2004; Coen and Lukenbach, 2000; Gerritsen et al., 1994)

Urbanization of estuaries along the Atlantic coast has lead to the destruction of ecological niches and the loss of many important species, including *C. virginica*. Presently, wild oysters in the lower HRE are only found in small pockets; no large reefs have been found on shorelines or by

benthic mapping (Medley, 2010; Franz, 1982). Along with overharvesting, habitat degradation was a primary driver for the loss of *C. virginica* within the HRE. For over 300 years, raw sewage, organic pollutants, and trace metals were frequently dumped into the HRE with no regulation (Brosnan et al., 2006). Following the passing of the Clean Water Act (1972), water quality has improved, but physical destruction (i.e., marsh loss, dredging, bulkheading) of the habitat continues, as does persistent pollutants in the sediment and water column. The resulting changes in dissolved oxygen, nutrient and sediment loadings within the HRE have altered the ability of *C. virginica* to survive within this urbanized habitat. While the current environmental conditions within the HRE are improved over the past decades, this environment is still quite altered from that which was present when oysters last flourished (Yozzo et al., 2004; Wakeman and Themelis, 2001; Franz, 1982). High sedimentation rates can inhibit oyster reef growth, and affect settlement of spat (Volety et al., 2009). Oyster reefs require low sedimentation levels to survive and grow upwards, and as such, the high sedimentation rates within the lower HRE due to dredging may affect future growth of oysters.

In addition to physical destruction and nutrient enrichment, the lower HRE is one of the most impacted estuaries on the East Coast in terms of organic and inorganic pollutants (Cerrato et al., 2006). PCBs and PAHs have been found at several sites at levels exceeding NOAA's ER-M (Effects Range Median) guidelines (Dimou et al., 2006; Feng et al., 1998). Several essential and non-essential trace metals, including cadmium (Cd), copper (Cu), mercury (Hg), and zinc (Zn), are found in elevated levels in sediments throughout the HRE (Yozzo et al., 2004; Feng et al., 1998; Crawford et al., 1994). It is unknown whether or not the level of contamination of metal contaminants, in tandem with organic pollutants and eutrophication, would lead to toxicity and

mortality in bivalve biota within the HRE. Both organic contaminants and trace metals have been shown to effect metabolism in oysters and other bivalves, causing mortality, reproductive failure, and decreased growth rates, as well as alterations in cellular responses (i.e., lysosomal destabilization) (Cherkasov et al., 2007; Ringwood et al., 1998; Roesijadi, 1996).

Accumulation of trace metals can affect key physiological functions within oysters, including those responsible for growth and metabolism. Oysters are able to bioconcentrate metals up to several orders of magnitude higher than ambient concentrations before experiencing toxicity (O'Connor, 2002; Mouneyrac et al., 1998). Once accumulated, metals can be stored within a variety of subcellular fractions, including heat denatured proteins (HDP), heat stable proteins (HSP), insoluble metal-rich granules (INS), organelles (ORG), and cell debris (CD) (Wallace et al., 2003), after which the metal can be sequestered, eliminated, or transferred along the food chain. These fractions can also be grouped into larger compartments; the metal sensitive fraction (MSF) consists of ORG and HDP and biologically detoxified metal (BDM) consists of INS and HSP. At low concentrations, metals may be stored in subcellular compartments or eliminated without negatively impacting the oyster (Geffard et al., 2002). Metals may be bound to metallothionein proteins or granular hemocytes in large quantities, which presumably render the metal biologically inert (Amiard et al., 2006; Wallace et al., 2003; Roesijadi, 1996). The exposure to, and storage of, metals within oysters can have drastic impacts on energy stores (Pridmore et al., 1990). Increased exposure to non-essential metals (i.e., Cd) has negative effects on growth and reproduction of oysters (Volety, 2008; Ringwood et al., 2004). The amount of energy reserves within the oyster, reproductive condition, and environmental parameters (e.g., food, temperature, salinity) can affect the amount of metallothioneins present within the oyster,

and thus the amount of metal that can be successfully sequestered (Erk et al., 2008; Amiard et al., 2006). If excess metal is present in the environment, accumulations from the environment can outpace detoxification mechanisms, leading to toxicity and mortality (Amiard et al., 2006).

Understanding the relationship between environmental contaminants and oyster health is a critical component in determining oyster survival and future reproduction within the HRE. Linking the effects of the environment (both physical and anthropogenic inputs) to changes in oyster physiology (i.e., energy stores, reproduction) will allow for a greater understanding of how oysters can be restored in urbanized estuaries. In many cases, a suite of contaminants as well as various environmental stresses are typically responsible for adverse effects on organisms and it is often difficult to discern which variables are the primary causes for stress. Oysters that sequester and eliminate toxins may deal with physiological trade-offs including lowered reproductive output, respiration and metabolic rates, and smaller size (Lanning et al., 2008; Hartwell et al., 1991). *Crassostrea gigas* living in a polluted estuary with high Cu and Zn concentrations were found to have lower glycogen content and reduced overall condition than those living in a cleaner area (Pridmore et al., 1990). Additionally, the binding of metals onto different subcellular fractions may adversely impact oyster metabolism and energy budgets, leading to mortality. Even at low concentrations, Cd has been found to bind to mitochondria which can lead to disruption of the electron transport system and alterations of the oysters' metabolism (Cherkasov et al., 2009). Considering the many different environmental variables (i.e., pollutants, dissolved oxygen, temperature and salinity) occurring within the HRE, a field study examining linkages between important environmental variables and anthropogenic inputs would allow for a more thorough analysis of the overall health of transplanted oysters, and its

relation to the accumulation of important non-essential trace metals. Field studies allow for exposure to a wide variety of environmental variables, including those altered by urbanization and habitat degradation (i.e., chlorophyll-*a* content, suspended particulates), and for researchers to possibly discern the myriad of effects of these variables on oyster physiology.

To the authors' knowledge, this study represents the first comparing oyster physiology to environmental and anthropogenic variables within the HRE. There are few studies examining oyster physiology within urbanized areas, and the multiple effects a suite of contaminants and environmental parameters can have on restoration and survival (Levinton et al., 2013; Volety et al., 2009; Wang et al., 2008; Weinstein, 1997). Many areas that use *C. virginica* in restoration efforts, for either ecological or commercial benefit, are located in more pristine (less urbanized) areas than the lower HRE (i.e., North and South Carolina, Florida) (Nelson et al., 2004; Coen and Luckenbach, 2000). The HRE encompasses one of the most urbanized areas in the United States (New York City and its boroughs), and many different environmental parameters (i.e., seston characteristics, dissolved oxygen) may be impacted. However, previous studies of oysters in other estuaries (i.e., Cerco and Noel, 2010, Volety et al., 2009, Oliver et al., 2001) may be used to compare how oyster physiology relates to environmental parameters and how data may be analyzed to illustrate these effects.

The objectives of this study were to (1) determine the effects of habitat degradation (defined by lower dissolved oxygen, increased sedimentation, and elevated contaminant levels) on the physiology of juvenile *C. virginica*, (2) determine the total body burden of Hg and Cd, and subcellular accumulation of Cd in juvenile oysters placed at various sites (as representative non-

essential trace metals found in elevated levels within the lower HRE), and (3) to determine if there are any relationships between declining physiological conditions (i.e., condition indices, stored levels of energetic biochemical compounds within tissue, elevated levels of oxygen usage), environmental conditions (i.e., particle loads, chlorophyll-*a* abundance, oxygen levels), and body burdens of Cd or Hg. A large-scale transplant study was used in order assess the relationship between varying environmental parameters and oyster physiology. It was hypothesized that oysters placed at the more impacted sites (those with highest contaminant burdens, lower food quality/quantity, and high sediment loads) would have lower overall condition, lower amounts of energy reserves (carbohydrates, lipids, and proteins), and higher body burdens of Cd and Hg. Additionally, the lower physiological functions of oysters at these impacted sites will show a strong relationship to the environmental parameters defining the site, than oysters at less degraded sites.

METHODS

Field deployment of oysters

Oysters were placed at the field sites from July 8- October 8, 2009 and July 13- October 22, 2010. Several chronically contaminated sites within the HRE were chosen, based on (1) potential or current usage in oyster restoration projects within New York Harbor, (2) probability that native oysters were historically found at the site (Waldman, 1999; Kurlansky, 1996; Franz, 1982), and (3) presence of anthropogenic inputs, including eutrophication, metals, and organic compounds (Figure 1). These sites also represented both a small spatial scale (within Jamaica

Bay), and a large spatial scale (Lower HRE). Soundview Park (*SVP-09*; Bronx River, Bronx NY) and Keyport Harbor (*RB-09*; Raritan Bay, Keyport NJ) are located at the northern and southern ends (respectively) of the Lower HRE. Within Jamaica Bay (Brooklyn/Queens NY), three creeks were chosen (in order, mouth of the bay towards back of the bay): Paedergat Basin (*PB-09*), Fresh Creek (*FC-09*), and two locations within Spring Creek (*SCS-09* and *SCN-09*). Within Spring Creek, a northern end (SCN, further from the bay) and a southern end (SCS, closer to the bay) were chosen to determine if the amount of flushing from Jamaica Bay affects physiology. In 2010, a larger spatial scale was attempted, with a "clean" site (located outside the HRE) being observed as well as degraded sites within the HRE. Soundview Park (*SVP-10*) and Spring Creek (*SCS-10*) were repeated at different microhabitats within the site location, as these were sites were the subject of several pilot oyster restoration studies (Grizzle et al., 2013; Zarnoch et al., *unpublished*). An additional site at Floyd Bennett Field (*FBF-10*, Rockaway Inlet, Brooklyn NY), located at the mouth of Jamaica Bay, was selected along with a site south of the HRE along the Atlantic coast of New Jersey (*TK-10*; Great Bay, Tuckerton NJ). In total, ten sites were used over the two year experiment, with each site having a four month exposure period (2009 or 2010). The impacted sites have been shown to have elevated concentrations of pollutants, including essential and non-essential metals (Bopp et al., 2006; Wirgin et al., 2006; Feng et al., 1998; Adams et al., 1996; Seidemann, 1991). Tuckerton has been used as a local reference site (outside the HRE) in other studies concerning metal uptake by invertebrates (Khoury et al., 2009) (Figure 1).

Juvenile oysters (shell length 12.5 ± 0.2mm in 2009; 14.5 ± 0.2 in 2010) were obtained from a hatchery outside the HRE (Aeros Cultured Oyster Company, Southold NY) each year, and were

transported on ice to the various sites. All oysters were placed at sites within 24 hours after removal from the hatchery waters. At each site, three replicate polyethelyne mesh bags (Aquatic Ecosystems®, FL) were each filled with 350 juvenile oysters, secured to cinderblocks (to elevate off the sediments) and placed in the upper subtidal zone. At TK, the mesh bags were suspended from a dock into the subtidal zone as there was no hard bottomed area to place cinderblocks.

Following deployment at each site (7/8/09 or 7/13/10), oysters were subsampled bi-weekly until October (10/8/09 or 10/23/10) for a total of six (2009) or seven (2010) sampling events (2009 sampling was ended earlier in October due to weather constrictions). The bags were retrieved during a low tide, all bags were opened and dead oysters removed. A random subsample of 55 juvenile oysters was removed, and oysters redistributed once again into triplicate bags. All oysters were placed on ice, and immediately transported back to the laboratory (The College of Staten Island).

At each site, several environmental variables were discretely measured during each sampling event. Temperature (^{o}C), salinity (ppt), and dissolved oxygen (mg/L) were recorded using a YSI Pro-20 handheld water quality probe. Though discrete measures do not yield the most accurate description of the site, placing water quality sondes at each site was not an option due to the constraints of the project. Additional water samples were collected and brought back to the laboratory on ice to be filtered for total suspended particulates and chlorophyll *a* (total and size fractionated).

Once at the laboratory, oysters were either placed in a tank of clean artificial seawater (set at ambient temperature and salinity) to depurate, or immediately flash-frozen (using dry ice) or stored in a -80^{o}C freezer. Twenty-five oysters were immediately frozen and used to analyze overall condition index and biochemistry (carbohydrates, lipids, and proteins). An additional twenty oysters were flash frozen to be used to analyze the activity of the electron transport system. Eight oysters were depurated for 24 hours in artificial seawater, then flash-frozen and used to determine total mercury body burdens or total and subcellular body burdens of cadmium. All assays were performed either at The College of Staten Island or transported on dry ice to Baruch College.

Physiological endpoints

Condition index was determined using the methods of Crosby and Gale (1990), using the total, dry shell and dry tissue weights (g). Tissue was dried at 65^{o}C for a minimum of 3 days, and was then ground into a fine powder and pooled per sampling event (n=25) to be used for biochemical analysis. Dried tissue was kept in an oven at 65^{o}C until analyzed for carbohydrate, protein and lipid levels. Total lipid concentration in dried tissue was determined using chloroform-methanol extraction technique (Bligh and Dyer, 1959). The percentage of protein in dried tissues 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). Total carbohydrate concentration in dried tissue was determined using the phenol-sulfuric acid method (Dubois et al., 1956).

To estimate energy usage, analysis of the electron transport system (ETS) was completed using modified methods of Madon et al. (1998). Briefly, oysters 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 10,000*g* for 20 minutes 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: ETS μmol O_2 h^{-1} g DW^{-1}= {(E_{corr} * V_{hom} * 60/t * 1 * V_{rxn}) / (V_{inc} * DW * 31.8)} where E_{corr} is the corrected sample absorbance, 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).

Metal accumulation and subcellular fractionation

For Cd determination, four oysters were defrosted, shucked, and the wet weight (g) was determined after gently blotting the tissue dry with a Kim-wipe. Whole oysters were then homogenized with TRIS buffer (pH 7.6, 1:4 wet weight:volume) using a tissue homogenizer (Kinetamitca AG Polytron PT MR2100), for at least three passes of 20 seconds, until all tissue was broken up. A subsample ($1/6^{th}$ the total volume) was then removed and placed into a pre-weighed acid-washed scintillation vial to determine total body burden (TOT). Oyster tissue was separated into operationally defined subcellular fractions based on the methods of Wallace et al. (2003). Sequential centrifugation and heat treatment steps were used to separate the remaining

oyster tissue into operationally-defined fractions (INS, HSP, ORG, HDP, CD; Wallace et al., 2003), each placed into a separate pre-weighed acid-washed scintillation vial (Figure 2). When determining Cd concentrations, all fractions (including TOT) were placed in a 65^{o}C drying oven for a minimum of three days, and the dry weight determined (g). All fractions except for CD were then digested with trace-metal grade HNO_3, dried down on a hot-plate, and resuspended in 2% HNO_3 (Brown and Luoma, 1995). Samples were either analyzed at The College of Staten Island (2010 samples) using an Atomic Absorption Spectrophotometer (Perkin Elmer 3100 AAS), or by the Aquatic Toxicology Lab at the University of Medicine and Dentistry of New Jersey (UMDNJ) using an Atomic Absorption Spectrophotometer (Perkin Elmer 5100 Zeeman GF-AAS) to estimate the quantity of Cd ($\mu g\ g\ DW^{-1}$) in each fraction.

To determine Hg concentrations, four oysters were defrosted, shucked, and total wet weight determined (g). Again, whole oysters were homogenized with TRIS buffer (pH 7.6, 1:4 wet weight:volume) using a tissue homogenizer (Kinetamitca AG Polytron PT MR2100), for at least three passes of 20 seconds, until all tissue was broken up. All homogenate was then dried at 65^{o}C for a minimum of three days, and the dry weight determined (g). Oyster tissue was then digested with 1:4 nitric acid: sulfuric acid in a 60^{o}C hot water bath. Afterwards, the addition of potassium permanganate and potassium persulfate 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).

Water quality

Water samples (1-3 L, stored on ice) from each site was filtered through a 180μm mesh sieve, and onto a pre-weighed 1.8 μm GF/F glass fiber filter to collect total suspended matter. The filter was then dried in a 60^{o}C oven to determine the total particulate matter (TPM), and subsequently ashed in a muffle furnace at 450^{o}C to determine the ash-free dry weight [used to calculate organic content (%OC), particulate inorganic matter (PIM) and particulate organic matter (POM)] (Bayne, 2002). Additional water samples at each site (stored on ice) were filtered first through a 180μm sieve, and a subsample (100-500 ml) was passed through a 0.45μm nitrocellulose filter to determine total chlorophyll-*a*. The remaining (100-500 ml) 180μm-filtered water was then filtered through a 28μm sieve onto a 5μm filter to determine size-fractionated (5-28μm) chlorophyll-*a*. All filters were immediately frozen, and later digested in 90% acetone overnight (in the dark), centrifuged, and the supernatant analyzed on a UV-spectrophotometer (GENESYS 10) via the methods of Parsons et al. (1985).

Statistics

Data was analyzed to compare biochemistry, metal accumulation and subcellular distribution, and environmental variables at each site and to identify relationships between these variables. Regressions were used to determine linkages between variables. Both one-way ANOVA (site) and two-way ANOVA (site*date) were used to analyze differences in physiological functions. Post-Hoc tests (Tukey or Unequal N HSD) were performed to identify all significant differences (Zar 1999). Data was transformed if necessary to meet assumptions of ANOVA. When the assumptions of ANOVA could not be met, Kruskal-Wallis ANOVA (Dunn's Post-Hoc Test) or Friedman ANOVA were used.

RESULTS

Environmental parameters

Temperature, salinity, dissolved oxygen

Temperature was not significantly different among sites over the sampling season (ANOVA, $p>0.05$). A seasonal trend was seen with respect to water temperature, but it was not statistically significant. Salinity was significantly different among several sites over the sampling season (ANOVA, $p<0.0001$). The sites closest to ocean inlets, FBF-10 and TK-10, were significantly more saline over the sampling events than all other sites. However, there was no effect of date, as values did not fluctuate during the sampling season (ANOVA, $p>0.05$). There was no significant difference between the discrete dissolved oxygen levels at any sites (Kruskal-Wallis, $p>0.05$). All values were above 2 mg/L (threshold for hypoxia; Lenihan and Peterson, 1998) except for SCS-09 (1.43 mg L^{-1}, 8/21/09) and SVP-10 (1.75 mg L^{-1}, 7/26/10). However, these were short term hypoxic events; by the next sampling event dissolved oxygen values had rebounded to acceptable conditions (>2 mg L^{-1}). Detailed environmental measurements can be found in Table 1.

Seston characteristics

Mean TPM values over the sampling season differed between sites (ANOVA, $p<0.0001$). The highest TPM was found at SVP-10 (122.28 ± 51.19 mg/L) and SCN-09 (84.9 ± 24.06 mg/L). PB-09 had the lowest concentration of TPM, 10.08 ± 2.6 mg/L (Figure 3). There was an effect of site, date, and the site*date interaction on TPM (ANOVA, $p<0.0001$).

The organic fraction of TPM (%OC) ranged from 10.4% ± 1.34 (SVP-10) to 49.6% ± 10.1 (PB-09) (Figure 4). There was significantly lower percentage of organic content in the seston at SVP-10 than FC-09, PB-09, and SCS-09 (Kruskal-Wallis ANOVA, $p<0.001$). Both particulate organic matter (POM) and particulate inorganic matter (PIM) are significantly different amongst sites (ANOVA, $p<0.001$; data not shown). The sites with high particle loads (SVP-10, SCN-09) also had high PIM, with PIM ranging from 1-9.6 times higher than POM at sites.

Significantly higher concentrations of chlorophyll-*a* were found at SCN-09 (48.71 ± 10.33 μg L^{-1}) and RB-09 (47.98 ± 9.07 μg L^{-1}) than at FC-09 (14.66 ± 2.17 μg L^{-1}) and TK-10 (10.86 ± 1.32 μg L^{-1}) (Kruskal-Wallis, $p<0.001$) (Figure 5). Significant seasonal differences were seen with respect to site, date, and the site*date interaction (Friedman ANOVA, $p<0.0001$). During September and October there was more variation in chlorophyll-*a* concentrations between sites than during July and August (Friedman ANOVA, $p<0.0001$).

Sediments were analyzed for metal content in the upper 2cm (surficial sediments were swept away before sampling). Metal content for four metals- Ag, Cd, Cu, and Zn- were found at all sites during the sampling period. Silver content ranged from 0.22 μg g $sediment^{-1}$ at TK to 6.32 μg g $sediment^{-1}$ at SVP. Cadmium content ranged from 4.96 μg g $sediment^{-1}$ at TK to 31.37 μg g $sediment^{-1}$ at SCN. SCN also had the highest amount of Cu (1531.11 μg g $sediment^{-1}$) and Zn (2066.87 μg g $sediment^{-1}$). SCS had the lowest amount of Cu (213.57 μg g $sediment^{-1}$) and Zn (302.58 μg g $sediment^{-1}$) (Figure 6).

Physiological endpoints

Overall health and growth

There were significant differences between the overall condition of juvenile oysters at all sites (Kruskal-Wallis, $p<0.0001$). There was a significant difference in condition indices with respect to site, date, and the site*date interaction (Friedman ANOVA, $p<0.0001$). The average condition index ranged from 6.04 ± 0.12 (SVP-10) to 10.04 ± 0.15 (FBF-10) (Figure 7).

Daily shell growth rates (mm d^{-1}) were calculated from the average length measurements taken during each sampling event at each site. Average daily growth rates of oyster shells ranged from 0.152mm d^{-1} (TK-10) to 0.411 mm/d (FBF-10) (Figure 8). Shell metrics (length, dry weight) were compared to tissue metrics (dry weight, condition index) as well (data not shown).

Tissue biochemistry

Carbohydrate contents of dried tissue (μg mg DW^{-1}) were significantly different between sites over the season (Kruskal-Wallis, $p<0.0001$). Carbohydrate amounts were between 36.66 ± 3.23 μg mg DW^{-1} (SVP-10) and 97.94 ± 4.49 μg mg DW^{-1} (SCN-09) (Figure 9). There was an effect of site, date, and the site*date interaction on carbohydrate concentrations (Friedman ANOVA, $p<0.0001$).

Lipid concentrations of oyster tissue (μg mg DW^{-1}) were significantly lower in oysters transplanted to SVP-10 (75.73 ± 3.63 μg mg DW^{-1}) than all other sites (ANOVA, $p<0.001$). The highest concentration of lipids was found in oysters at SCS-10 (102.52 ± 2.95 μg mg DW^{-1})

(Figure 10). There was an effect of site, date, and the site*date interaction on lipid concentrations (Friedman ANOVA, $p<0.0001$).

Protein concentrations were significantly different (Kruskal-Wallis, $p<0.01$), but a post-hoc analysis revealed no differences among sites ($p>0.05$). Protein concentrations in dried tissue ranged from 493 ± 5.88 μg mg DW^{-1} (SVP-10) to 528.13 ± 11.83 μg mg DW^{-1} (RB-09). An effect of site, date, and the site*date interaction was seen with respect to protein concentrations but differences between sites could not be teased apart (Friedman ANOVA, $p<0.0001$) (data not shown).

Estimating aerobic potential of the oysters illustrated significant differences between sites over the sampling season (Kruskal-Wallis, $p<0.0001$). Oxygen potential ranged from 42.65 ± 7.6 μmol O_2 h^{-1} g DW^{-1} (SCS-10) to 460.38 ± 47.73 μmol O_2 h^{-1} g DW^{-1} (SVP-09) (Figure 11). An effect of site, date, and the site*date interaction was seen with respect to aerobic potential (Friedman ANOVA, $p<0.0001$).

Metal accumulation

Total Cd accumulation in oysters were estimated, and after a four month field deployment there was a significant difference between sites (ANOVA, $p<0.0001$). Total Cd body burdens of oysters at SVP-09 (25.01 ± 7.37 μg g DW^{-1}), SVP-10 (25.43 ± 6.33 μg g DW^{-1}), and RB-09 (23.61 ± 6 μg g DW^{-1}) were significantly higher than at SCS-09 (4.09 ± 0.92 μg g DW^{-1}) and SCN-09 (5.70 ± 2.54 μg g DW^{-1}) (Table 2, Figure 12).

Within the subcellular fractions, accumulation of Cd in insoluble granules (INS) was significantly higher at SVP-10 (4.72 ± 1.76 μg g DW^{-1}) and RB-09 (3.68 ± 0.73 μg g DW^{-1}) than at SCS-09 (0.36 ± 0.03 μg g DW^{-1}) (Kruskal-Wallis, $p<0.01$). There was no difference between sites with respect to the amount of Cd within the HSP fraction (Kruskal-Wallis, $p>0.05$). Cadmium accumulation within both ORG and HDP was significantly higher at RB-09 (5.47 ± 1.74 μg g DW^{-1} ORG; 8.50 ± 3.41 μg g DW^{-1} HDP) than SCS-09 (0.99 ± 0.15 μg g DW^{-1} ORG; 1.04 ± 0.24 μg g DW^{-1} HDP) (Kruskal-Wallis, $p<0.02$). Estimated accumulation of Cd in all subcellular fractions can be found in Table 2. When the fractions were grouped into larger subcellular compartments (MSF and BDM), there was a significant difference in the estimated accumulation of Cd within MSF (SCS-09 had the lowest accumulation; RB-09 the highest); there was no difference with BDM (Kruskal-Wallis, $p<0.02$ MSF, $p>0.05$ BDM) (Data not shown).

Estimated total body burdens were used to estimate the percentage of total Cd bound to each subcellular fraction. There was no significant difference between sites with respect to the percentage of Cd bound to the INS, HSP, or CD fractions. With respect to the percentage bound to INS, oysters at RB-09 had the highest percentage bound to insoluble granules (19.11 ± 5.67%), while FBF-10 had the least percentage (4.95 ± 0.82%). SCS-10 had the highest percentage of metal bound to HSP (14.82 ± 7.77%), while SVP-09 had the least (3.06 ± 0.1%). FC-09 had the least amount of metal bound to the CD compartment, only 18.17 ± 10.51%; FBF-10 had the most with 63.23 ± 1.64% of Cd associated with the CD compartment. There was a significant difference between sites with respect to Cd bound to ORG and HDP. FC-09 had a significantly higher percentage of Cd bound to ORG (30.96 ± 3.99%) than SVP-09, SVP-10, FBF-10, or SCN-09 (ANOVA, $p<0.0001$). Both SCN-09 (42.08 ± 4.69%) and RB-09 (33.5 ±

4.87%) had a significantly higher percentage of Cd associated with HDP than SVP-10 or FBF-10. Estimated percentages of Cd bound to subcellular fractions can be found in Table 3.

The estimated percentage of total Cd that accumulated in the MSF subcellular compartment was significantly higher at RB-09 (57.62%) and FC-09 (63%) than SVP-10 (17.58%), FBF-10 (29.98%), and SVP-09 (32.27%) (ANOVA, $p<0.05$). There were no differences between the percentage of Cd associated with the BDM compartment (Kruskal-Wallis, $p>0.05$) (Figure 13).

There was no detectable accumulation of Hg after four months at each site.

DISCUSSION

Site conditions: spatial and temporal variability of environmental parameters

Physiological studies that use *in-situ* experiments allow for researchers to examine the impacts of multiple environmental and anthropogenic variables on an organism (Vincent et al., 1994). Oyster survival and reproduction depends on multiple factors, including physical (i.e., hydrodynamics, sedimentation), environmental (i.e., temperature, salinity, oxygen), anthropogenic inputs (inorganic and organic), and biological (i.e., diseases) (Wang et al., 2008). Many regions undertaking large-scale oyster restorations (Apalachicola Bay, FL; Chesapeake Bay, MD/VA) employ the use of large scale field-based pilot reefs or computer models to determine the viability of the restoration (Wang et al., 2008; Klinck et al., 1992; Grabowski et

al., 2007). To date, no such projects have occurred within the HRE; however using a focused pilot study (such as the current study) allows for baseline information to be gathered, detailing the effects of this heterogeneous habitat on oyster physiology.

As predicted, when examining the environmental conditions at each site both spatial (between sites) and temporal variations were observed. Temperature did not vary significantly between sites, but did fluctuate seasonally; salinity was not seasonally variable, but spatial differences were observed {between the estuarine creeks (SCS, SCN, PB, FC) and the sites closest to ocean inlets (TK, FBF)}. All temperature and salinities observed throughout the study were well within the reported tolerance range for *C. virginica* (Shumway, 1996). Oysters are able to tolerate shifts in salinity and temperature that usually occur in a shallow estuarine environment, and normally would not lead to stress, and a reduction of overall health (Heilmayer et al., 2008; Shumway, 1996). However, when in tandem with an already weakened condition (due to anthropogenic inputs and habitat degradation), shifts in temperature and salinity may cause changes in physiology which would impair oyster health (Cherkasov et al., 2010; Fisher et al, 2000).

It has been shown that environmental parameters often influence physiological measurements. It is well documented that condition index and aerobic potential are closely related to temperature (Li et al., 2006; Lannig et al., 2006; Shumway, 1996). In this study, aerobic potential (measured by the ETS assay) was significantly related to temperature at all sites over the sampling season ($p<0.0001$, $R^2=0.33$) (Data not shown). Though overall condition and tissue biochemistry varied throughout the sampling season (July-October), there was no significant relationship of any other

physiological endpoint with temperature or salinity. After one month at sites (August), oysters placed at SVP, RB, and FC had significantly higher aerobic potential; however, as the temperatures cooled in September, SVP oysters still maintained a higher metabolic rate than oysters at all other sites. With temperatures declining, it would be expected that energy usage would decline as well (as the oyster begins to shut down metabolically); however, additional stressors at the site (including poor food quality/quantity and pollutants) would require the oyster to maintain a higher metabolic rate. Condition index and tissue biochemistry was not significantly related to temperature, but a seasonal trend was still observed, as condition index, carbohydrate and lipid concentration all showed an effect of site, date, and the site*date interaction (Figures 7,9,10).

Seston characteristics, including organic and inorganic particulates, varied temporally and spatially during the study. This was expected, as TPM is dependent on not only the hydrodynamics of the system but the weather patterns (Webster and Lemckert 2002). Seston characteristics of estuaries and shallow bays can change rapidly with weather condition, and can impact the discrete measurements of TPM and chlorophyll-*a* taken at each time point (Pourvreau et al., 2000; Cranford et al., 1998). Changes in the percentage of inorganic particles in TPM can be greatly exacerbated by tidal resuspension (Cranford et al., 1998), a phenomena which was particularly noted at SVP. While care was taken to not resuspend sediments when gathering water samples for TPM, the weather conditions and tidal resuspension may have increased TPM at some sites. Additionally, increased rain during the preceding days has been known to increase sediment input from upriver (pers. obsv.).

Effects of the environment on physiology

Habitat destruction (including dredging and filling in marshes) has destroyed areas of the HRE that once had thriving oyster populations (i.e., Soundview Park and Raritan Bay; Franz, 1982). There is still some suitable habitat within the HRE, and small 'pockets' of wild oysters found scattered on shorelines (Medley, 2010; pers. obsv). While previous studies have shown survivorship to be high at certain locations (i.e., Jamaica Bay, Levinton et al., 2011; Raritan Bay, Ravit et al., 2012) survivorship does not necessarily equate to healthy juveniles, which will be able to grow and reproduce and propagate the next generation (in order to substantiate a healthy reef ecosystem). A comparison of multiple sites with respect to changing environmental parameters and physiological endpoints allows for a more comprehensive examination of how juvenile *C. virginica* will respond to the HRE.

Using length measurements of oysters taken at each site, a daily shell growth rate (mm d^{-1}) was estimated (Figure 8). The highest daily growth rates were at sites within Jamaica Bay (FBF, SCS, FC), while the lowest growth rates were at TK and SVP. No relationship was found between daily shell growth rates and chlorophyll-*a* season averages, nor TPM. When the estimated shell growth rates of sites within the HRE were compared to a less impacted site (Oyster Bay, LIS), it was observed that the daily shell growth rate within the HRE was between 36%- 97% of the estimated rate seen at the LIS site (Bricelj et al., 1992). The oysters used by Bricelj et al (1992) were comparable in size to the oysters used in the current study, as was the season. When compared to a more impacted estuary, with low DO and high TPM, growth rates of oysters at HRE sites ranged from 75% to over 200% of the growth rates observed at the St. Mary's estuary (Chesapeake Bay; Gonda-King et al., 2010). It could be inferred that oysters

placed in the HRE experience conditions somewhere between an less impacted estuary (LIS) and a highly impacted estuary (St. Mary's), and oysters respond to changing environmental parameters by altering shell growth rates. Within the HRE, Medley (2010) found that wild oysters within the lower HRE did not differ significantly with respect to annual growth rates or condition index.

The wild oysters surveyed were adults (0.06 mm d^{-1} growth rate); comparatively, juveniles used in the present study had a faster growth rate (0.24 mm d^{-1}). Previous studies have observed that juvenile oysters often have faster daily growth rates than adults (Medley, 2010; Abbe et al., 2000). Additionally, oysters placed at Jamaica Bay sites during this study had comparable growth rates to other studies (Zarnoch and Schreibman, 2012); sites with lower oyster growth were often associated with areas with higher TPM and lower chlorophyll-*a* (SVP, TK). Shell growth was not correlated with condition index, indicating that this is not a good measure of overall health. With respect to overall condition and survivorship, Levinton et al. (2011) noted that juvenile oysters placed at sites within the HRE differed significantly in their survivorship and growth, with high survivorship reported for Jamaica Bay and Raritan Bay sites. The current study also found that oysters at sites within Jamaica Bay had higher condition indices and overall health, indicating that sublethal stressors were not as influential at these sites than others.

Shell growth rates can be influenced by several environmental factors, and can in turn influence oyster health. Thinner, smaller shells will impact future growth and proliferation of a reef, as shells may be easily crushed by predators and physical processes at the sites. Shell on a reef accretes upwards via the settlement and growth of spat on top of older shells; if the shells are

thin, brittle, and smaller, the reef will not grow well and oysters' survival may be impacted (Soniat et al., 2004). Shell matrix contains organic and inorganic molecules (Almeida et al., 1998). Shell deposition can be influenced by the suspended particulates in the estuary, as both organic particles and inorganic molecules (i.e., trace metals) can be suspended and filtered by the oyster. Trace metal concentrations varied at sites, with SVP and RB having the highest metal concentrations (this study; Paulson, 2005). Additionally, temperature, salinity, and sediment trace metals can affect chemical composition of shells, leading to thinner shells (Higuera-Ruiz and Elorza, 2009; Almeida et al., 1998). It can be concluded that oysters within the HRE display site-specific differences in growth rates, which may be due to site-specific changes in particulates and possibly trace-metal concentrations.

Physiological endpoints show a trend in overall health and reserves of energy yielding biochemical compounds (i.e., carbohydrates). Soundview Park had a significantly lower condition index than the sites located in Jamaica Bay (Figure 3). Jamaica Bay is a larger body of water that only exchanges with the Atlantic Ocean through the narrow Rockaway Inlet (FBF, Figure 1). Therefore, the creeks towards the back of the bay (i.e., SCS, SCN) have "poor mixing" with the incoming ocean water (Benotti et al., 2007). Due to the bathymetry and mixing of the bay, sites towards the back of the bay (in tidal creeks) tend to have higher concentrations of chlorophyll-*a* (in some cases, algal blooms dominate, Sirois and Fredrick, 1978). The condition index in these areas was significantly higher than at sites with lower chlorophyll-*a* values, and more water flow (RB, TK). While it is known that high food concentrations (evidenced by higher chlorophyll-*a* and organic content of seston can lead to higher condition

indices (Carmichael et al., 2004; Sará and Mazzol, 1997), the observed dichotomy seen here between sites closer to ocean inlets and those sites further from ocean inlets is of note.

The organic content of seston at each site is an important driver predicting the health of juvenile oysters. Within the HRE, organic content was found to have a significant relationship with respect to feeding behavior of adult *C. virginica* (Zarnoch et al., 2013, *unpubl.*). During this study, condition index was significantly correlated with the percentage of organic matter in TPM (p=0.0005, R^2=0.2; Figure 14). Oysters filter the water column for food particles, rejecting inorganic matter (into pseudofeces) and assimilating carbon from organic particles (Bayne, 1998). Sites with high particle loads, but low organic content (i.e., SVP-09, SVP-10) had oysters with lower condition indices throughout the season, while sites with higher organic content (PB, SCS, SCS) had higher condition indices (Figure 14). Research in similar high-energy bays has shown that resuspended sediments can contain organic particulates (i.e., microphytobenthos, detritus) that can be used by oysters as food (Barillè et al., 1997). By processing high amounts of poor food, oysters waste energy that could be put towards growth and meat condition. Tissue biochemistry was not correlated with the organic content of the seston (p>0.05), suggesting that even when food quality is poor, it is not influencing the storage of energetically-important molecules within oyster tissue.

The poor food quality at some sites may affect the growth and condition of the oysters by impacting shell growth or reproductive tissue; Paterson et al. (2003) found that oysters placed in tidal creeks had higher condition index, growth, and wet weight when particulate organic content was higher. In this study, oysters at SVP, RB, and TK (sites with lower condition index) were all

observed to have much thinner, more brittle shells than other sites {Medley (2010) also reported thin shells in oysters from sites around the HRE}. On the other hand, sites with higher condition indices (FBF, SCN, SCS, PB, and FC) had oysters that developed reproductive tissue in larger individuals; gonad tissue has been noted in "spat" oysters (less than one year) in both *C. virginica* and the related *C. gigas* (pers. obsv.; Royer et al., 2008). Shell length and dry mass were analyzed after four months at field sites (Figure 15), at there was a positive relationship between the two variables (Multiple regression, p=0, R^2=0.76). There were significant site-specific differences with respect to the slopes of regressions of shell length and dry mass (ANCOVA, p<0.0001). Three distinct groups are separated out: SVP, RB, and TK have similar length/dry mass ratios, with smaller, lighter shells; SCS, SCN, PB, and FC are similar, with heavier and longer shells; finally, FBF had significantly longer and heavier shells than all other sites (Figure 15). The sites with smaller, thinner shells also had lower condition indices, which supports findings in previous studies (Carmichael et al., 2004). Quahogs and soft-shell clams in a eutrophic estuary (Cape Cod, MA) were found to have increasing length/ dry mass ratios as food quantities increased. Carmichael et al. (2004) related this increase in size back to an increase in nitrogen to the system (land-derived nitrogen), which increased chlorophyll-a content, which in turn increased secondary production of the clams. Oysters with the highest length/ dry mass ratios were from sites that had highest chlorophyll-*a* content, and are known to be eutrophic (from increased nitrogen inputs into Jamaica Bay; Benotti et al., 2007). This suggests that oysters are benefitting from increased nitrogenous inputs into Jamaica Bay by incorporating algal biomass into tissue and shell growth.

Linking metal accumulations to environmental and physiological parameters

Trace metal concentrations within the HRE are among the most elevated in the country, and include several metals above the national average (Kimbrough et al., 2010). In particular, elevated Cd and Hg concentrations may lead to physiological problems as they are non-essential trace metals. As is with a field study, a single contaminant cannot be assumed to be the sole cause for physiological alterations of oysters; this study examined the combined effects of an impacted estuary, including contaminant trace metals. However, examining the relationships between accumulated metals and physiological processes may provide insight into what specific environmental or anthropogenic processes are responsible for the changes in physiology.

Total body burdens of Cd were significantly higher at SVP and RB than within Jamaica Bay (SCS, SCN, PB, FC) or TK. Total body burdens were normalized for size (μg Cd per gram dry tissue), but site-specific differences in growth rates may alter how much Cd is accumulated, and how much can be detoxified via INS or HSP. Small oysters have greater metabolic activity, and thus can accumulate Cd more rapidly when exposed (Abbe et al., 2000). Once the Cd is accumulated, it can be sequestered within insoluble granules (metal-rich granules) or metallothionein-like proteins (Wallace et al., 2003). If the environmental concentration of Cd is low enough that the majority of metal accumulated can be stored and detoxified within oyster cells, no physiological stresses would be observed and growth may still be high (i.e., SCS-09). However, when Cd accumulation overloads detoxification mechanisms ("spillover", Campbell et al., 2007), and a larger amount of Cd is bound to organelles or enzymes, physiological stresses will be observed (such as the lower growth rates, poor physical condition, mortality observed at SVP). The rapid growth rates observed at Jamaica Bay sites also suggest that the adverse abiotic

conditions present at sites were not affecting shell growth, as rates were similar in previous studies of juvenile *C. virginica* growth rates (Zarnoch and Schreibman, 2012; Bricelj et al., 1992).

While some changes in physiology may be due to seasonal changes in temperature and seston (i.e., aerobic potential), additional changes in energy usage, tissue biochemistry and overall condition may be due to Cd accumulation. It is possible that accumulations of organic contaminants (PAHs, PCBs) lead to physiological alterations (either alone, or in tandem with metal accumulation), but further examination is required to solidify that statement. With respect to Cd accumulation during this study, the percentage of Cd bound to the metal-sensitive compartments was higher within Jamaica Bay sites. When oysters cannot detoxify accumulated burdens, by sequestering Cd into INS or HSP fractions, metal may bind to the sensitive components, HDP and ORG. Binding of Cd to the sensitive fraction will lead to changes in physiology, at both the subcellular and cellular levels (see Chapter 4; Sokolova et al., 2005). After four months at field sites, oyster tissue concentrations of carbohydrates, lipids, and proteins were not related to Cd body burdens, but this may change after a longer time frame or higher concentration of Cd. The concentrations of Cd bound to subcellular compartments may be more useful than the percentage of total Cd in each compartment for determining future toxicity for *C. virginica* at field sites. The amount of Cd bound to MSF was higher at SVP-09 and SVP-10, but the percentage of total Cd found in the MSF compartment was lowest at these sites, indicating that oysters are able to detoxify Cd better than oysters in Jamaica Bay (where a higher percentage was found in MSF; Figure 13). Sokolova et al. (2005) found that physiologically relevant concentrations may be more informative than the percentage (of the total amount of Cd) accumulated in the compartment; exposure of *C. virginica* to elevated Cd concentrations resulted

in less than 10% of total Cd being associated with ORG, but dysfunction of specific organelles (i.e., mitochondria) was still observed.

With respect to Hg at the study sites, there was no detectable level of Hg within oyster tissue after four months exposure at each site. Environmental concentrations of Hg are elevated at several sites in the HRE, where concentrations exceed ERM values (NOAA, 1995). Tissue burdens of oysters were below the detection limit of the machine, which suggests that oysters either (1) did not accumulate Hg from sites, or (2) did accumulate Hg, but it was not above the detection limit of the machine (0.25 $\mu g\ L^{-1}$). There is no previous data on juvenile oysters and Hg accumulation within the HRE. Other field studies have found accumulations for C. virginica in the field ranging from 0.026-0.462 $\mu g\ g\ DW^{-1}$ (Gulf of Mexico; Apeti et al., 2012). Other oyster species have had similarly low body burdens of Hg (*Saccostrea cucullata*= 0.07 ± 0.015 $\mu g\ g\ DW^{-1}$, Pan and Wang, 2011; *Crassostrea tulipa*= 0.13-0.21 $\mu g\ g\ DW^{-1}$, Otchere et al., 2003). It is possible that oysters do not accumulate Hg quickly and to high concentrations within cells. Mercury is also able to be detoxified readily, either by binding with MTLPs (Amaird et al., 2006) or with lipids, which can get passed on during reproduction or tissue loss. In a concurrent laboratory study, juvenile oysters were exposed to varying concentrations of Hg, and even at levels well above the EPA maximum for Hg in estuarine systems (0.009μM; US EPA 1999, 2001) no physiological changes were observed (see Chapter 4). Overall condition, protein levels, and energy usage (ETS) were not significantly different in oysters exposed to elevated Hg concentrations than those in control situations. However, it is possible that subcellular changes in physiology, such as changes in key enzyme pathways, were altered during exposures (Zhang et al., 2010). During this field study, Hg accumulation may have been undetectable, but changes

in physiology (i.e., declines in carbohydrate and lipid concentration, increased energy usage; Figures 9-11) may be due to the impacts on Hg on subcellular enzymatic pathways.

CONCLUSION

Juvenile oyster physiology is strongly influenced by the site-specific nature of abiotic factors. Environmental parameters (i.e., TPM, %OC, chlorophyll-a) vary widely across the HRE, and thus influence where oysters will survive and grow the best. This will be very important when making management decisions for future oyster restoration projects, as long-term viability and survival of oysters is a key trait of a successful restoration. Shell growth rates, condition index, and aerobic potential are all acceptable metrics for analyzing physiological responses to the environment, as after a short time frame (four months) there were appreciable site-specific differences. However, lipid and protein content did not respond as quickly to environmental influences, and are not as useful when analyzing oyster health at sites. Subcellular accumulation of Cd influences oyster health, as sites with lower Cd body burdens had healthier oysters (i.e., higher condition indices). The location of subcellular binding is important for future toxicity; sites with lower Cd environmental concentrations had more metal bound to the MSF compartment, which may result in future toxicity after a longer exposure period.

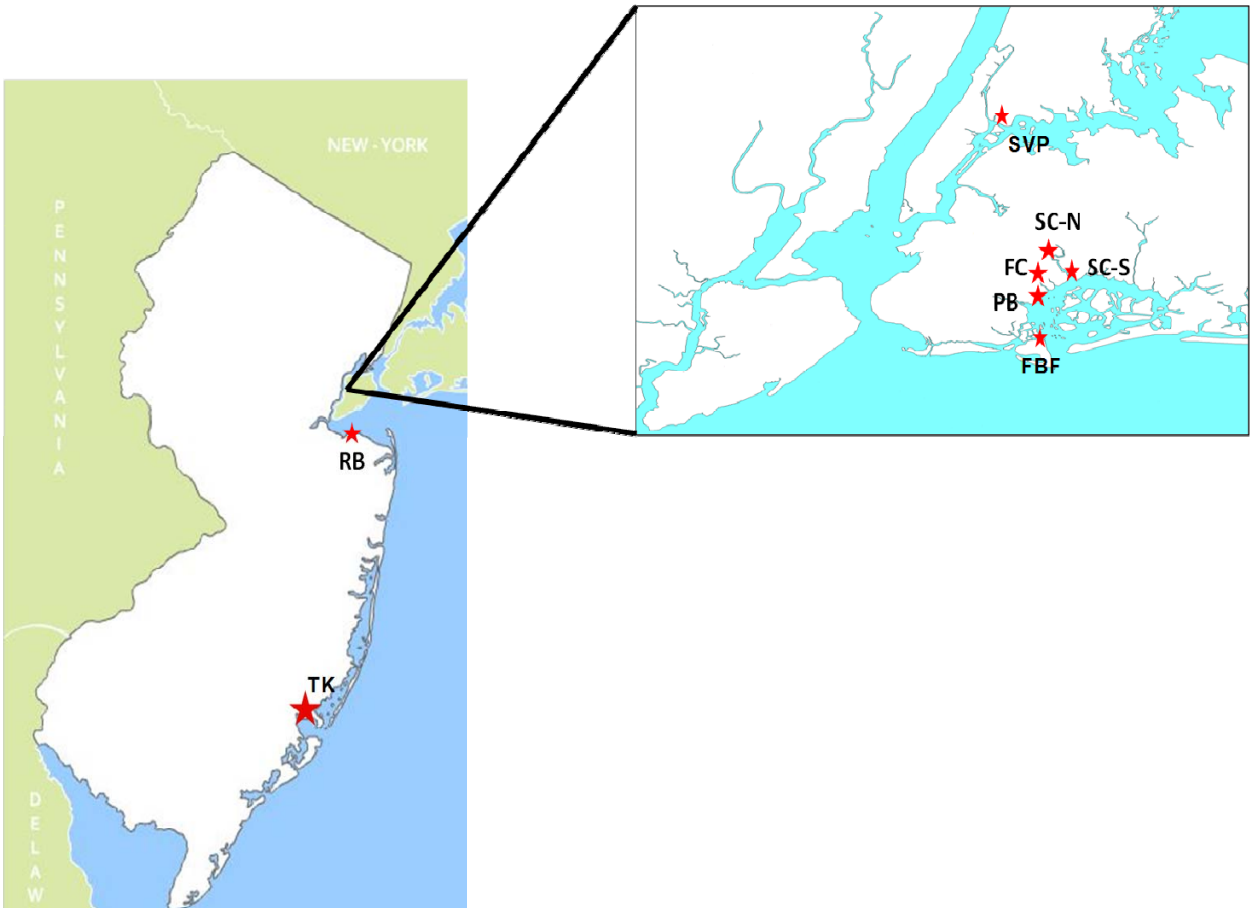

Figure 1: Map of study sites 2009-2010. SVP= Soundview Park, Bronx River, NY. SC-N= Spring Creek (North), Jamaica Bay, NY. SC-S= Spring Creek (South), Jamaica Bay, NY. FC= Fresh Creek, Jamaica Bay, NY. PB= Paedergat Basin, Jamaica Bay, NY. FBF= Floyd Bennett Field, Rockaway Inlet, NY. RB= Keyport Harbor, Raritan Bay, NJ. TK= Great Bay, Tuckerton, NJ.

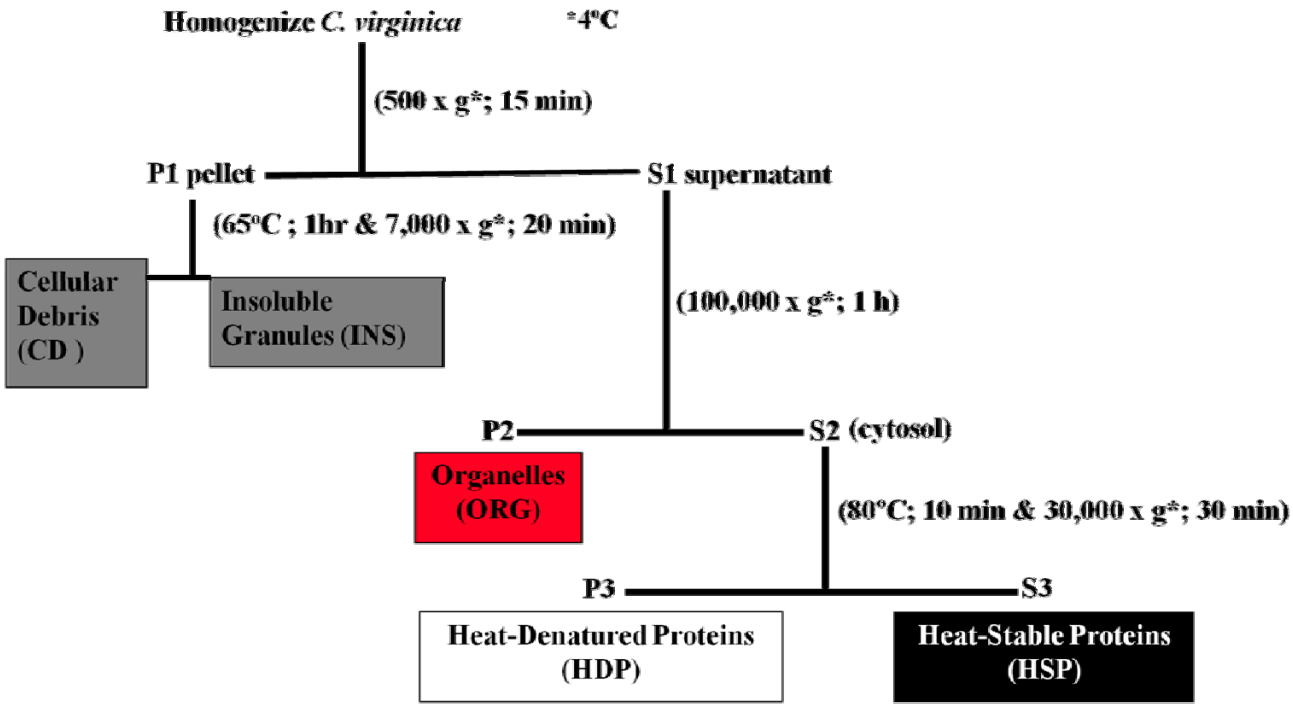

Figure 2: Diagram of subcellular fractionation procedure (after Wallace et al., 2003). CD= cell debris; INS= Insoluble Granules (i.e., metal rich granules); ORG= Organelles; HDP= Heat-Denatured Proteins (i.e., enzymes); HSP= Heat-Stable Proteins (i.e., Metallothionein-like proteins). Temperature, spin intensity, time indicated for each step, resulting in either pellet (P) or supernatant (S).

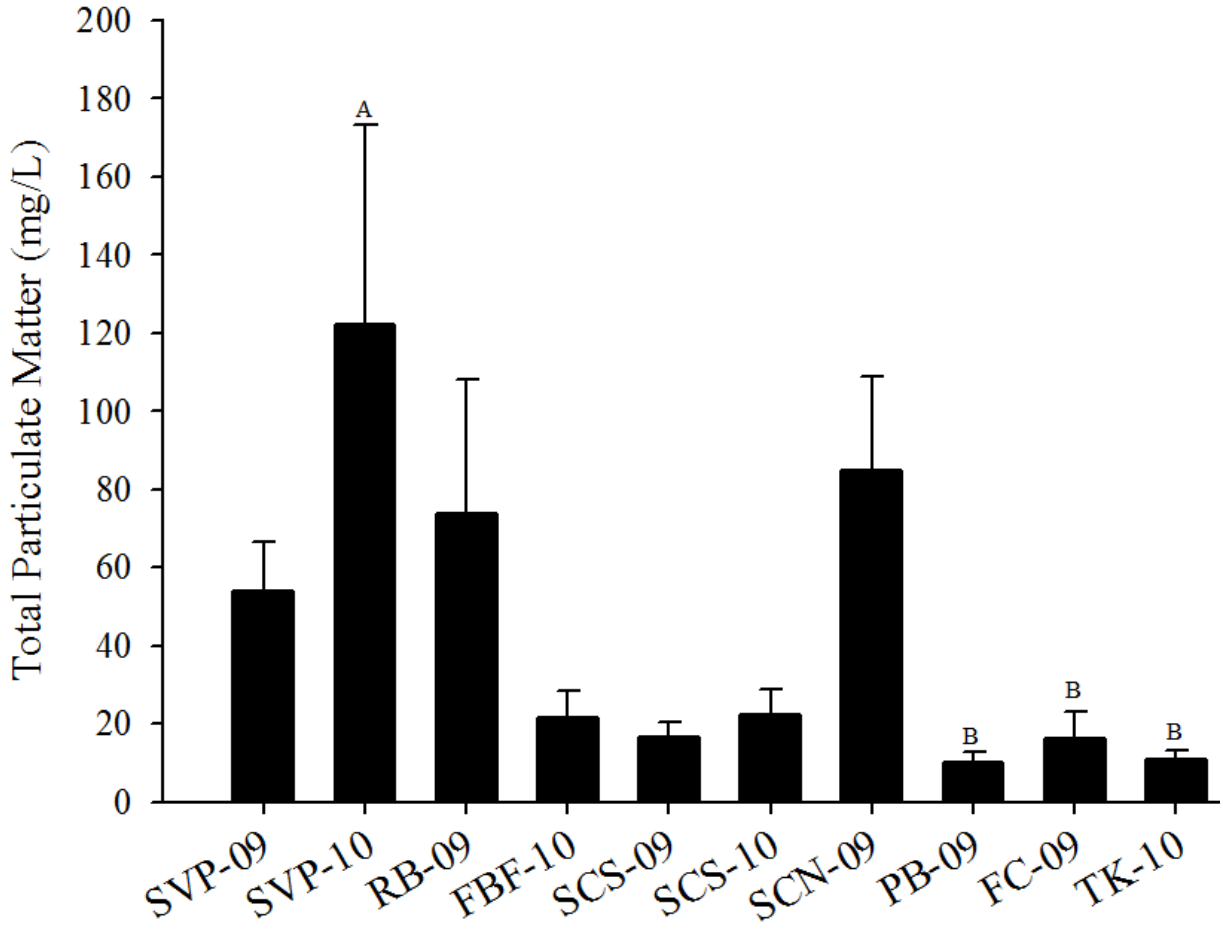

Figure 3: Total Particulate Matter, at each site over the sampling season (July-October, 2009 or 2010). Bars represent mean (n=15-21) ± SE. Letters indicate significant differences between sites (ANOVA, $p<0.0001$, Tukey's Post Hoc).

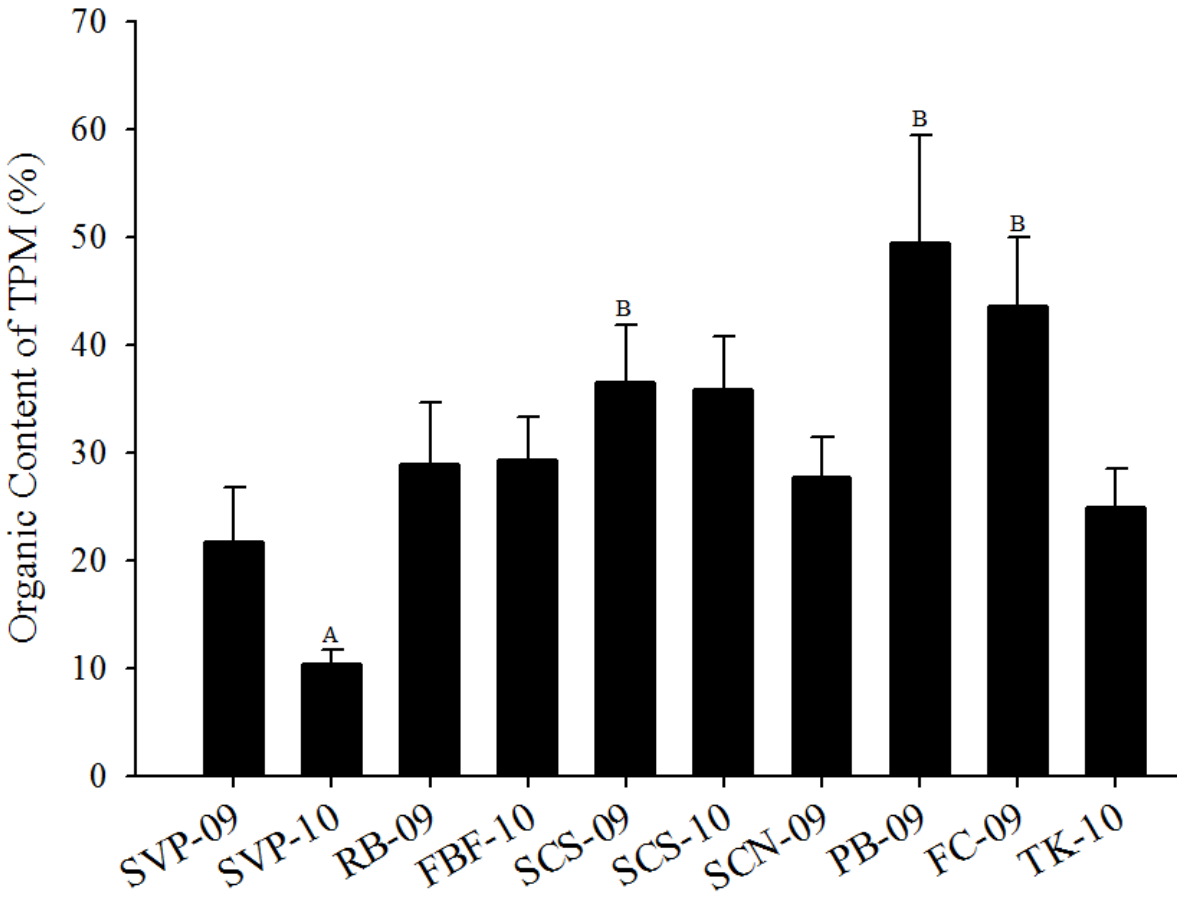

Figure 4: Organic content of TPM at each site over the sampling season (July-October, 2009 or 2010). Bars represent averages (n=15-21) ± SE. Letters indicate significant differences between sites (Kruskal-Wallis, p<0.001, Dunn's Post Hoc).

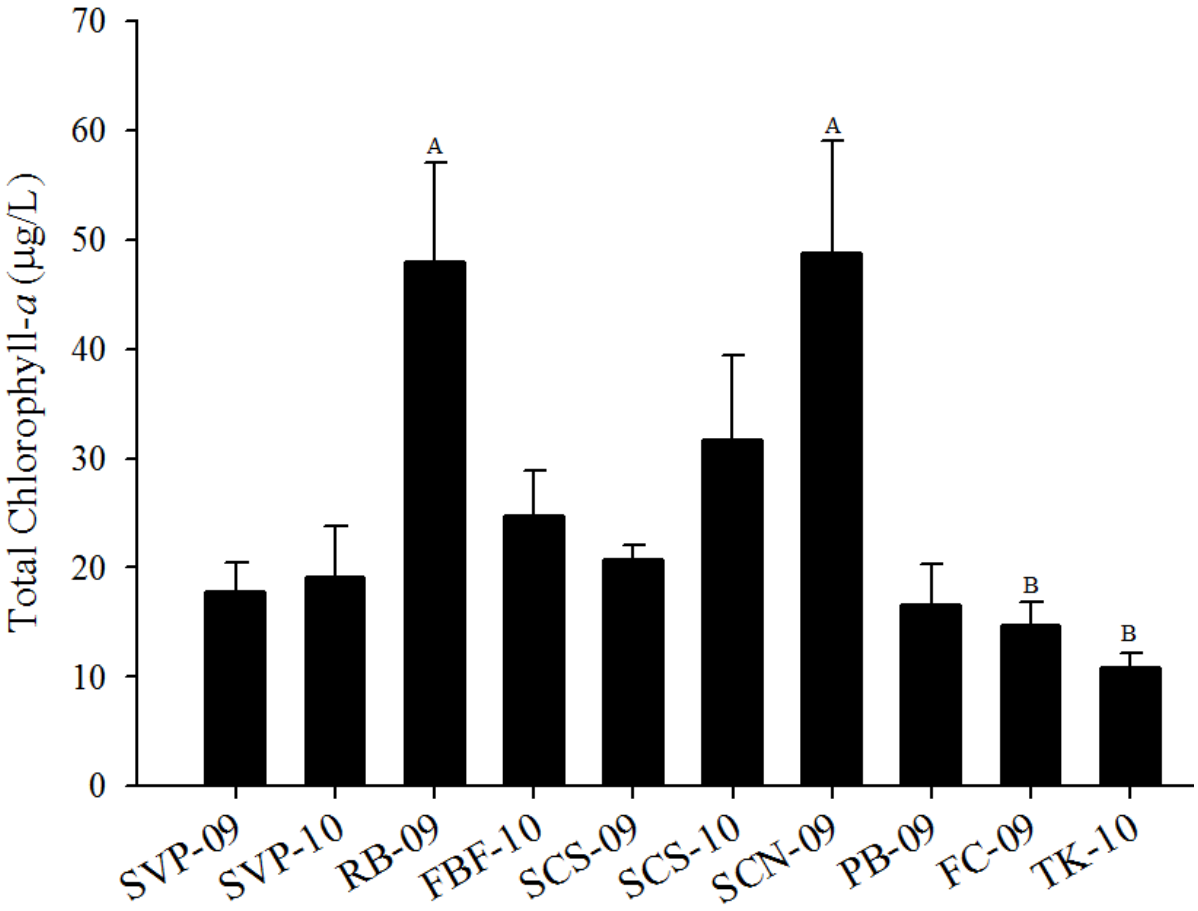

Figure 5: Total chlorophyll-*a*, at each site over the sampling season (July-October, 2009 or 2010). Bars represent mean (n=15-21) ± SE. Letters indicate significant differences between sites (Kruskal-Wallis, p<0.0001, Dunn's Post Hoc).

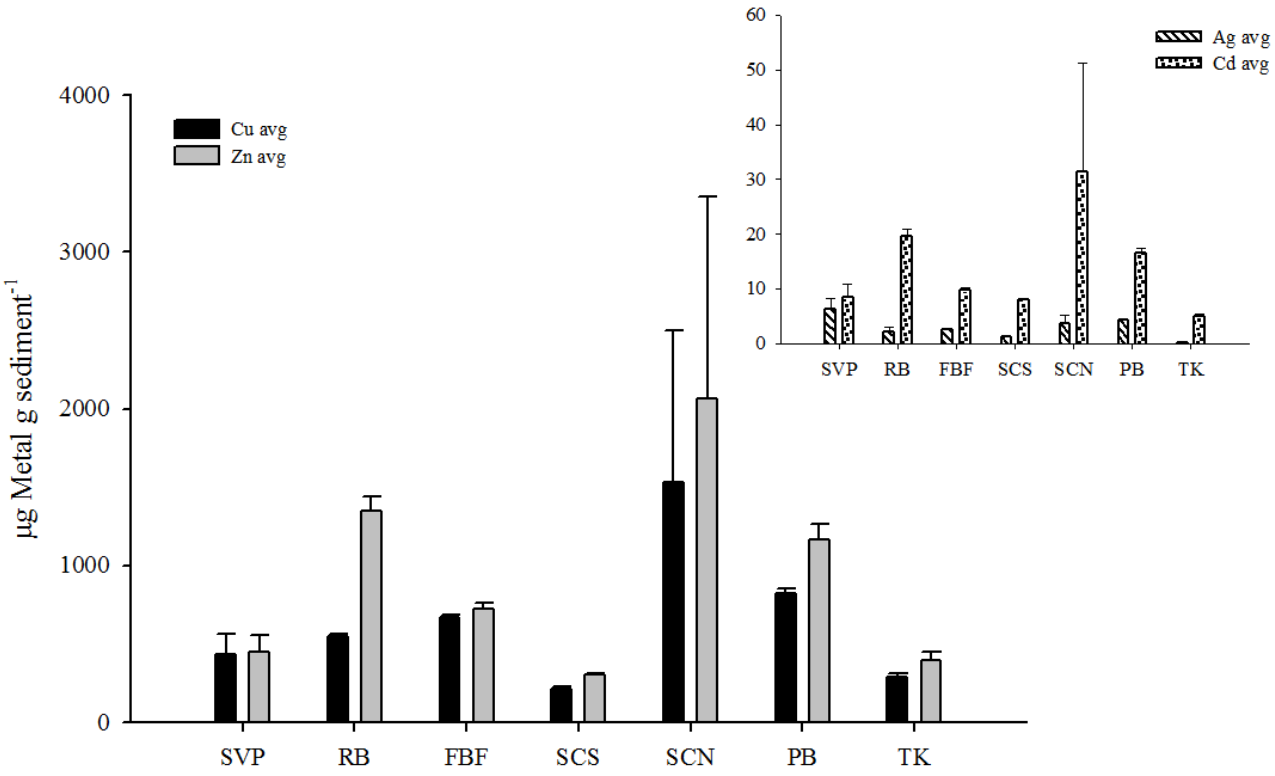

Figure 6: Metal concentrations (mg Metal g sediment-1) in the upper 2cm of sediments at each site, 2009-2010. Four prevalent metals are shown (Ag, Cd, Cu, Zn). Inset graph has smaller scale (10-fold lower). Bars represent averages ± 1 SE.

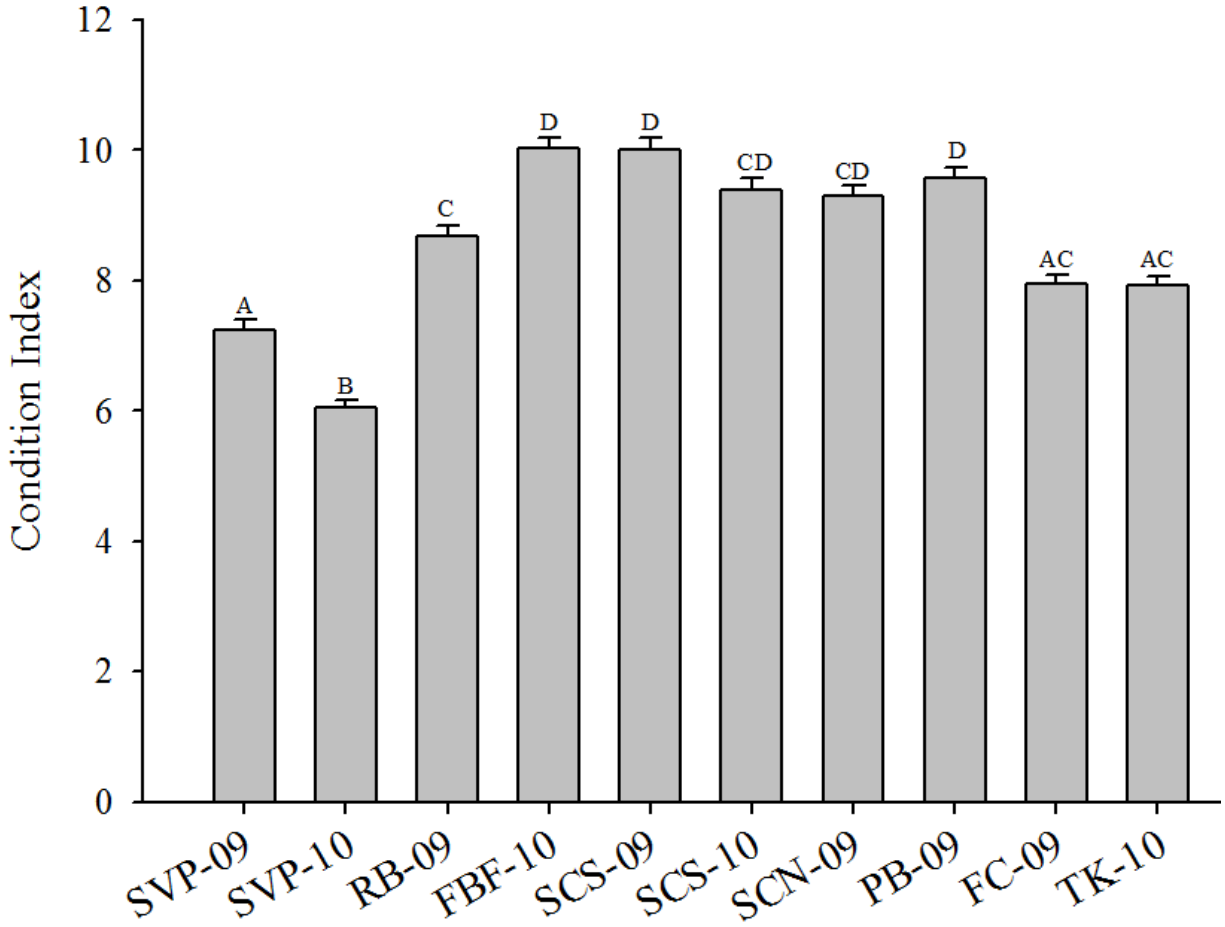

Figure 7: Mean condition index at each site over the sampling season (July-October, 2009 or 2010). Bars represent mean (n=125-175) ± SE. Letters indicate significant differences between sites (Kruskal-Wallis, p<0.0001, Dunn's Post Hoc).

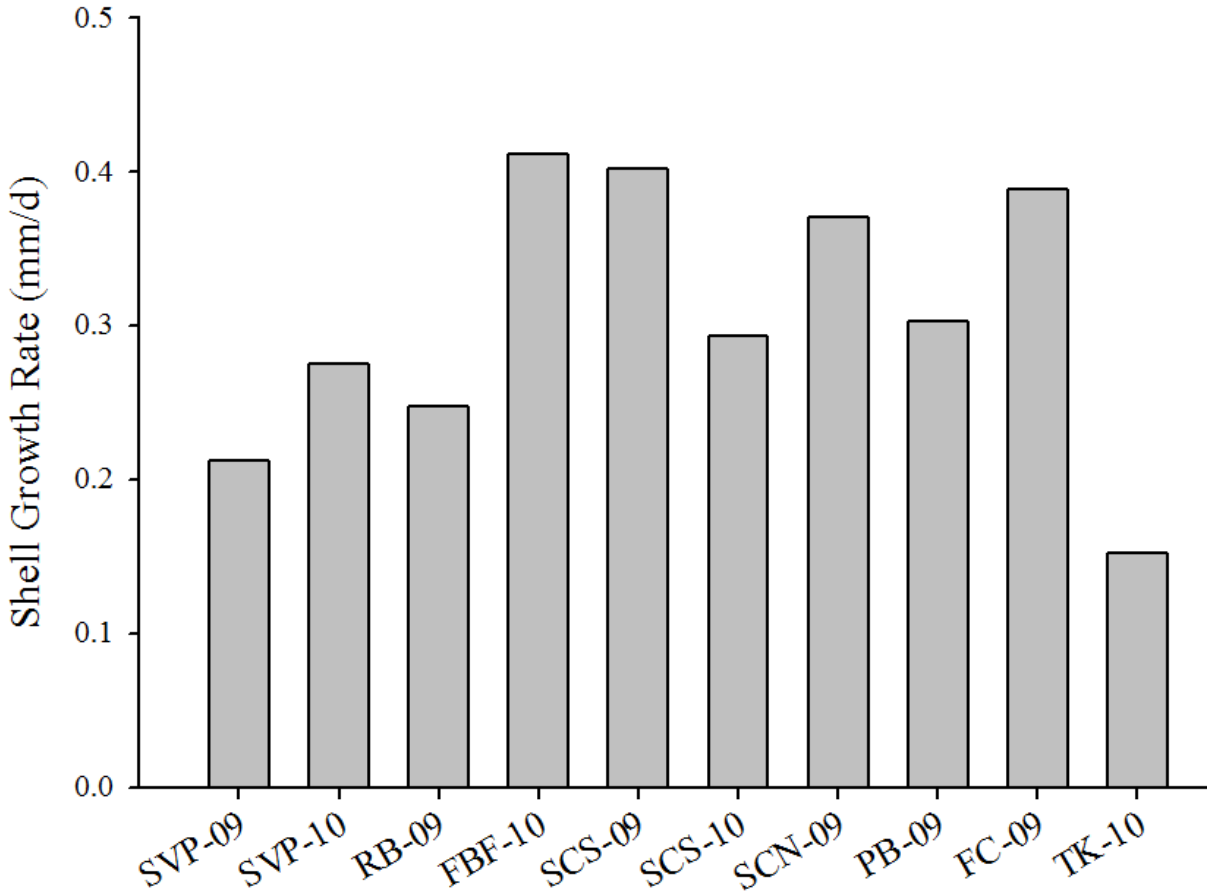

Figure 8: Daily shell growth rate at each site (mm/d), based off the average shell length taken at each sampling event. Bars represent the difference between mean shell length (n=20) at the first and last sampling event, divided by the number of days between first and last sampling events.

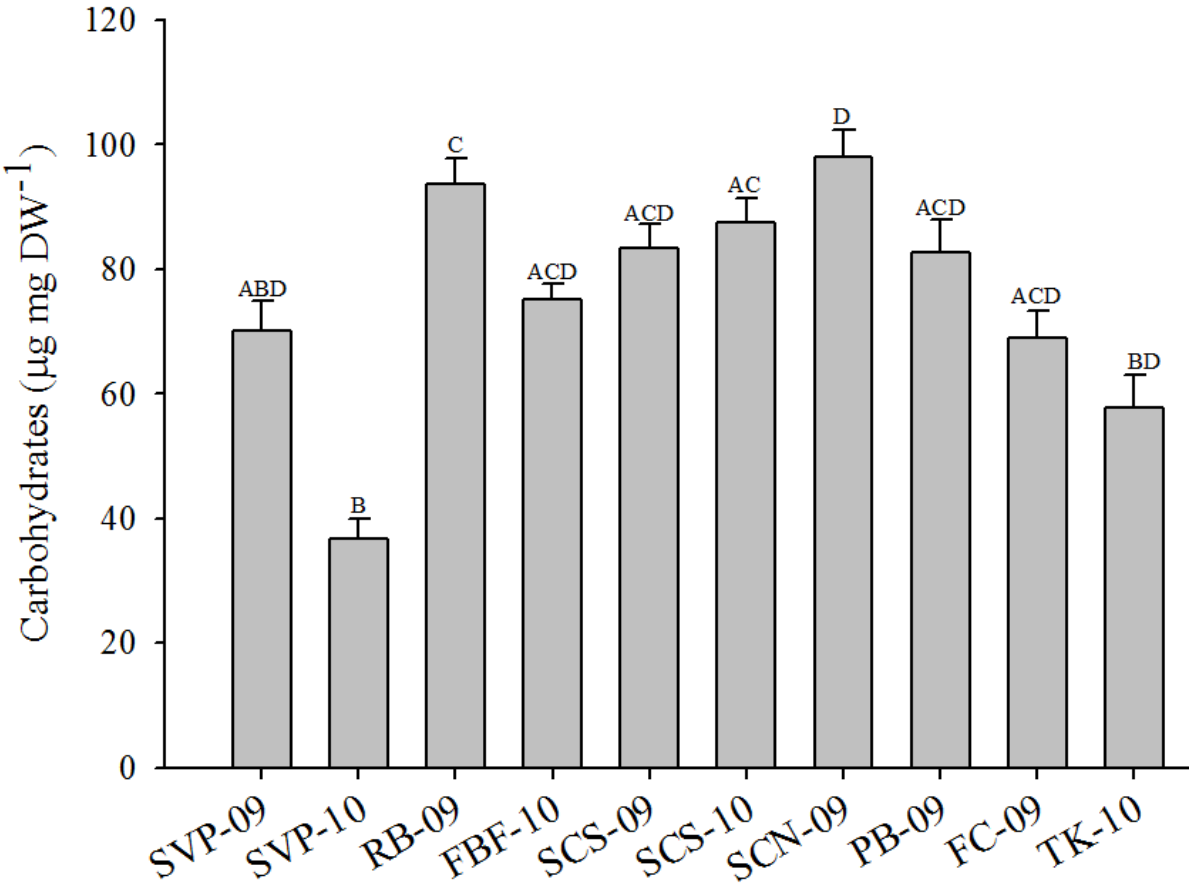

Figure 9: Mean carbohydrate tissue concentration (µg mg DW^{-1}) at each site over the sampling season (July-October, 2009 or 2010). Bars represent mean (n=15-21) ± SE. Letters indicate significant differences between sites (Kruskal-Wallis, p<0.0001, Dunn's Post Hoc).

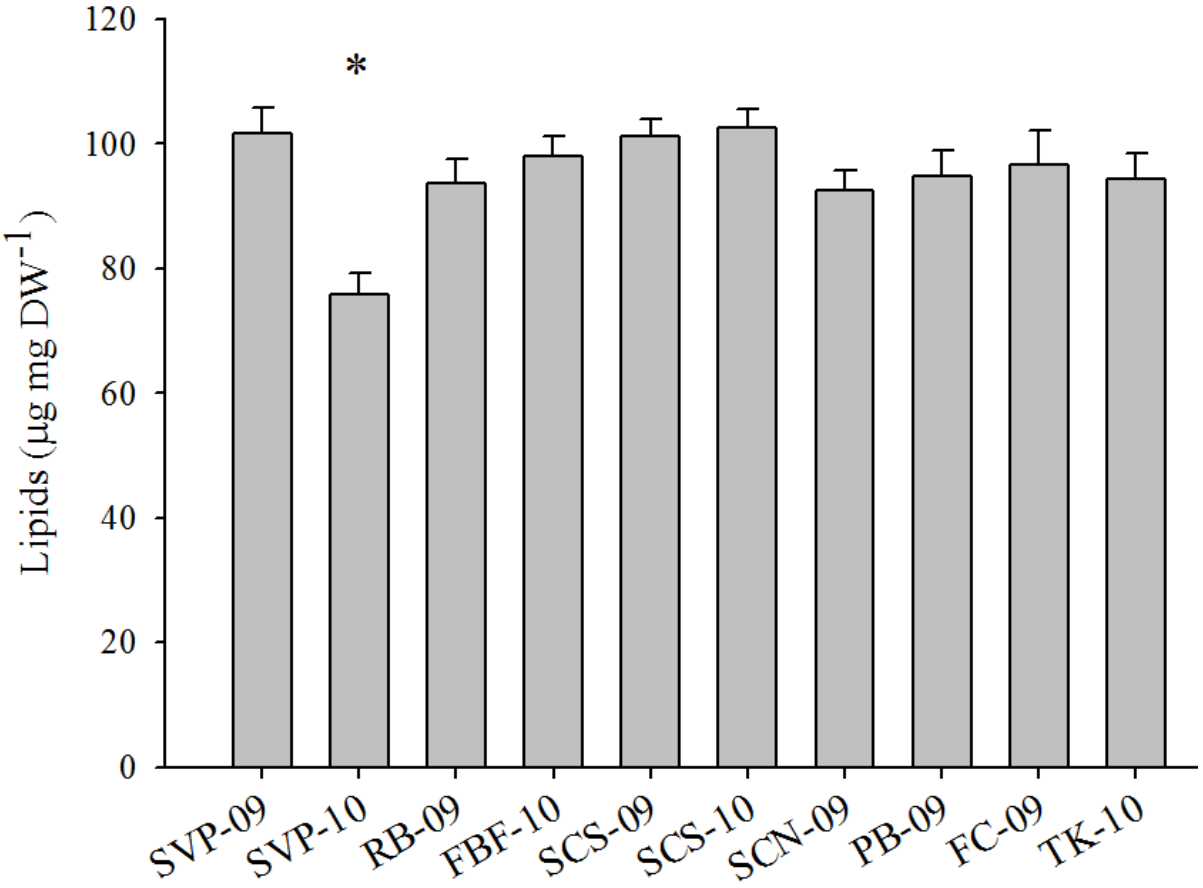

Figure 10: Mean lipid tissue concentration (µg mg DW^{-1}) at each site over the sampling season (July-October, 2009 or 2010). Bars represent mean (n=15-21) ± SE. Asterisk (*) indicates a significant difference between sites (ANOVA, p<0.001, Tukey Post Hoc).

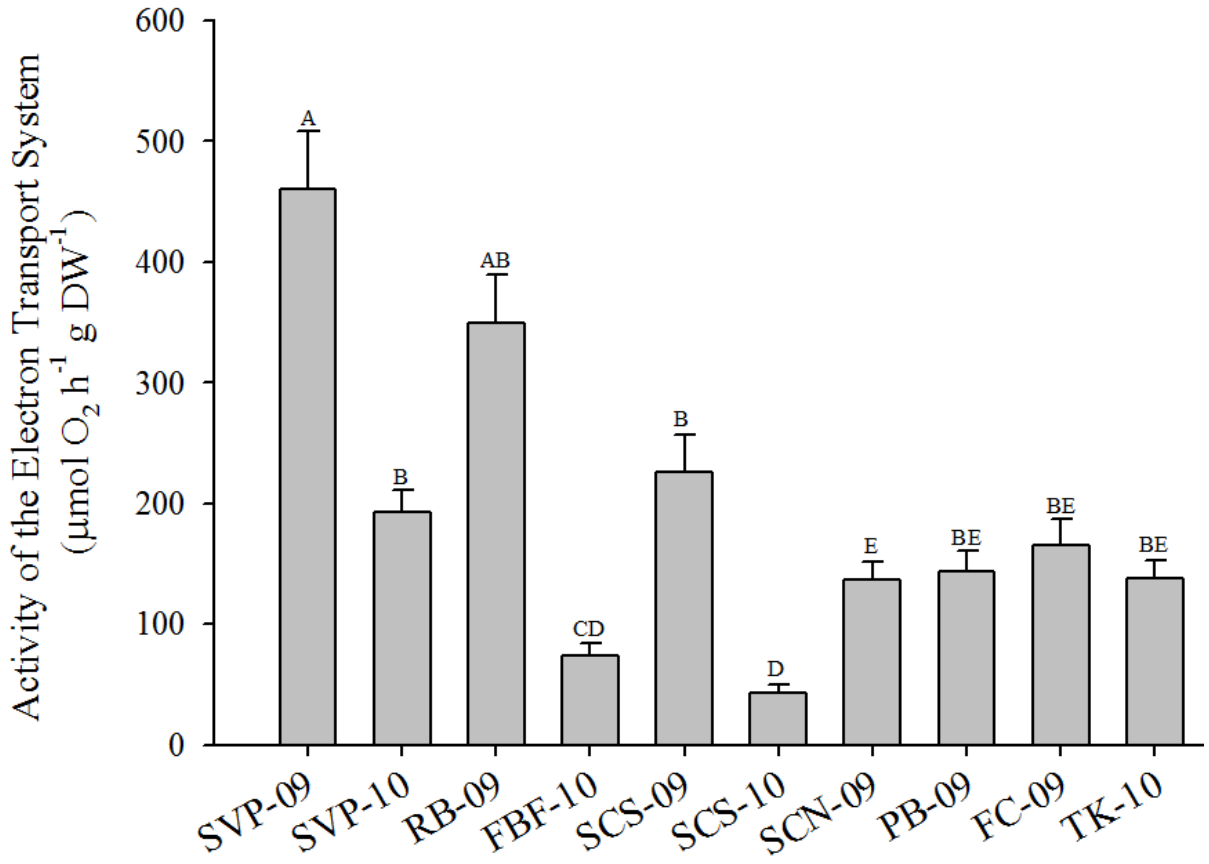

Figure 11: Mean aerobic potential of oysters, as measured by the ETS assay, at each site over the sampling season (July-October, 2009 or 2010). Bars represent mean (n=100-160) ± SE. Letters indicate significant differences between sites (Kruskal-Wallis, p<0.001, Dunn's Post Hoc).

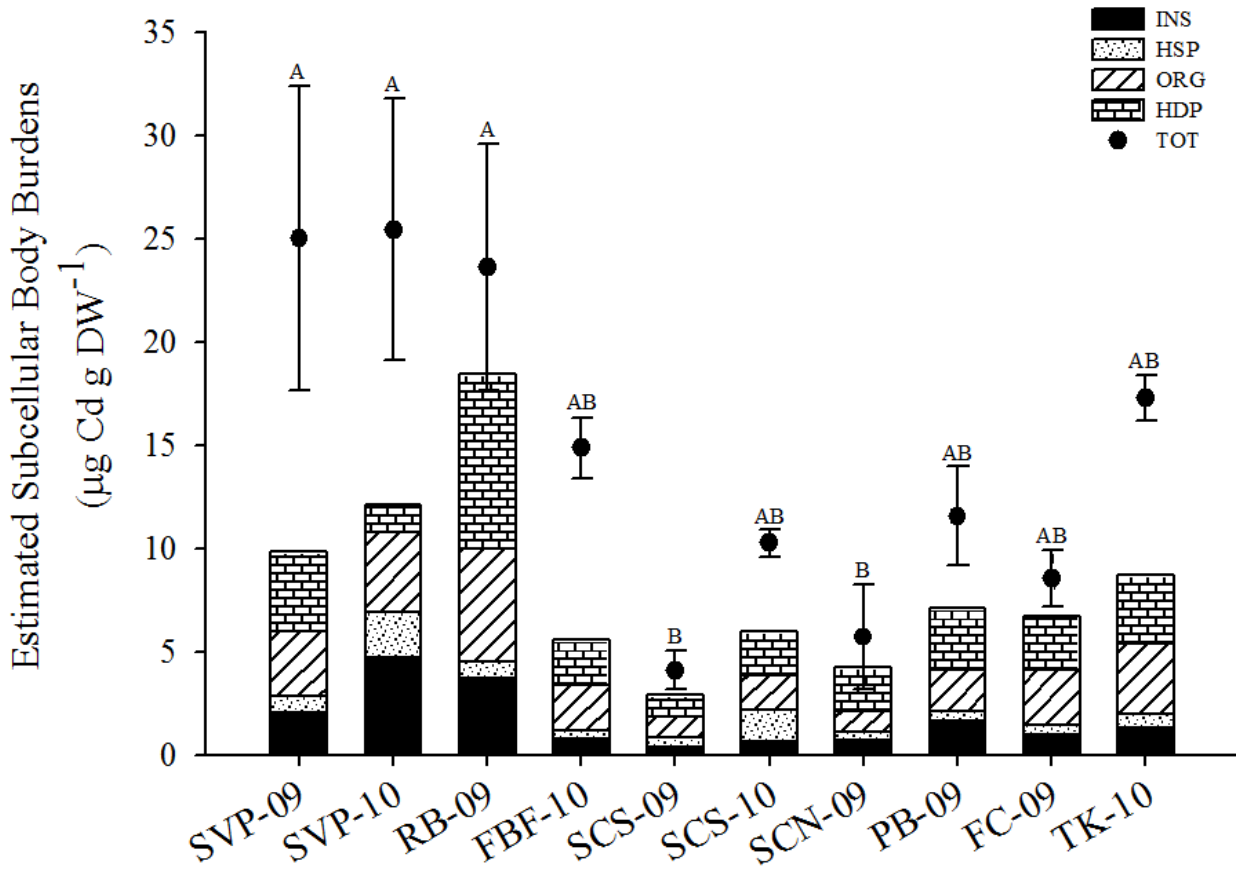

Figure 12: Total and subcellular accumulation (µg g DW^{-1}) of Cd in juvenile oysters after four-months at field sites. Bars represent (from bottom to top) the mean accumulation (n=4) within INS, HSP, ORG, and HDP. The circles above indicate the total accumulation of Cd within oyster tissue (n=4) ± SE. Letters indicate significant differences in total Cd accumulation between sites (ANOVA, p<0.0001, Tukey's Post Hoc).

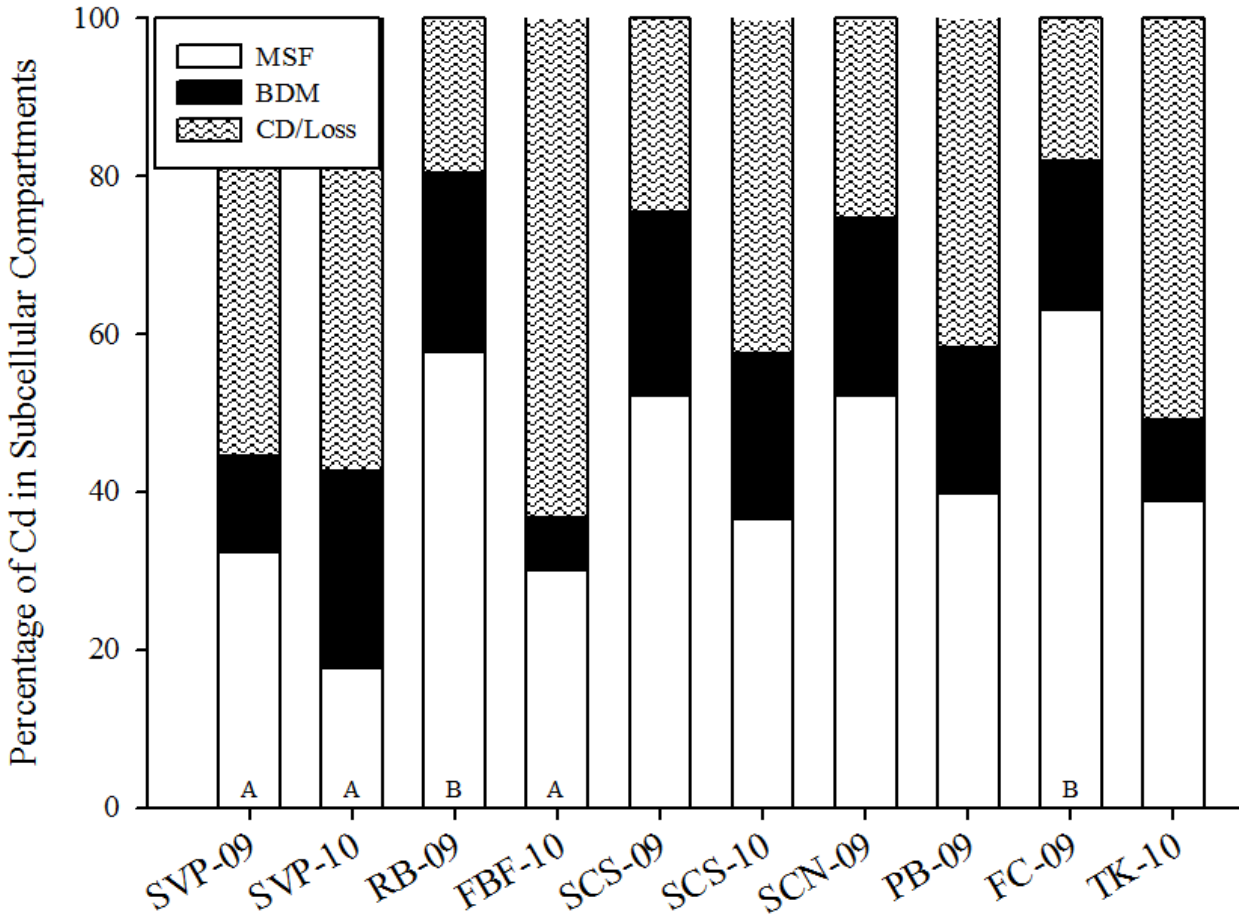

Figure 13: Percent of total Cd associated with MSF, BDM, or CD subcellular compartments. White bars represent MSF, black bars represent BDM, and patterned bars represent CD (or lost during processing). Letters represent significant differences between the percentage of Cd bound to MSF compartment (ANOVA, $p<0.05$, Tukey's Post Hoc). There were no significant differences between sites with respect to Cd accumulation to BDM or CD compartments ($p>0.05$).

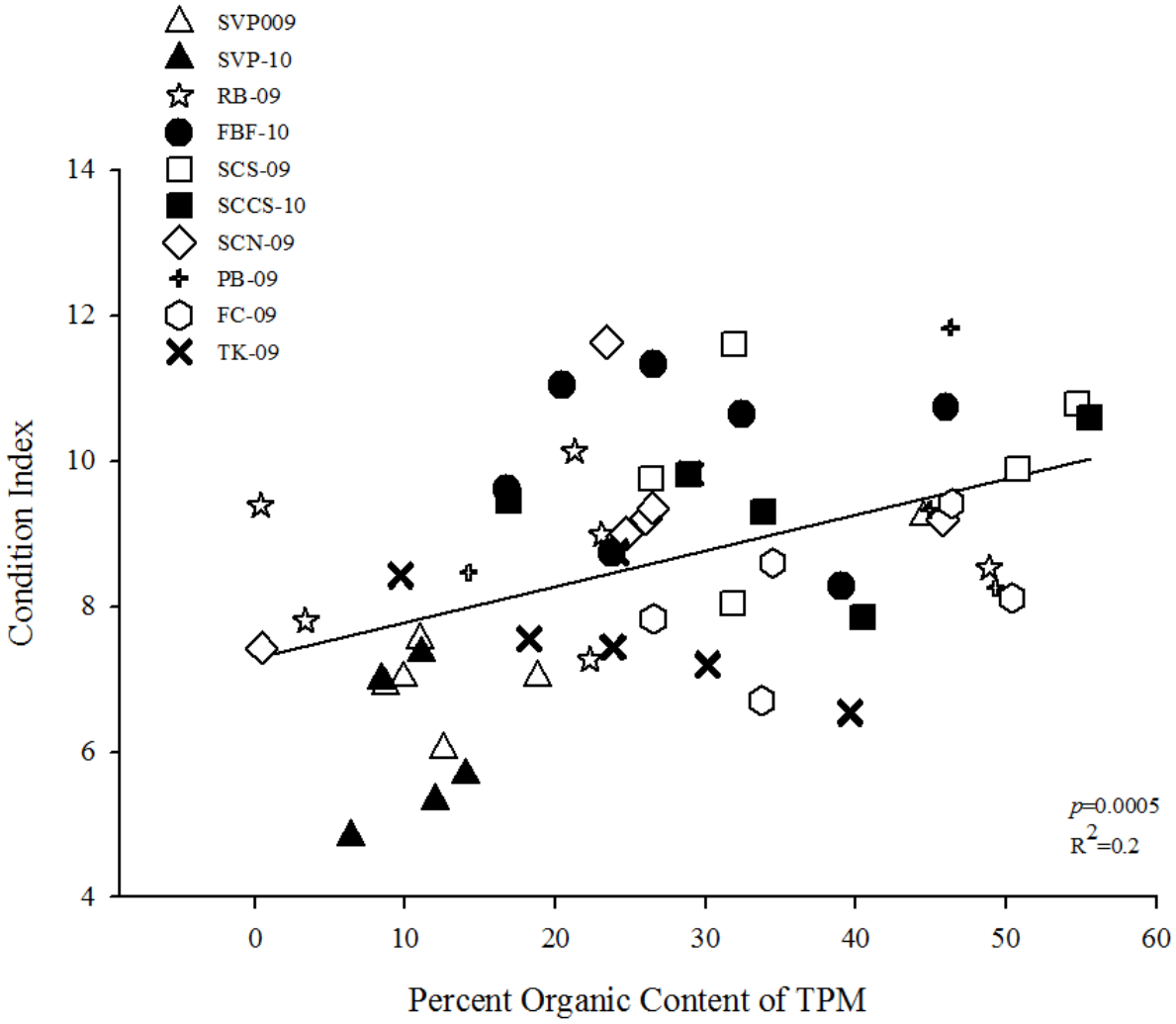

Figure 14: Relationship between the organic content of TPM at sites and condition index of juvenile *C. virginica* (2009-2010). Open symbols represent sites used in 2009; closed symbols represent sites used in 2010. Multiple regression p=0.0005, R^2=0.2.

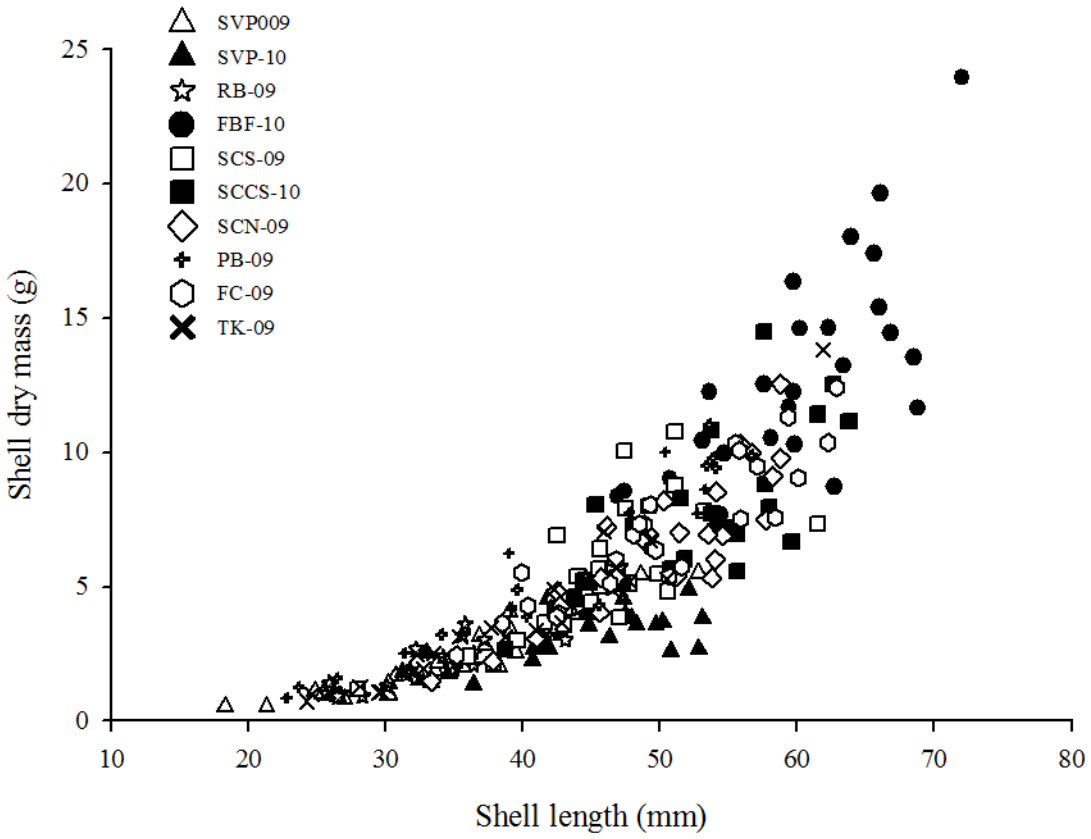

Figure 15: Relationship between shell length (mm) and shell dry mass (g). Symbols illustrate different sites used 2009-2010. When all data was analyzed together, there is a significant positive relationship between length and dry mass (p=0, R^2=0.76). When the sites were analyzed individually, and slopes of the regressions compared, there were site specific differences (ANCOVA, p<0.0001)

Site	July	August		September		October	
	24-Jul	8-Aug	21-Aug	8-Sep	21-Sep	8-Oct	22-Oct
	TEMPERATURE (^{o}C)						
SVP-09	19.9	21.9	25.1	22.7	20.4	16.5	
SVP-10	23.5	23.6		22.4		18	16.5
RB-09	23.9	27.9	31.3	25.4	20.7	16.5	
FBF-10	25.6	24.9	23	20.1	23.1	18.3	14.2
SCS-09	28	24.3	25.5	22.6	17.3	14.1	
SCS-10	24.9	29.3	21.9	21.1	23.2	19.2	12.3
SCN-09	24.7	23.3	23.3	20.9	18.1	14.8	
PB-09		25.2	26.2	23.8	18.8	13.1	
FC-09	26.4	24.8	23.8	23.3	19.1	13	
TK-10	27.6	27.6	24.7	25.5	21.1	16	15.1
	SALINITY (ppt)						
SVP-09	24	23	15	21	22	20	
SVP-10	25	25		23		30	18
RB-09	21	15	15	16	20	25	
FBF-10	***28***	***30***	***31***	***32***	***30***	***30***	***31***
SCS-09	25	25	25	24	25	30	
SCS-10	26	26	28	29	27	29	29
SCN-09	24	20	20	21	25	29	
PB-09	25	25	25	24	26	31	
FC-09	25	23	20	22	26	27	
TK-10	***30***	***30***	***29***	***30***	***33***	***33***	***32***
	DISSOLVED OXYGEN (mg/L)						
SVP-09	3.72	3.2	5.51	4.18	4.1	7.01	
SVP-10	1.75	3.76		6.5		6.12	9.63
RB-09	7.63	11.96	17.63	9.69	5.26	7.35	
FBF-10	9.61	7.21	6.09	8.01	10.61	7.05	8.66
SCS-09	7.57	3.59	1.43	6.17	10.53	9.14	
SCS-10	8.63	12.01	6.11	7.07	12.03	8.4	10.19
SCN-09	6.43	5.55	2.99	6.17	8.52	9.06	
PB-09		6.67	5.68	7.71	6.72	8.27	
FC-09	8.78	2.08	3.23	6.46	5.72	7.76	
TK-10	7.36	7.36	8.72	7.32	7.78	7.69	7.66

Table 1: Temperature (^{o}C), salinity (ppt), and dissolved oxygen (mg/L) discrete measures at each site over sampling period (July-October, 2009 or 2010). Italicized numbers are significantly different (ANOVA, $p<0.0001$) for salinity. There were no significant differences with respect to temperature or dissolved oxygen. Blank entries indicate no measurement was taken during that sampling event.

	Estimated Body Burdens (μg Cd g DW^{-1})				
Site	TOT	INS	HSP	ORG	HDP
SVP-09	25.01 ± 7.37 **A**	2.05 ± 1.06 **A**	0.76 ± 0.21	3.13 ± 0.82	3.88 ±0.82
SVP-10	25.43 ± 6.33 **A**	4.72 ± 1.76	2.17 ± 0.92	3.88 ± 1.6	1.32 ± 0.35
RB-09	23.61 ± 6 **A**	3.68 ± 0.73 **A**	0.82 ± 0.24	5.47 ± 1.74 **A**	8.50 ± 3.41 **A**
FBF-10	14.87 ± 1.46	0.75 ± 0.15	0.4 ± 0.02	2.25 ± 0.38	2.19 ± 0.19
SCS-09	4.09 ± 0.92 **B**	0.36 ± 0.03 **B**	0.49 ± 0.34	0.99 ± 0.15 **B**	1.04 ± 0.24 **B**
SCS-10	10.25 ± 0.66	0.61 ± 0.02	1.57 ± 0.9	1.69 ± 0.18	2.1 ± 0.36
SCN-09	5.7 ± 2.54 **B**	0.69 ± 0.28	0.4 ± 0.18	1.02 ± 0.5	2.12 ± 0.87
PB-09	11.56 ± 2.41	1.61 ± 0.2	0.48 ± 0.24	2.04 ± 0.65	2.96 ± 1.18
FC-09	8.55 ± 1.36	0.94 ± 0.19	0.52 ± 0.08	2.64 ± 0.51	2.65 ± 0.39
TK-10	17.3 ± 1.11	1.28 ± 0.1	0.71 ± 0.39	3.4 ± 0.28	3.3 ± 0.37

Table 2: Mean estimated total and subcellular body burdens of Cd in juvenile *C. virginica* after field deployment. Values listed are means (n=4) ± SE. All values are standardized per dry tissue weight of the fraction. Upper case letters represent significant differences between sites within given fraction (column) (Kruskal-Wallis, p<0.02, Dunn's Post Hoc).

Estimated Percentage of Total Cd Accumulation within Subcellular Fractions

Site	%INS	%HSP	%ORG	%HDP	%CD
SVP-09	9.25 ± 3.83	3.06 ± 0.1	14.37 ± 2.93 ^{B}	17.9 ± 2.92	55.42 ± 7.83
SVP-10	17.44 ± 4.09	7.62 ± 1.25	14.17 ± 2.27 ^{B}	4.54 ± 0.74 ^{A}	57.35 ± 7.37
RB-09	19.11 ± 5.67	3.62 ± 0.55	24.12 ± 4.45	33.5 ± 4.87 ^{B}	19.65 ± 11.1
FBF-10	4.95 ± 0.82	2.46 ± 0.09	14.97 ± 1.48 ^{B}	15.01 ± 1.48 ^{A}	63.23 ± 1.64
SCS-09	10.19 ± 2.22	13.01 ± 9.17	26.71 ± 4.94	25.42 ± 1.14	24.67 ± 11.25
SCS-10	6.03 ± 0.4	14.82 ± 7.77	16.32 ± 0.76	20.23 ± 2.54	42.6 ± 10.68
SCN-09	14.5 ± 3.98	8.04 ± 1.36	13.39 ± 1.46 ^{B}	42.08 ± 4.69 ^{B}	25.33 ± 4.4
PB-09	14.93 ± 2.04	3.57 ± 1.18	16.43 ± 2.15	23.31 ± 5.25	41.75 ± 6.67
FC-09	12.37 ± 3.17	6.46 ± 1.23	30.96 ± 3.99 ^{A}	32.04 ± 4.38	18.17 ± 10.51
TK-10	7.37 ± 0.34	3.78 ± 1.88	19.67 ± 0.97	19.18 ± 1.98	50.94 ± 3.92

Table 3: Estimated percentage of Cd within each subcellular fraction. Values listed are mean percentages (n=4) ± SE. Percentages were estimated using the total accumulation (see Table 2), and the CD fraction was assumed to be the difference between the sum of subcellular percentages (INS, HSP, ORG, and HDP) and the total body burden (Cain et al. 2004). Upper case letters represent significant differences between sites within given fraction (column). (ANOVA, p<0.0001, Tukey Post Hoc)

CHAPTER 3

THE EFFECTS OF CHRONIC HABITAT DEGRADATION ON ADULT *CRASSOSTREA VIRGINICA*: ARE CHANGES IN SUBCELLULAR PHYSIOLOGY LINKED TO METAL ACCUMULATION OR ENVIRONMENTAL INFLUENCES?

INTRODUCTION

Eastern oysters, *Crassostrea virginica*, build extensive inter-and subtidal reefs throughout the Atlantic coastline. While previously abundant within the Hudson-Raritan Estuary (HRE), the bivalve has since declined due to habitat alterations, pollutant inputs, and overharvesting (Franz, 1982). There are restoration efforts underway to restore this ecologically important bivalve, but most focus on the input of spat-on-shell, not the addition of broodstock (Levinton et al., 2011; Zarnoch and Schreibman, 2012). Adult oysters within the HRE are subject to a multitude of stressors within their degraded habitats including increased sedimentation, harmful and nuisance algal blooms, declines in dissolved oxygen, and inorganic or organic pollutants. If spat placed into the HRE are able to survive and grow to an appropriate size, they still must be able to reproduce and propagate the continuance of a reef in order to restore habitat and functionality to the HRE.

Oysters are sequential hermaphrodites and will not turn into females until they reach a critical size threshold (Eble and Scro, 1996; Thompson et al., 1996). In recent surveys of the HRE, most of the adults examined were revealed to be either males or indeterminate sex (Medley, 2010; B. Ravit, pers. comm.). It is possible that many of these oysters do not achieve the size necessary to produce ova, especially at lower salinity sites (Medley, 2010). Additionally, oysters spawn gametes directly into the water column, coordinating release over the entire reef. If the population of oysters is low, there will be less gametes in the water, and it is less likely that fertilization will be successful (Thompson et al, 1996).

Reproduction efforts may be estimated by quantifying the amount of Vitellogenin-like (Vtg) proteins present in the oyster tissue. Vitellogenesis, the formation of egg yolk, occurs prior to spawning, and is highly influenced by the overall health of the oyster (Thompson et al., 1996). In northeast and midatlantic estuaries, including the HRE, one major spawning period occurs mid-summer, with a second smaller spawning period occasionally observed in fall (pers. obsv.; Kennedy, 1996; Thompson et al., 1996). Vtg proteins have not been measured in *C. virginica* yet, but have been successfully used as a metric for reproductive effort in several other bivalve studies (Gagnè et al., 2003; Matozzo and Marin, 2007).

The overall health, and thus reproductive health, of an oyster can be adversely affected by both organic and inorganic pollutants, and the environmental changes associated with these inputs (i.e., fluctuations in dissolved oxygen levels, increases in chlorophyll-*a* content). Temperature and salinity changes are often associated with spawning cues, as are biological molecules (i.e., pheromones; Shumway, 1996; Thompson et al., 1996). Several anthropogenic inputs are known to be endocrine disrupting and may cause induction of Vtg proteins, including non-essential toxic metals (cadmium, mercury, zinc) and organic contaminants (i.e., estradiols; Gagnè et al. 2002). Within the HRE, several metals and organic compounds are known to be found in elevated amounts (NOAA, 1993, 1995).

The objective of this study was to determine how co-occuring stressors associated with a degraded habitat within the HRE, such as poor water quality, increased sedimentation, and contaminants, will affect the health and physiology of adult *C. virginica*. A transplant study was performed to compare oyster physiology at a degraded area of the HRE (Soundview Park, Bronx

NY) and a less degraded site outside the HRE (Great Bay, Tuckerton NJ). Oysters came from a hatchery in Long Island, NY (Oyster Bay) and were placed at each site for a one-year period. Overall condition and biochemistry (including Vtg proteins) were used to assess health and possible reproductive status. The accumulation of non-essential trace metals, including Cd and Hg, was estimated as a measure of anthropogenic impact at the sites. An additional experiment collected wild oysters found at SVP, which were used to assess the long-term impacts on oysters at a highly degraded site within the HRE by determining metal accumulation and physiological health. It was hypothesized that oysters transplanted to an impacted site would have lower overall condition and Vtg proteins, and higher accumulation of Cd and Hg, than oysters at an less impacted site.

METHODS

Oyster placement and field measurements

Adult *C. virginica* were obtained from a local hatchery (Frank M. Flowers & Sons, Oyster Bay, NY). Oysters were transported from the hatchery to field sites on ice, and placed in the water within 12 hours. Two sites were chosen: Soundview Park, Bronx NY (SVP) and Great Bay, Tuckerton NJ (TK) (Figure 1). SVP is regarded as a 'highly degraded' site within the HRE, with increased sedimentation (resulting in higher amounts of particulate matter), poor food quality, periodic hypoxia, and elevated sediment metal concentrations (see Chapter 2 for more details). Located outside the HRE, TK is considered a more pristine site, as it is away from urbanization and marsh degradation, and is highly flushed with water from the Atlantic Ocean (Little Egg

Inlet/ Great Bay). This site does not experience hypoxic events, has lower sediment metal concentrations, and better food quality; however, the site can still have high periods of sedimentation (see Chapter 2 for more details). At each site, 500 adults were placed, with 100 adults per mesh bag (polyethelyne mesh bags; Aquatic Ecosystems®, FL). At SVP, each replicate bag was secured on top of a cinderblock with zipties (to elevate the oysters off the sediments), and placed in the subtidal zone (bags were only exposed during mean low tide). At TK, replicate bags were hung from a dock so that during mean low tide, the bags were just above the water line.

Sites were sampled once a month from June 2010- June 2011 (no samples were taken between November 2010 and April 2011, as the oysters were inactive and the sites would have a layer of ice which prevented sampling). During each sampling event, bags were removed from the water and any epiphytes and encrusting organisms (tunicates, algae) were scrubbed off. The bags were opened, and oysters checked for mortality (any dead oysters were removed). A random subsample of thirty oysters was removed during each sampling event. These oysters were immediately placed on ice and transported back to the laboratory. Remaining oysters were replaced into replicate bags and placed back into the subtidal zone. Once at the laboratory, twenty oysters were immediately frozen on dry ice and placed at -80^{o}C until analysis (condition index). An additional ten oysters were placed into a 37 L tank filled with artificial seawater (DI water and Reef Crystals®) set at ambient temperature and salinity of the site. After 24-36 hours, these oysters were frozen at -80^{o}C and used for metal analysis and biomarker assays.

During each sampling event, discrete measurements of water quality were taken with a hand-held YSI probe {temperature (^{o}C), dissolved oxygen (mg L^{-1})}. Salinity (ppt) was measured with a refractometer. Water samples were collected in triplicate to analyze the seston characteristics (total particulate matter, organic content) and chlorophyll-*a* content. Water samples were placed on ice and transported back to the laboratory, and analyzed via the methods of Bayne (2002) and Parsons et al. (1985) (see Chapter 2 for detailed methodology).

Wild oysters were collected in June 2011 from intertidal rocks at SVP. Additional oysters were transplanted from the hatchery (see above) to SVP. Both transplanted and wild oysters were held in cages in the subtidal zone until October 2011. Monthly, oysters were removed and used to determine physiological endpoints.

Physiological endpoints:

All assays were performed either at The College of Staten Island or transported on dry ice to Baruch College. Condition index was determined using the methods of Crosby and Gale (1990), using the total, dry shell and dry tissue weights (g). Additional oysters were dissected and a portion of the digestive gland and gonad tissue (if present) was removed to determine total protein and the amount of Vtg protein (Gagnè et al., 2006). Aerobic potential of the oysters was estimated using gill tissue (Lannig et al., 2006). Protein concentration of the digestive gland, concentrations of Vtg proteins, and the aerobic potential of the gill tissue were determined using biomarker assays and a microplate spectrophotometer (Molecular Devices SpectraMax M_2e).

Vitellogenin-like Proteins: Alkali-labile protein method

Vtg protein was analyzed using the method of Gagne et al. (2003), with modifications. Oysters were defrosted and shucked, with a section of the digestive gland and gonad tissue (if present) being removed for analysis (approximately 1g wet weight). The tissue subsample was added to 4mL 25mM HEPES/NaOH buffer (pH 7.4, containing 125mM NaCl, 1mM dithiothreiol, 1mM EDTA), and homogenized (OMNI Thq tissue homogenizer; 3 passes of 20s each, at 18-21 x1000 RPM). A subsample of the homogenate (2mL) was removed and centrifuged (30 min, 12000g, 4^{o}C). The supernate was transferred to a 2mL centrifuge tube (1.3mL supernate) and combined with 700µL acetone. The mixture was thoroughly vortexed, and then centrifuged again (5min, 10000g, 4^{o}C). The pellet resulting from the centrifugation was resuspended in 1mL 1mM NaOH, and placed into a 60^{o}C hot water bath for 30 minutes (vortexing intermittently).

Following heat treatment, the pellet was vortexed thoroughly, and the reaction was set up in a 96-well microplate. In each well, 100µL of the tissue mixture (from heat treatment step), 20µL Molybdenum reagent (ammonium molybdate tetrahydrate + H_2SO_4), and 8µL ANSA reagent {Fiske-Subbarow reducer + sodium metabisulfite ($Na_2S_2O_5$) + sodium sulfite (Na_2SO_3)} were mixed (five replicate wells were set up for each oyster). The microplate was read by a spectrophotometer set at 660nm (Gagnè et al., 2003; Stanton, 1968; Blaise et al., 1999). Blanks were set up for each run, and used to correct for the absorbance of the instrument. A standard curve of potassium phosphate (KH_2PO_4) was used for comparison.

Total proteins

Total protein was analyzed following the methodology of Bradford (1976) and using a Coomassie Plus assay kit (Thermo Scientific). Briefly, a subsample (500µl) of the homogenate from above (Vtg proteins) was added to a 2mL centrifuge tube, with 250µL 1mM NaOH. The centrifuge tubes were then thoroughly vortexed. The reaction was set up in a 96-well microplate (five replicate wells per oyster). In each well, 10µL of tissue homogenate was added, and 300µL of the Coomassie Plus reagent (Coomassie Blue). The plate was shaken for 30 seconds, and allowed to incubate at room temperature for ten minutes. Then, the reaction was read on the spectrophotometer at 595nm, and compared to a standard curve using bovine serum albumin standards (Thermo Scientific).

Aerobic potential: electron transport system assay

To estimate energy usage, analysis of the electron transport system (ETS) was completed using modified methods of Madon et al. (1998). A piece of gill tissue (approximately 1g wet weight) was excised from the oyster (same oyster used for Vtg proteins and total protein analysis). All samples were run using a microplate (five replicate wells per oyster), at 490nm on the spectrophotometer. (See Chapter 2 for detailed methodology).

Metal accumulation: Cd and Hg

Additional oysters were dissected for metal accumulation. Each oyster was opened, and a piece of the digestive gland removed (approximately 1g wet weight; 0.13 ± 0.6g dry weight) for each analysis (Geffard et al., 2002). To determine total burdens of Cd, samples were digested in nitric acid and analyzed on a Atomic Absorption Spectrophotometer (Perkin Elmer 5100 Zeeman GF-

AAS; Aquatic Toxicology Lab, UMDNJ) following the digestion protocol of Brown and Luoma (1995). To determine total burdens of Hg, samples were digested with nitric and sulfuric acid, and 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) (See Chapter 2 for detailed methodology).

In addition to the transplanted oysters, wild oysters were sampled in June 2011 and a subsample of digestive gland tissue was excised and digested with nitric acid for analysis with inductively coupled plasma mass spectrophotometer (Perkin Elmer Optima 3300XL ICP-OES) at the Interstate Environmental Commission Laboratory for trace metal analysis (same digestion procedures as for the GF-AAS). Hg burdens of digestive glands were also analyzed for wild oysters (FIMS).

Statistics

Two-way ANOVAs were used to analyze differences between sites, at different time points, for physiological (condition index, protein, Vtg), anthropogenic (Cd and Hg accumulation) and environmental (TPM, %OC, chlorophyll-*a*) variables. Differences between sites were determined using one-way ANOVA. All significance levels were set at $p<0.05$ (Zar, 1999). If necessary, data was transformed (log-10 or arcsine) to meet the assumptions of ANOVA; nonparametric tests (Kruskal-Wallis and Friedman's ANOVA) were used if data could not meet the assumptions. All analysis was performed with Statistica 7.1 (StatSoft Inc ®) and Sigma Plot 10 (Systat Software Inc ®).

RESULTS

Environmental Parameters

Temperature was not significantly different between SVP and TK throughout the year (ANOVA, $p>0.05$), although there were seasonal fluctuations in temperature at each site (Figure 2). Temperature at SVP ranged from 8.8-23.6^{o}C, and from 10.1-28.7^{o}C at TK. Salinity (Figure 2) and dissolved oxygen (Figure 3) were significantly different between sites (ANOVA, $p<0.0001$, $p<0.05$, respectively). Salinity ranged from 18-25ppt at SVP, and 27-32 at TK. No hypoxic or anoxic events were recorded, as dissolved oxygen ranged from 3.6-9.6 mg L^{-1} at SVP and 5.9-9.3 mg L^{-1} at TK.

Analysis of seston characteristics revealed that TPM was significantly different across time at both SVP (Kruskal-Wallis ANOVA, $p<0.01$) and TK ($p<0.01$). TPM at SVP was highest during August 2010 (322.7 ± 8.1 mg L^{-1}) and lowest during June 2011 (16.4 ± 0.7 mg L^{-1}). TPM at TK was lower, ranging from 4.6 ± 0.2 mg L^{-1} (June 2010) to 21.7 ± 0.6 mg L^{-1} (September 2010). There was an effect of site, date, and the site*date interaction on TPM; SVP had significantly higher TPM on all dates except June 2011 (Friedman's ANOVA, $p<0.0001$) (Figure 4).

Organic content of the seston at each site varied little throughout the seasons (Figure 5). Organic content was significantly lower at SVP the entire year except for May 2011 (Friedman's ANOVA, $p<0.0001$). At SVP, organic content varied significantly from 8.43 ± 0.2% (August 2011) to 20.1 ± 0.8% (May 2011) (Kruskal-Wallis ANOVA, $p<0.05$). Organic content at TK was higher, ranging from 17.6 ± 1.7% (November 2010) to 30.1 ± 1.7% (August 2010). There

was an effect of date on TPM concentrations at TK (Kruskal-Wallis ANOVA, $p<0.01$), but these differences were unable to be teased apart (Dunn's Post Hoc, $p>0.05$) (Figure 5).

Chlorophyll-*a* concentrations were significantly different between sites, and over time (Friedman's ANOVA, $p<0.0001$). Chlorophyll-*a* was significantly higher at SVP June, July, and November 2010, and May, June 2011. At SVP, chlorophyll-*a* concentrations differed significantly across dates, from 2.6 ± 0.4 μg L^{-1} (October 2010) to 21.6 ± 0.4 μg L^{-1} (July 2010) (Kruskal-Wallis ANOVA, $p<0.05$). Concentrations were generally lower at TK, ranging from 3.7 ± 0.1 μg L^{-1} (June 2011) to 18.53 ± 1.1 μg L^{-1} (September 2010). Chlorophyll-*a* at TK was significantly higher during late summer/early fall (August/September 2010) than during June 2011 (Kruskal-Wallis ANOVA, $p<0.01$).

Physiological Endpoints

Condition indices of oysters at SVP were significantly lower than those at TK in July, October, and November 2010, and April and June 2011 (Figure 7). There was an effect of site, date, and the site*date interaction on condition index (ANOVA, $p<0.0001$). SVP oysters ranged from 2.19 ± 0.16 to 8.10 ± 0.55 in condition indices, with a significant drop in condition between June and July 2010 (ANOVA, $p<0.0001$) (Figure 7). TK oysters displayed a higher overall condition, ranging from 5.37 ± 0.30 to 7.64 ± 0.45. A significant drop in condition occurred again between June and July 2010, and again between November 2010 and May/June 2011 (ANOVA, $p<0.0001$) (Figure 7).

Total protein in the digestive gland was not significantly different between SVP and TK, on any date (ANOVA, $p>0.05$). However, at each site there were significant seasonal changes (ANOVA, $p<0.0001$). SVP had significantly higher protein content (μg protein per mg dry tissue) in September 2010 (71.18 ± 2.6 μg mg DW^{-1}) than in October 2010 (46.25 ± 1.7 μg mg DW^{-1}), April 2011 (49.8 ± 3.1 μg mg DW^{-1}), May 2011 (48.96 ± 8.7 μg mg DW^{-1}), and June 2011 (52.33 ± 4.8 μg mg DW^{-1}) (ANOVA, $p<0.0001$). At TK, there was a significant decline in protein from June 2010 (72.49 ± 6.4 μg mg DW^{-1}) to July 2010 (50.63 ± 1.7 μg mg DW^{-1}), but no further fluctuations were seen after July (ANOVA, $p<0.0001$) (Figure 8).

Concentration of Vtg proteins within oyster tissue was not significantly different between sites (ANOVA, $p>0.05$), but at each site significant seasonal changes were observed (Figure 9). At SVP, Vtg increased to a maximum of 2.74 ± 0.2 μg ALP mg $protein^{-1}$ in October 2010, a significant increase over July (1.23 ± 0.1 μg ALP mg protein-1) and September 2010 (1.05 ± 0.3 μg ALP mg $protein^{-1}$) (ANOVA, $p<0.01$). At TK, Vtg reached a maximum concentration in October 2010 as well (3.62 ± 0.4 μg ALP mg $protein^{-1}$), rising significantly from July 2010 (1.56 ± 0.3 μg ALP mg $protein^{-1}$) (ANOVA, $p<0.05$) (Figure 9). When examining the percentage of total protein that is comprised of Vtg proteins, significant seasonal shifts are present within each site but not between sites (ANOVA, $p>0.05$) (Figure 10). At SVP, the percentage of total protein comprised of Vtg proteins ranged from 14.3 ± 4% (September 2010) to 59.6 ± 5.3% (October 2010). October was significantly higher than June, July, and September 2010 (ANOVA, $p<0.001$). At TK, the maximum concentration was 69 ± 10% in October 2010, which

was significantly higher than the lowest concentration (29 ± 5.6%) in June 2010, as well as July 2010 and May 2011 (ANOVA, $p<0.01$) (Figure 10).

The activity of the electron transport system (ETS) was analyzed throughout the year at each site, and no significant differences were found at any time point between the two sites (ANOVA, $p>0.05$) (Figure 11). However, there was a significant effect of date, and the site*date interaction, on ETS activity (ANOVA, $p>0.0001$). At SVP, aerobic potential ranged from 105.71 ± 27.64 μmol O_2 h^{-1} g DW^{-1} in September 2010 to 21.79 ± 2.08 μmol O_2 h^{-1} g DW^{-1} in April 2011. Aerobic potential in September was significantly higher than both April 2011 and October 2010 (32.14 ± 9.77 μmol O_2 h^{-1} g DW^{-1}; Kruskal-Wallis, $p<0.05$). Oysters at TK had the highest aerobic potential in June 2010 (109.57 ± 27.75 μmol O_2 h^{-1} g DW^{-1}), which was significantly higher than July and October 2010, and April, May, and June 2011 (ANOVA, $p<0.0001$) (Figure 11).

Metal accumulation

At SVP, Hg accumulation significantly increased through time (Kruskal-Wallis, $p<0.01$) (Figure 12). The amount of Hg found in digestive gland tissue (μg Hg g DW^{-1}) was higher in June 2011 (0.319 ± 0.41 μg Hg g DW^{-1}) than in June 2010 (0.093 ± 0.004 μg Hg g DW^{-1}). At TK, there was no Hg detected within tissues from June 2010-October 2010 (no samples were taken from November 2010; the detection limit of the instrument is set at 0.25 μg L^{-1}). However, from April-June 2011, Hg increased steadily. By June, Hg concentrations in the digestive gland were 0.284 ± 0.016 μg Hg g DW^{-1}, significantly higher than the previous months (ANOVA, $p<0.01$). There was an effect of date ($p<0.0001$) and site*date interaction ($p<0.05$) on Hg accumulation

(ANOVA); during the spring of 2011, Hg accumulation was significantly higher at TK than SVP in May 2011 (ANOVA, $p<0.05$) (Figure 12).

There was no significant difference between Cd accumulation in oysters placed at SVP and TK (ANOVA, $p>0.05$) (Figure 13). There was a significant effect of the site*date interaction, with seasonal differences in Cd concentration at SVP (ANOVA, $p<0.01$). Cadmium concentrations within digestive gland tissues from oysters transplanted to SVP varied significantly from 34.93 ± 5.94 μg Cd g DW^{-1} (August 2010) to 81.32 ± 8.49 μg Cd g DW^{-1} (November 2010). Accumulations were significantly higher in November 2010, May and June 2011 than in June and August 2010 (ANOVA, $p<0.001$) (Figure 13). Accumulation within TK oysters was not significantly different over time (ANOVA, $p>0.05$), with an average Cd concentration in tissues of 48.32 ± 2.93 μg Cd g DW^{-1}.

Relationships between physiology, metal burdens, and environmental conditions at sites

There was no significant relationship between Hg body burdens and physiology at either site. At SVP, condition index was negatively related to Cd burdens ($p=0.04$); as Cd increased in the digestive gland, condition indices declined (Figure 14). Neither site showed relationships between physiological endpoints and temperature, salinity, dissolved oxygen, or chlorophyll-*a* content. Relationships between seston characteristics and physiological endpoints were found at both SVP and TK. At SVP, total protein content was positively related to the particle concentration of the water column (TPM); as the amount of TPM in the water column increased, so did the protein content in dried tissue ($p=0.01$; Figure 15). At TK, the aerobic potential of

oysters was positively related to the percentage of organic material associated with TPM (p=0.02; Figure 16).

Wild oysters

Wild oysters were not significantly different in size (shell length, mm) than the transplanted oysters, and did not grow significantly throughout the summer (Kruskal-Wallis, p>0.05). Average length of wild oysters was 91.10 ± 2.1mm, and was 94.51 ± 3.6mm for transplanted hatchery oysters. Wild oysters had significantly lower condition indices in July and August than June or September (ANOVA, p<0.01). During the same time period, transplanted oysters also had significantly lower condition indices in July (ANOVA, p<0.01). However, transplanted and wild oysters did not significantly differ in condition indices at any month throughout the summer (ANOVA, p>0.05) (Figure 17)

Wild oysters displayed similar physiological measurements as the transplanted hatchery oysters. Protein content of oyster tissue was not significantly different between wild and transplanted oysters (ANOVA, p>0.05). While there were no differences between months with respect to the protein content of transplanted oysters, wild oysters had significantly higher protein content during June (93 ± 5.9 μg mg DW^{-1}) than any other month (ANOVA, p<0.001) (data not shown). Vtg proteins were also measured, and no significant differences were found between wild oysters and transplanted oysters throughout the summer (ANOVA, p>0.05). Transplanted oysters had significantly more Vtg protein in August and September than June or July (2.98 ± 0.2 μg ALP mg $protein^{-1}$), wild oysters did not display any significant changes over the season, with the average concentration of 2.08 ± 0.64 μg ALP mg $protein^{-1}$ (data not shown).

Aerobic potential of wild oysters was significantly higher in September than in June or July (ANOVA, $p<0.0001$) (Figure 18). Wild oysters increased their aerobic activity each month, from 73.9 ± 22.8 μmol O_2 h^{-1} g DW^{-1} in June to 240 ± 39.4 μmol O_2 h^{-1} g DW^{-1} in September, while transplanted oysters peak aerobic potential occurred in July (247.9 ± 32.6 μmol O_2 h^{-1} g DW^{-1}). Transplanted oysters had significantly higher aerobic potential in July, but during the rest of the summer there was no significant difference between wild and transplanted oysters (ANOVA, $p<0.001$).

Wild oysters were assayed on an ICP-MS to determine a wide variety of trace metal accumulation in the digestive gland. Significantly higher accumulations of silver (Ag) and Hg were found in wild oysters than transplanted oysters (Student's t-test, $p<0.05$) (Figure 19). The highest accumulated metal was Cu, the least accumulated was Hg.

DISCUSSION

Adult oysters placed at two sites, one at a degraded location within the HRE (SVP) and one outside the HRE in a more pristine location (TK), were significantly different in their overall condition and metal accumulation throughout the year, but there were no differences detected at the subcellular level (protein content, Vtg proteins, and aerobic potential) (Figures 7-13). Both whole-body (condition index) and subcellular (protein) physiology were influenced by abiotic parameters (TPM, Cd). The influence of abiotic conditions on physiological responses is

important when assessing reproduction. When wild oysters at SVP were compared to transplanted oysters, differences in condition index and aerobic potential were observed during the summer, indicating that oysters may have acclimated to the conditions found at SVP if spawned and developed in these conditions (Figures 17, 18), even with elevated accumulations of trace metals (Figure 19).

Environmental influences on adult C. virginica

The two sites had similar temperatures, but there were significant differences between salinity and dissolved oxygen. SVP is a less saline site, located at the northern end of the lower HRE (at the confluence of the East River and Bronx River), where salinity is lower (mean salinity 2009-2011=23ppt) than outside of the HRE. At TK, located within the Mullica River-Great Bay estuary, higher saline seawater from the Atlantic Ocean (via Little Egg Inlet) is mixed with estuarine waters (mean salinity 2010-2011= 31ppt). The salinity tolerance range for *C. virginica* is between 14-28ppt (Shumway, 1996) though oyster survival at salinities around 30 ppt is not uncommon; oysters naturally occurred in the marshes of Mullica-Great Bay estuary in the past and current oyster restoration sites are located within this estuary (for both ecological and commercial restoration; NJDEP/FWS, 2007). Elevated salinities can lead to reduced survival and growth, and reduced tolerance to contaminants and other anthropogenic inputs (Shumway, 1996). Although the temperature range at both sites was within the acceptable tolerance for *C. virginica*, the synergistic effects of temperature and salinity are often essential to oyster survival and reproduction (Shumway, 1996). Using combined temperature and salinity regimes, Heilmayer et al. (2008) found average condition indices to be between 4.9 and 6.9 for treatments similar to the conditions found at SVP (salinity 21-25ppt; temperature 15-30^{o}C). These

experiments took place in a closed system (not at a field site), indicating that other environmental variables were not present (i.e., contaminants, increased sedimentation), yet these condition indices were not very different from the values seen in the current study (4.8- 6.5 average condition at both sites; Figure 7). In contrast, the synergistic effects of elevated temperatures (similar to the HRE during summer months) and lower salinities (less than 7ppt) are detrimental to the overall condition of *C. virginica* (Heilmayer et al., 2008). These low salinities are not found at either SVP or TK, and thus do not pose a problem to the *C. virginica* transplanted or spawned at these sites. In contrast, a recent experiment comparing transplanted oysters (from the same hatchery used in the current study) and wild oysters at a low saline environment {Hastings-on-Hudson, Hudson River, NY; mean summer salinity= 8ppt (K. Kalchmayr, pers. comm.)} found oysters at this site had altered physiological responses (i.e., feeding rates) from oysters placed at SVP (data not shown).

Another environmental parameter that differed significantly between SVP and TK was the concentration of TPM (mg L^{-1}) in the water column. TPM was significantly higher at SVP than TK throughout the year. TPM concentrations (mg L^{-1}) at SVP ranged from 3-fold (April/May 2011) to 52-fold (August 2010) higher than TPM concentrations at TK during the same time frame (Figure 4). Increased TPM may be detrimental to oyster health, as more particles are encountered during feeding. Oysters must expend more energy to ingest and digest particulates (i.e., increased pseudofeces production; Barillè et al., 1997), which may or may not have nutritional value. Though TPM was higher at SVP, the organic content of TPM (Figure 5) was significantly higher at TK indicating that this site had more nutritious particles and less inorganic particles in the TPM. TPM at SVP was made up of mostly inorganic particulates (i.e.,

sediments) which are not biologically useful and are passed through as pseudofeces (Bayne, 2009). Increasing organic content of TPM has been linked to increases in clearance rate and physiological condition (Bayne, 2009). Organic content of TPM at TK was 1.5-3-fold higher than at SVP, and no seasonal fluctuations were observed at either site. Seston quantity and quality is known to influence several physiological parameters in oysters, including growth (shell length and tissue weight) and condition indices (Paterson et al., 2003). At SVP, even with high TPM values, there was no relationship between TPM and shell length, wet or dry tissue weight, and overall condition indices. Though there was no significant relationship between TPM and the amount of energy expended by the oyster (ETS assay), during times of high TPM (June and August 2010) aerobic activity was lower than all other months (Figures 4 and 11). This suggests that the oyster may reduce filtration activity in order to avoid energetic losses when inorganic TPM is overwhelming (Bayne, 2009). In contrast, oysters transplanted to TK did have a significant relationship with TPM; length (p=0.03) and the ratio of wet/dry tissue (p=0.05) were both negatively related to the amount of TPM indicating that TPM may be a stronger driver of oyster size here than at SVP (data not shown).

Both sites exhibit a dependence of oyster physiology on environmental parameters, which was also evident in previous experiments performed with juvenile oysters within the HRE (See Chapter 2 for in depth discussion). Seasonal fluctuations in physiology often correspond with seasonal fluctuations in environmental parameters, and several physiological measures (i.e., condition index, aerobic activity) are related to various environmental parameters (i.e., temperature, salinity). For example, a study in Australia with *C. gigas* (similar size/age to this study) also found significant seasonal fluctuations with respect to physiological endpoints (tissue

biochemistry, hemolymph activity), and relationships between these physiological endpoints and environmental parameters (Li et al., 2009a). Though the seasonal shifts and relationships demonstrated in this study may be expected, it still does not completely account for the reduction in condition and physiology of SVP oysters. The anthropogenic inputs at the site, and the synergistic effects of anthropogenic inputs and environmental fluctuations, may be more important than environmental parameters alone.

Metal accumulation

Transplanted oysters at SVP and TK were assayed to determine Cd accumulation throughout the year (Figure 13). Oysters at SVP showed a significant accumulation, with tissue burdens increasing throughout summer 2010 into spring 2011. Concentrations reached a maxima in November, 2010 (81.32 ± 8.5 μg Cd g DW^{-1}), and did not significantly decrease throughout the following spring. In contrast, while oysters at TK began with similar body burdens as SVP oysters, they did not significantly accumulate any metal throughout the study. Wild oysters collected at SVP had lower body burdens of Hg and Cd than transplanted oysters (Figure 19), suggesting resident populations may have "pre-acclimated" to site conditions thereby allowing for increased metal accumulation without experiencing toxicity (Klerks and Bartholomew, 1991).

Bendell and Feng (2009) noted that Cd concentrations in oysters placed at field sites for three years accumulated metal linearly (per oyster), though fluctuations in the μg Cd per gram dry weight were seen. These seasonal fluctuations were likely due to changes in tissue mass. During the present study, Cd increased linearly from June 2010- June 2011, though there were

fluctuations monthly, as tissue weight changed throughout the seasons (Figure 13). The present study also found that Cd was higher in colder months (October- May) than during summer months (similar to Bendell and Feng, 2009). In the HRE, waters do not generally warm until June-July. Oysters have lower metabolic activity below 10^{o}C (i.e., filtration rate; Loosanoff, 1958). Oyster at SVP had the highest metal concentrations during November, when water was cooler and metabolism slowed down; when waters warmed again in June, the oysters had not accumulated significantly more metal than in the winter (Figure 13). Cassis et al. (2011) found that Cd concentrations in *C. gigas* were also elevated in colder months, as was the particulate Cd available in the water column.

There was no relationship between any abiotic parameters and Cd body burdens at either site, indicating that environmental shifts did not influence Cd accumulation within oysters. Oysters can accumulate Cd through both dietary and dissolved pathways (Lekhi et al., 2008; Bendell and Feng, 2009). Though the organic content was significantly different between SVP and TK seston, it did not influence the accumulation of Cd at neither site, nor the amount of food available at each site (chlorophyll-*a* content). Lekhi et al. (2008) found that up to 50% of Cd accumulated by *C. gigas* at field sites could be attributed to dissolved Cd sources interacting with gill tissue during feeding. At SVP, increases in TPM are usually from resuspension of surficial sediments, which are very fine. Concentrations of Cd-laden particles were measured at 0.0026 μg Cd g dry seston^{-1} (data not shown); sediment concentrations of Cd at SVP were measured at 8.4 ± 2.4 μg Cd g dry sediment^{-1} (see Chapter 2). If Cd was to be resuspended and then ingested by filtering *C. virginica*, accumulation within tissues would increase, even if the chlorophyll-a content at the site was low (as the Cd is sorbed to inorganic particles; Langston et al., 1998).

Though physiology was not significantly related to TPM at either site, the interaction of increased TPM and elevated Cd levels may be detrimental to oyster health, resulting in a decline in overall condition. Similar field surveys with transplanted juvenile oysters at SVP indicated that TPM was a driving factor in oyster health and Cd accumulation, and TPM may also be important in adult oyster health (See Chapter 2 for more in-depth discussion).

The synergistic effects of the abiotic factors of each site and the physiological responses of the oysters to these factors may be very influential in overall metal accumulation. Abbe et al. (2000) found that transplanted *C. virginica* at a contaminated field site accumulated higher body burdens of Cd due to higher water temperatures which resulted in more metabolic activity and faster growth (thus more tissue accumulation of Cd). In this study, the temperatures at SVP and TK were not significantly different throughout the year; neither was dissolved oxygen (Figure 2 and 3). However, seston characteristics (TPM, chlorophyll-*a*) were significantly different between sites (Figures 4 and 6). Both sites have a similar amount of Cd found in sediments (but SVP transplanted oysters accumulated Cd throughout the exposure period, and TK oysters did not (Figure 13). Oysters at SVP also had significantly lower overall condition indices, suggesting that the site-specific environmental parameters, and the potential interactions of the biotic and abiotic, may be important in the accumulation of metals.

Reproduction of C. virginica within the HRE

At the present time, only small pockets of wild oysters are found within the HRE. Reproduction of the population has not been quantified; some studies have attempted to sex oysters and histologically examine reproduction (Zarnoch and Schreibman, 2012; Ravit et al., ***submitted***).

These studies have found a higher percentage of males at SVP and other impacted areas within the HRE. *Crassostrea virginica* is a dioecious, protandric species which spawns its gametes into the water column annually, where they fertilize into planktotrophic larvae (Eble and Scro, 1996; Newell and Langdon, 1996; Thompson et al., 1996). Larger, older oysters tend to be female, while smaller younger oysters are males (Eble and Scro, 1996). Sex determination is controlled by both endogenous (i.e., genes) and exogenous factors (i.e., temperature) (Santerre et al., 2013). At SVP, the average height of oysters surveyed there was 64 mm (Medley, 2010), at which the oysters may not be large enough or old enough to induce sex change and vitellogenesis. If the wild populations are mostly male, it may impact future populations; sex ratios in an oyster population are important for spawning and fertilization (Thompson et al., 1996).

With respect to the transplanted oysters, histological sex determination was not undertaken during this study. Analysis of Vtg protein in oysters at SVP at TK determined that there were no differences in Vtg concentration between sites at any time point (Figure 9). Vtg levels did fluctuate seasonally at each site. At SVP, a significant decrease in condition index coincided with a significantly decrease in the concentration of Vtg proteins, suggesting that the oysters had spawned. The levels of Vtg found during this study were similar to values reported in the literature for *Mya arenaria* (Gagne et al., 2003), *Tapes philippinarum* and *Cerastoderma glacum* (Matozzo and Marin, 2007) which were from impacted estuaries (Saint Lawrence River, Canada, and Lagoon of Venice, Italy, respectively). Similar values were also reported for the oyster *Crassostrea cortezinesis* (Hurtado et al., 2012) from northwestern Mexico, at an oyster farm. No previous studies have examined Vtg protein levels in *C. virginica*, though studies have been performed determining gametogenesis and sex determination in wild and transplanted oysters

(Wintermyer and Cooper, 2007; Li et al., 2010). In addition to the fluctuations seen in Vtg concentration, the percentage of total protein comprised of Vtg protein changes throughout the season (Figure 10). At SVP, the percentage of Vtg proteins increased as the water temperature cooled (September/October), but did not increase again the following spring. The consistently low levels of Vtg proteins may be an indication that oysters are not reaching reproductive capacity and this may be due to exogenous factors impacting oyster health.

If oysters at SVP did in fact spawn during June/July 2010 (as suggested by the significant decrease in condition index; Figure 7), the stress of spawning along with the stress caused by the environment may have caused the steady decline in physiology exhibited by oysters after August 2010. Several physiological processes are known to be reduced by the combined effects of one or more stressors (i.e., food deprivation and spawning stress; Li et al., 2009*b*). *Crassostrea gigas* were examined pre- and post-spawning with a significant stressor (starvation) and were found to have significantly lower condition index when starved post-spawning than pre-spawning (Li et al., 2009*b*). If oysters at SVP are exposed to significant stressors (i.e., increased sedimentation, contaminants, poor food quality and quantity) pre-spawning, they may not develop significant gonad tissue. In July, when condition index and Vtg protein declined (suggesting spawning), food quality was poor (Figure 5), temperature was elevated (Figure 2), and DO was lower (Figure 3). These stressors could have induced oysters to spawn, and also influenced future spawning events. If oysters at SVP are exposed to significant stressors post-spawning, they may not recover fully and regain adequate condition to grow and reproduce again. Both food quality/quantity and temperature are very important influences on subsequent reproduction in oysters (Bernard et al., 2011).

CONCLUSION

Adult oysters respond to site-specific abiotic influences with changes in overall condition and subcellular physiology. Oysters transplanted to SVP, an impacted site within the HRE, had significant decreases in condition index, protein, and Vtg compared to oysters placed at an unimpacted site (TK). Accumulation of non-essential trace metals increased over time, with significant accumulation of Hg and Cd by oysters placed at SVP. There was a negative relationship between Cd accumulation and condition index, suggesting that increased accumulation influenced overall condition of the oysters; however it is not the only abiotic factor responsible for physiological decline of oysters at SVP. Synergistic effects of multiple abiotic factors (i.e., increased TPM, lower OC%) had the strongest influence on oyster physiology, supporting the conclusion that oyster health is site-specific. With respect to reproduction, adult *C. virginica* placed at SVP possibly spawned in July, as the drop in both condition index and Vtg protein suggests. Oysters did not form more gonad tissue afterwards, and condition indices and Vtg proteins remained low throughout the year, despite increased metabolic activity. Metal accumulation and increased TPM may have affected reproduction by influencing gonad tissue development and Vtg proteins. Wild oysters at SVP exhibit physiological adjustments to chronic habitat degradation, including altered aerobic potential, which may be mechanisms tolerate to increased metal accumulations.

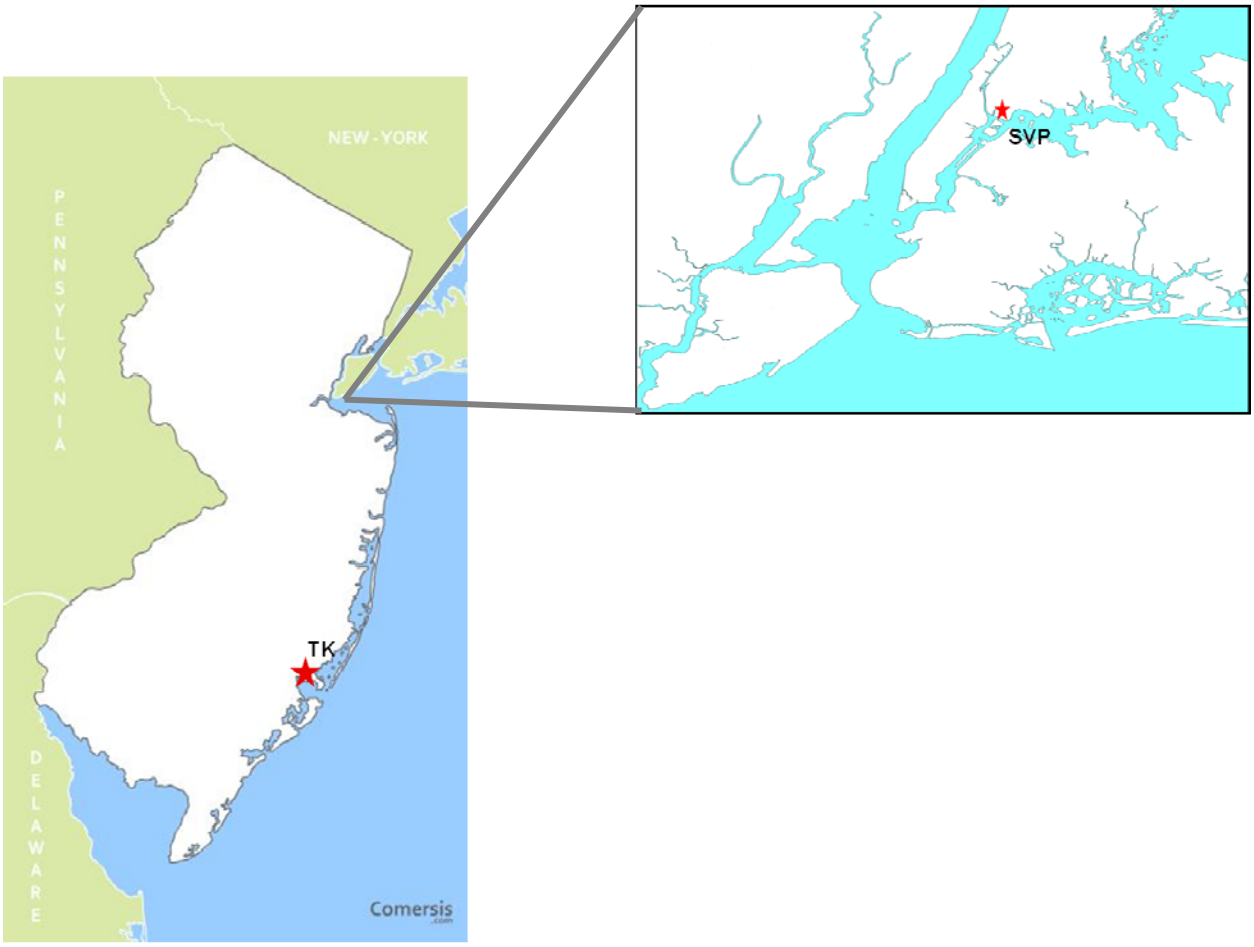

Figure 1: Map showing study sites from at Soundview Park, Bronx NY (SVP) and Great Bay, Tuckerton NJ (TK) from 6/14/10-6/18/11.

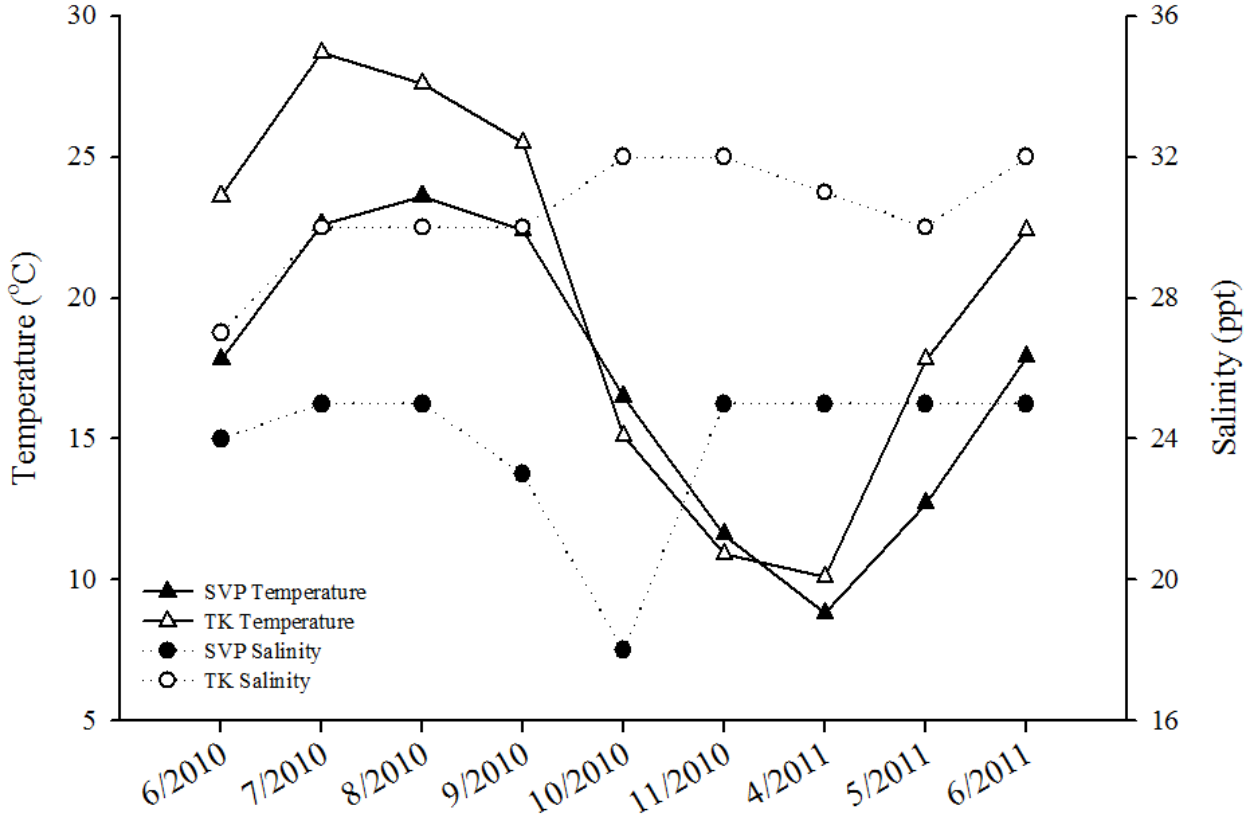

Figure 2: Temperature (°C) and salinity (ppt) measured at SVP and TK from 6/14/10-6/18/11. Filled symbols denote SVP, open symbols denote TK. Temperature was not significantly different between SVP and TK; salinity was significantly different between the two sites (ANOVA, $p<0.0001$).

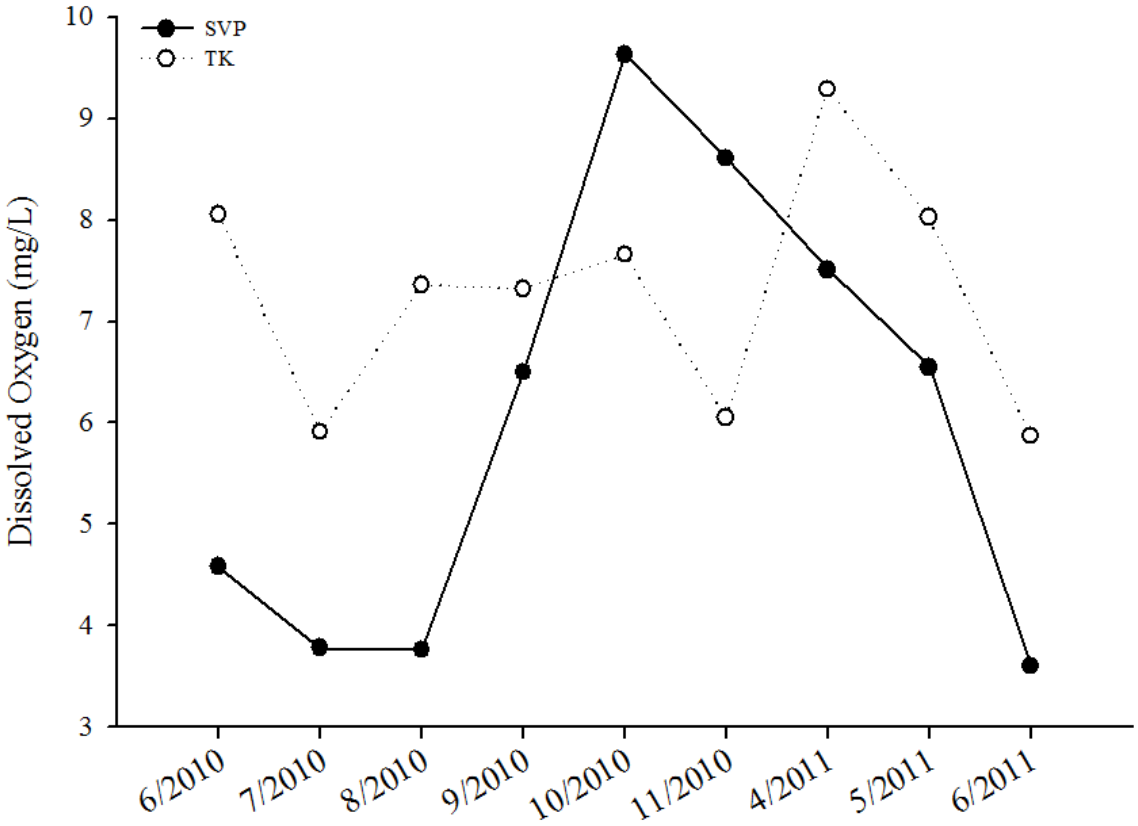

Figure 3: Dissolved oxygen (mg L^{-1}) measured at SVP and TK from 6/14/10-6/18/11. Filled symbols denote SVP, open symbols denote TK. Dissolved oxygen was significantly different between sites (ANOVA, $p<0.05$).

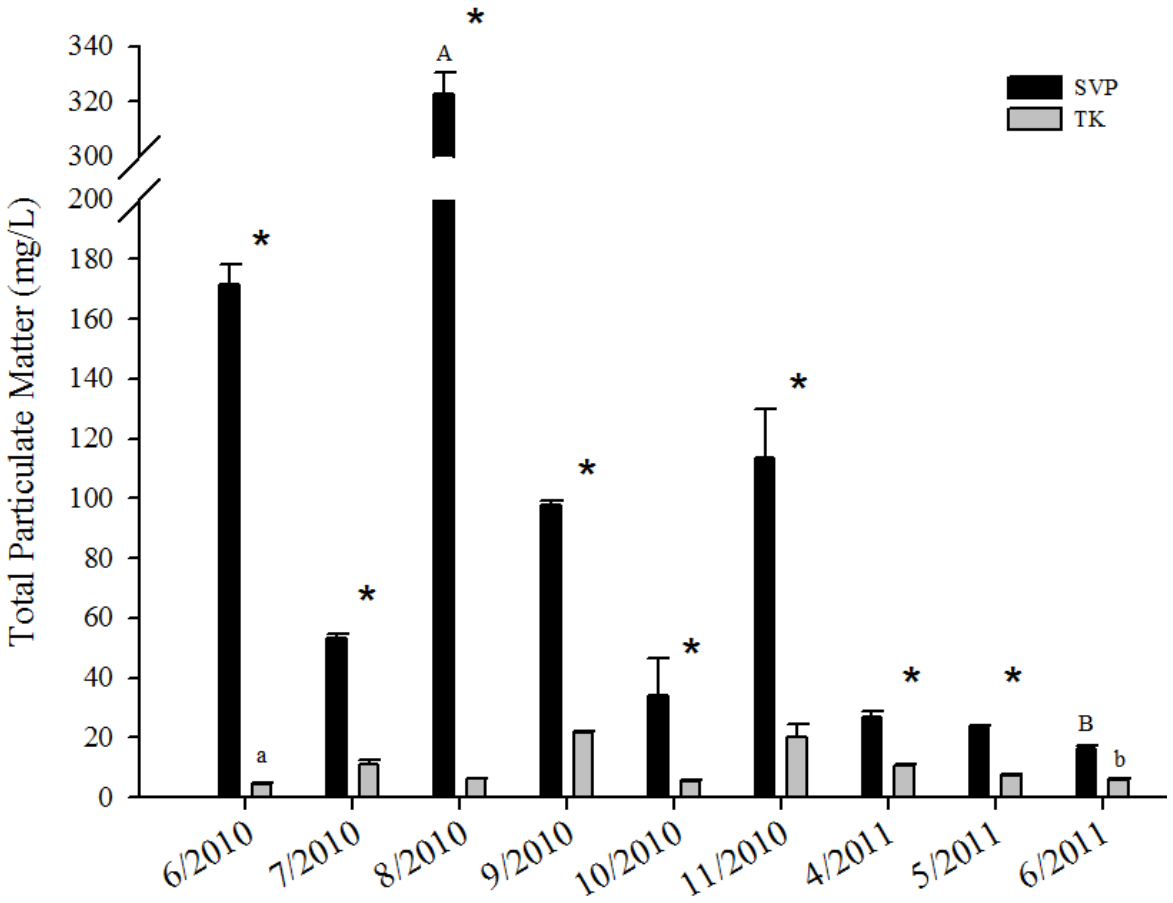

Figure 4: Total particulate matter (mg/L) measured at SVP and TK from 6/14/10-6/18/11. Black bars denote SVP, grey bars denote TK. Upper case letters indicate a significant difference between time points at SVP (Kruskal-Wallis, $p<0.01$); lower case letters indicate a significant difference between time points at TK (Kruskal-Wallis, $p<0.01$). Asterisk (*) indicates a significant difference between SVP and TK (Friedman's ANOVA, $p<0.05$).

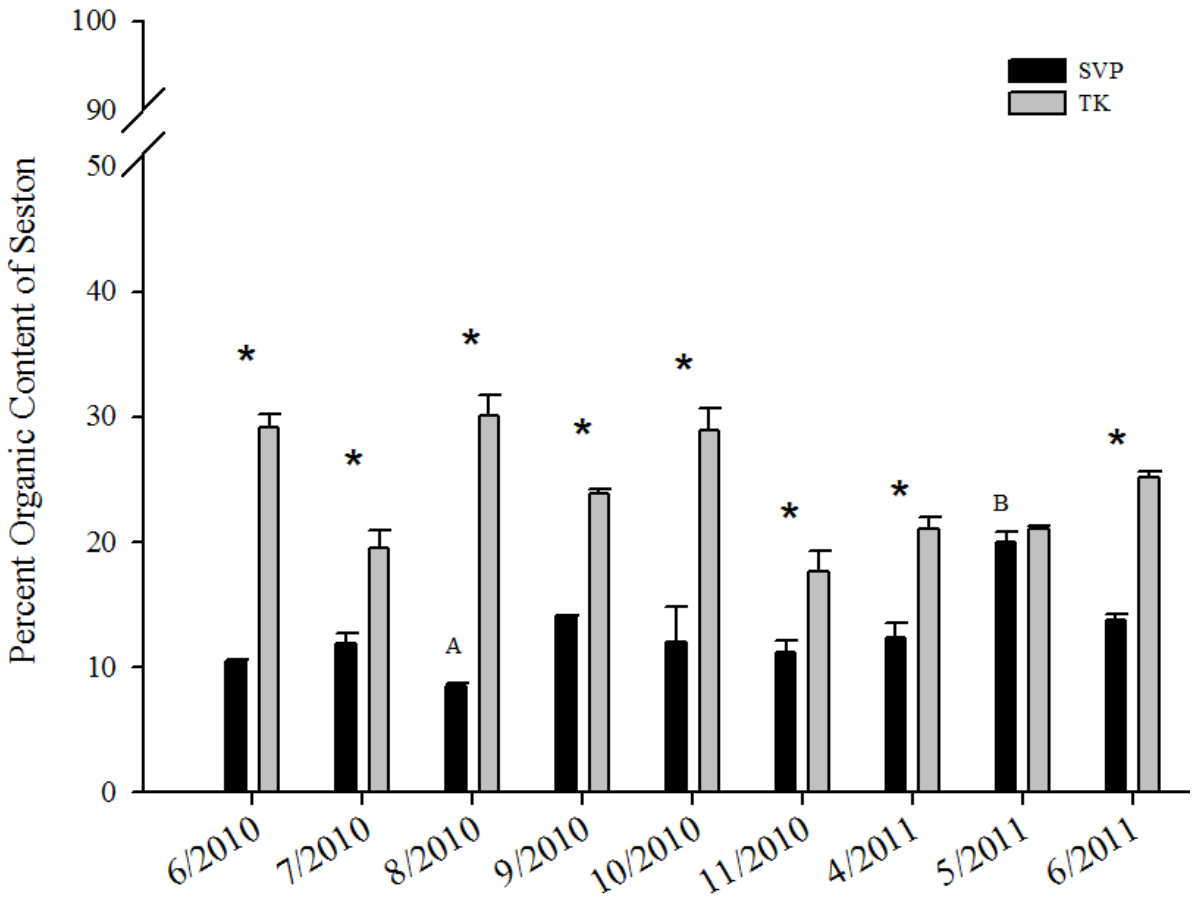

Figure 5: Organic content (percentage) measured at SVP and TK from 6/14/10-6/18/11. Black bars denote SVP, grey bars denote TK. Upper case letters indicate a significant difference between time points at SVP (Kruskal-Wallis, $p<0.05$). Asterisk (*) indicates a significant difference between TPM at SVP and TK (Friedman's ANOVA, $p<0.05$).

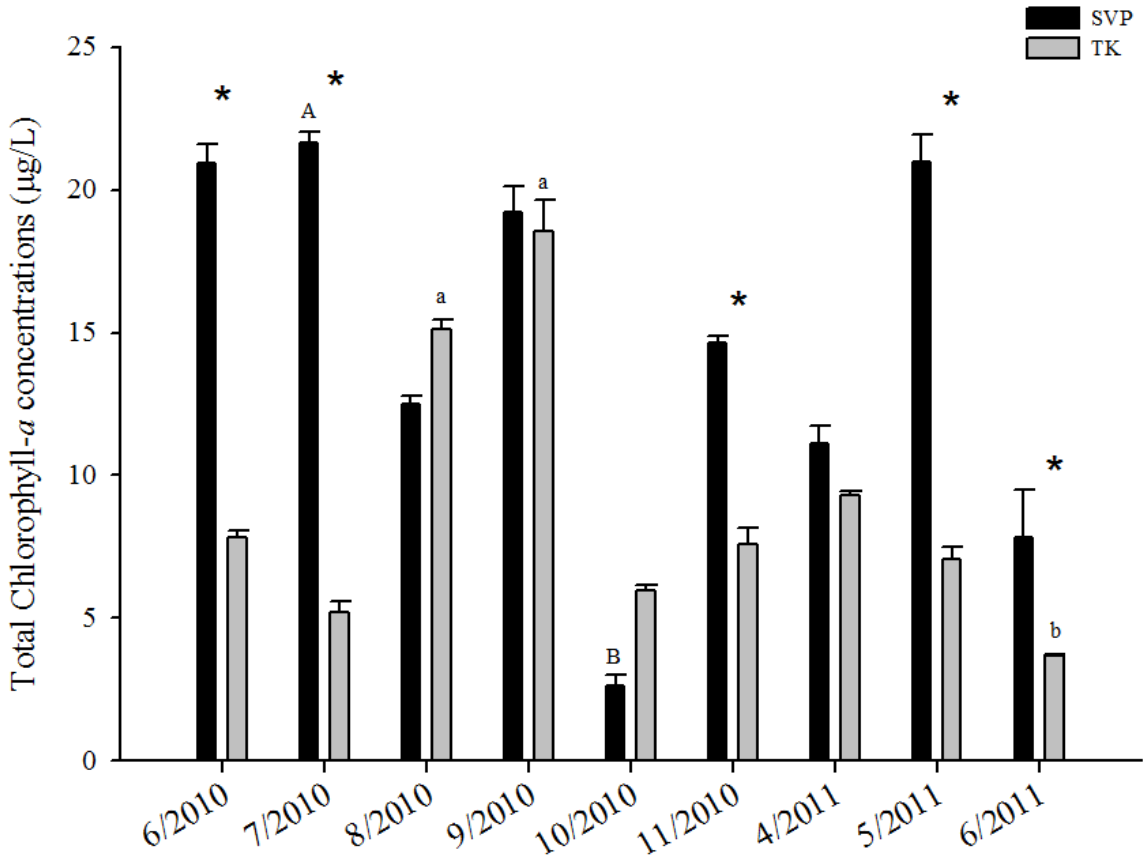

Figure 6: Chlorophyll-*a* concentration (µg L^{-1}) measured at SVP and TK from 6/14/10-6/18/11. Black bars denote SVP, grey bars denote TK. Upper case letters indicate a significant difference between time points at SVP (Kruskal-Wallis, $p<0.05$). Lower case letters indicate a significant difference between time points at TK (Kruskal-Wallis, $p<0.01$). Asterisk (*) indicates these dates (June, July, November 2010; May, June 2011) showed a significant difference between SVP and TK chlorophyll-*a* concentrations.

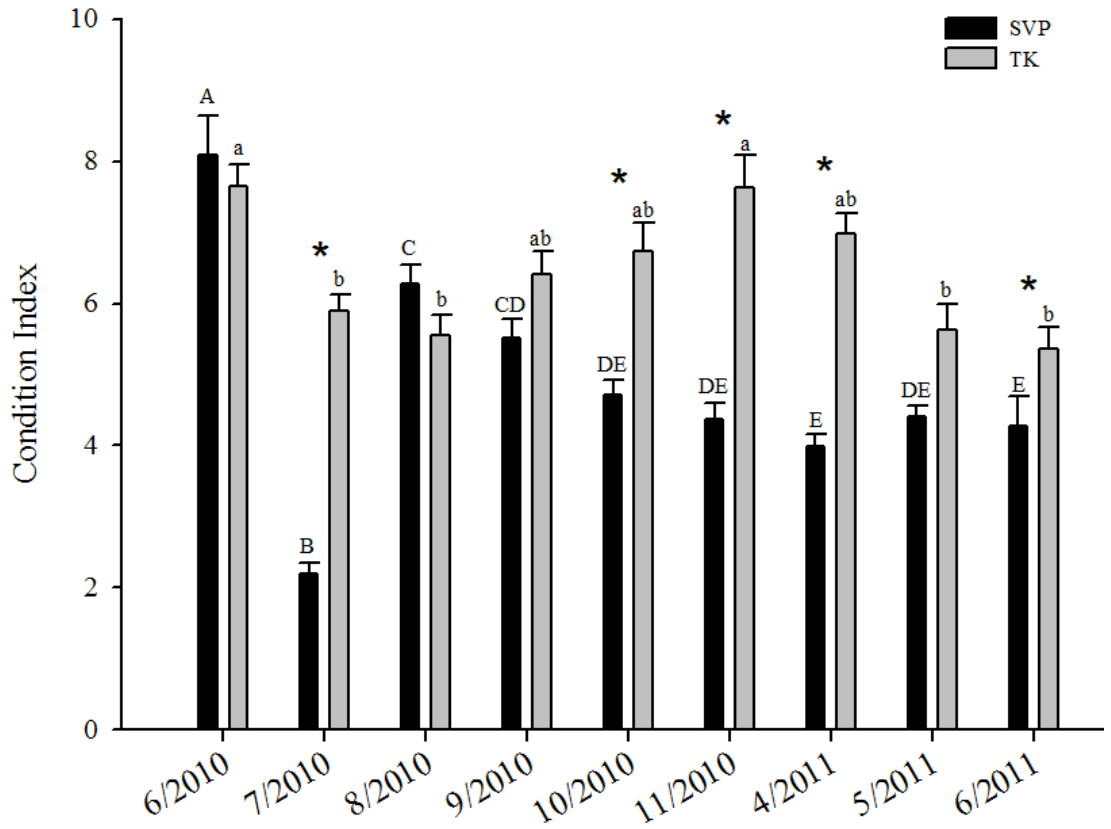

Figure 7: Condition index of oysters placed at SVP and TK, from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (ANOVA, $p<0.0001$, Tukey Post Hoc test). Lower case letters indicate significant changes through time at TK (ANOVA, $p<0.0001$, Tukey Post Hoc test). Asterisk (*) indicates these dates (July, October, November 2010; June 2011) had oysters with significantly higher condition indices at TK than SVP (ANOVA, $p<0.0001$).

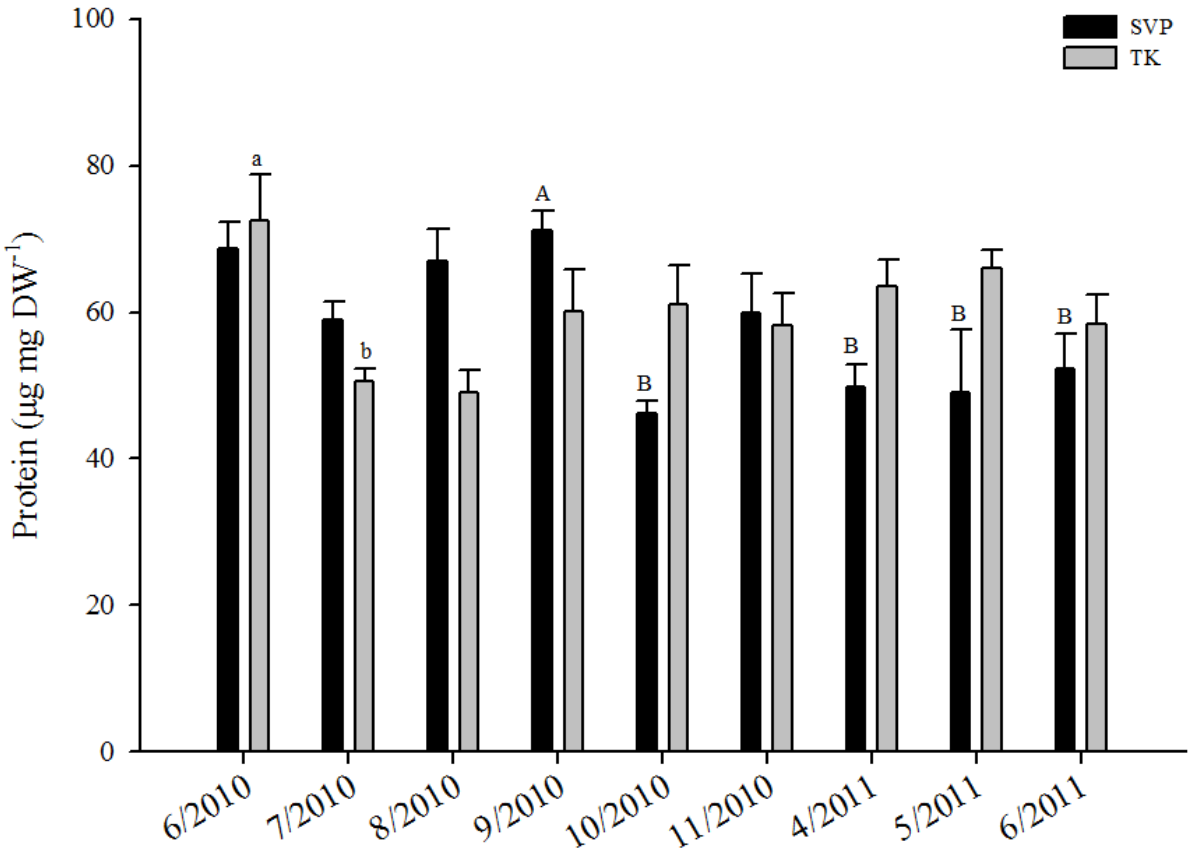

Figure 8: Protein (µg mg DW^{-1}) of oyster digestive glands. Oysters were transplanted to SVP and TK, from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (ANOVA, $p<0.0001$, Tukey Post Hoc test). Lower case letters indicate significant changes through time at TK (ANOVA, $p<0.0001$, Tukey Post Hoc test). There was no difference between sites at any time point (ANOVA, $p>0.05$).

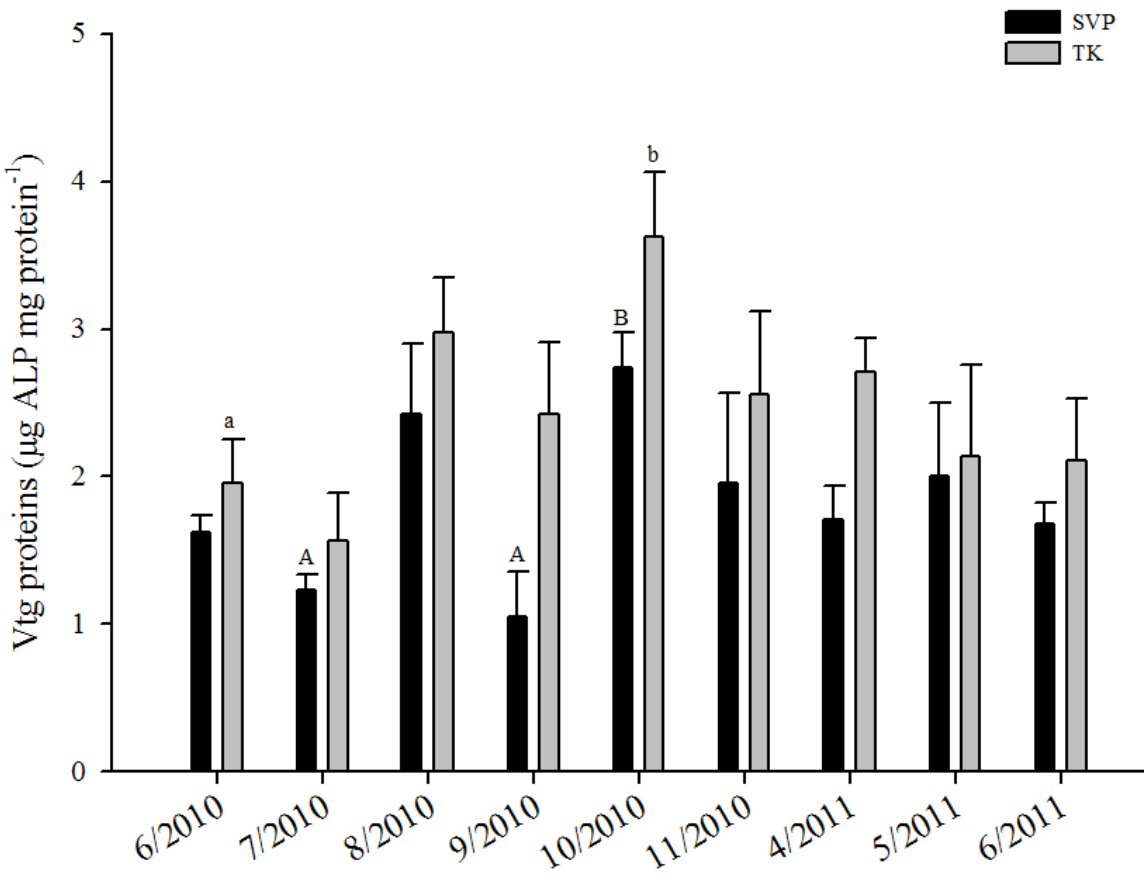

Figure 9: Vtg protein (µg ALP mg protein^{-1}) of oyster digestive glands. Oysters were transplanted to SVP and TK, from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (ANOVA, $p<0.01$, Tukey Post Hoc test). Lower case letters indicate significant changes through time at TK (ANOVA, $p<0.05$, Tukey Post Hoc test). There was no difference between sites at any time point (ANOVA, $p>0.05$).

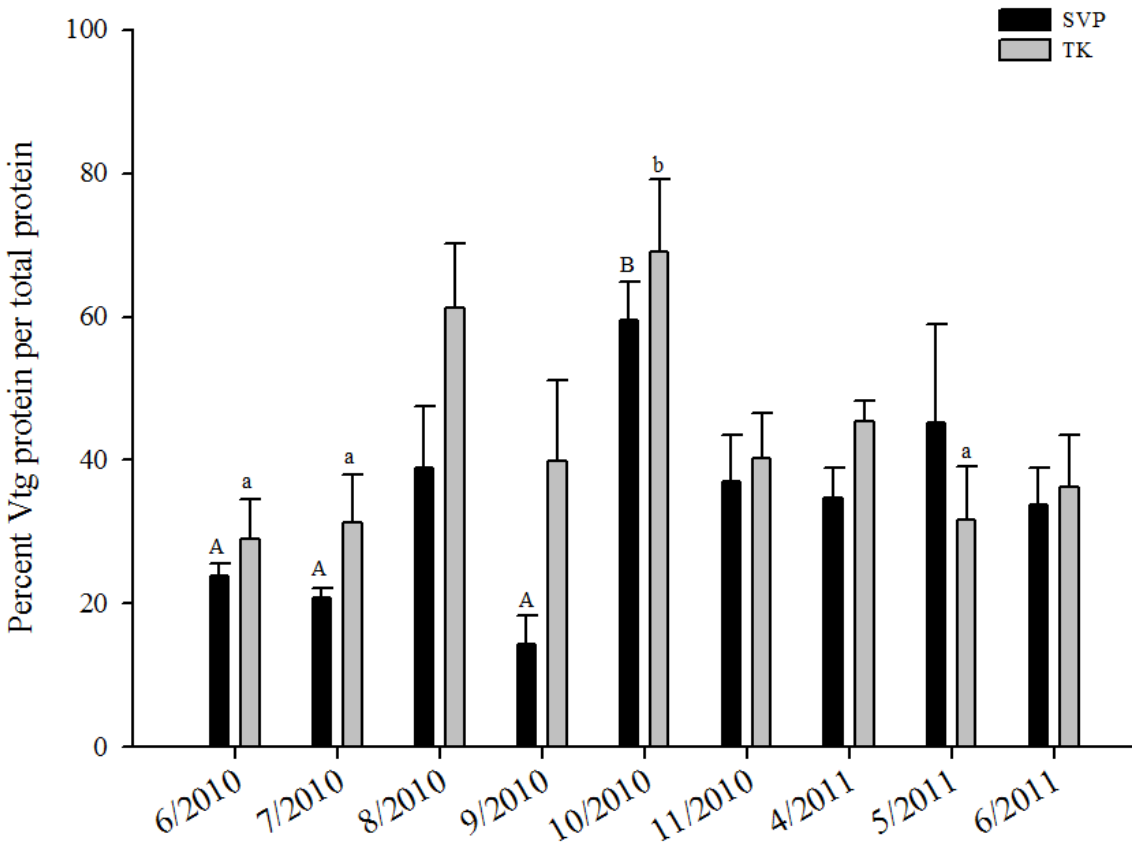

Figure 10: The percentage of total protein comprised of Vtg protein, examined in oysters placed at SVP and TK, from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (ANOVA, $p<0.001$, Tukey Post Hoc test). Lower case letters indicate significant changes through time at TK (ANOVA, $p<0.01$, Tukey Post Hoc test). There was no difference between sites at any time point (ANOVA, $p>0.05$).

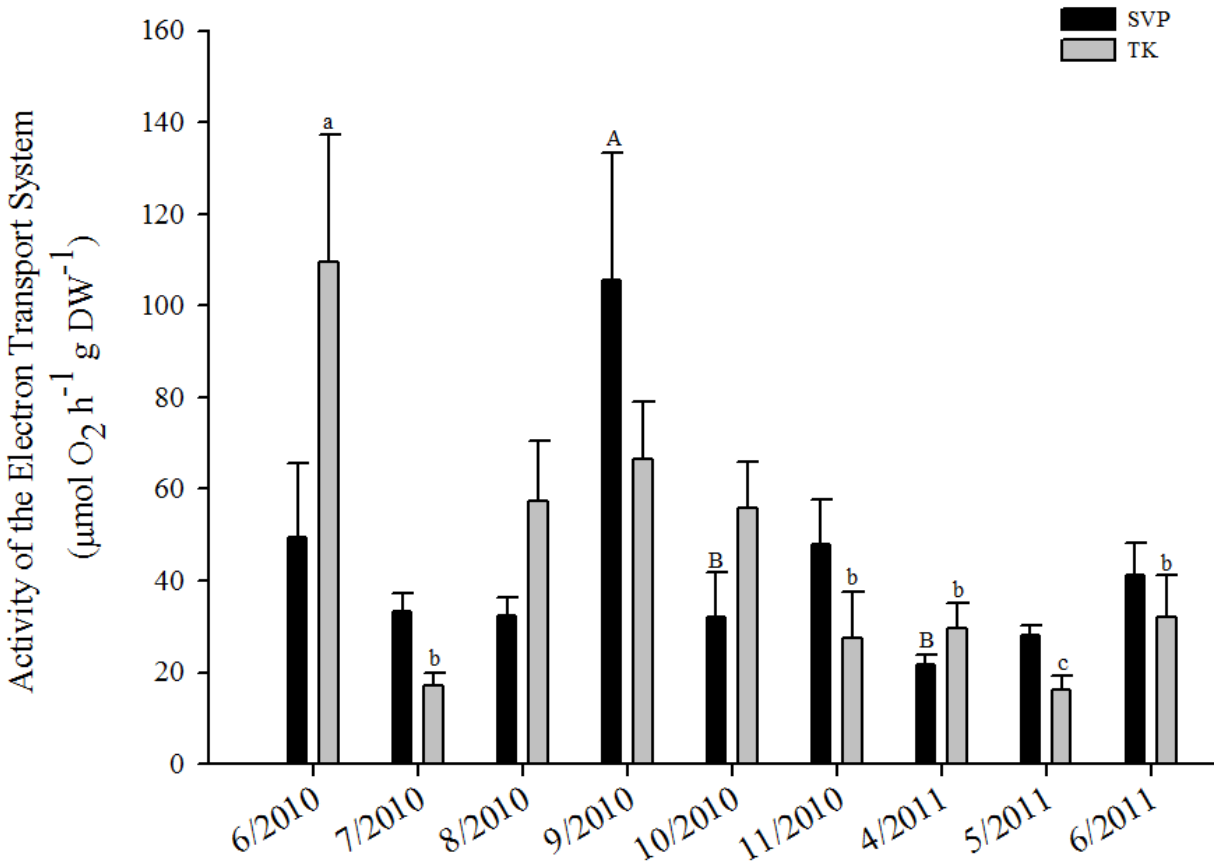

Figure 11: Aerobic potential (µmol O^2 h^{-1} g DW^{-1}) of oyster gill tissue. Oysters were transplanted to SVP and TK, from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (Kruskal-Wallis, $p<0.05$, Dunn's Post Hoc test). Lower case letters indicate significant changes through time at TK (ANOVA, $p<0.0001$, Tukey Post Hoc test). There was no difference between sites at any time point (ANOVA, $p>0.05$).

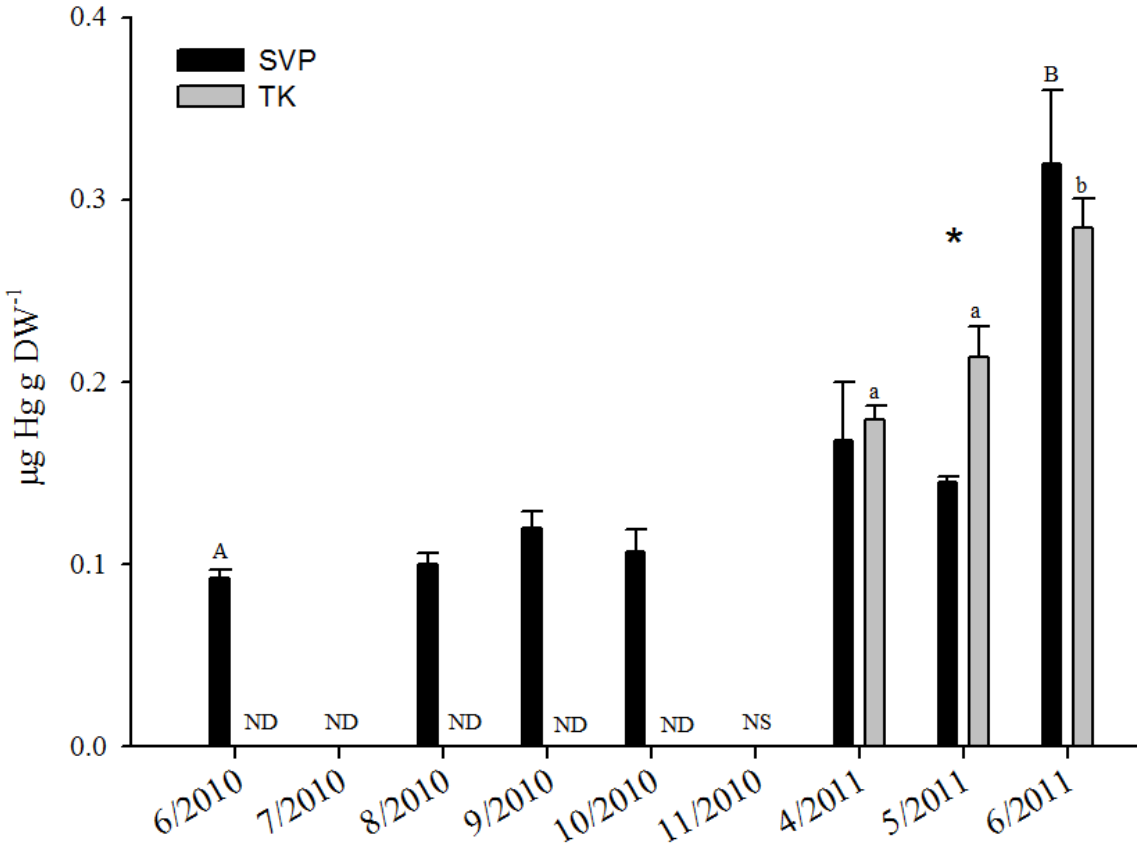

Figure 12: Hg accumulation within digestive gland tissues of adult *C. virginica* placed at SVP or TK from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (Kruskal-Wallis, $p<0.01$, Dunn's Post Hoc Test). Lower case letters indicate significant changes through time at TK (ANOVA, $p<0.01$, Tukey Post Hoc test). Asterisk (*) indicates a significant difference between SVP and TK in May 2011 (ANOVA, $p<0.05$). ND= no detection; samples from SVP (July 2010) and TK (June-October 2010) were below the detection limit of the machine (0.25 µg/L Hg). NS= no samples (November 2010).

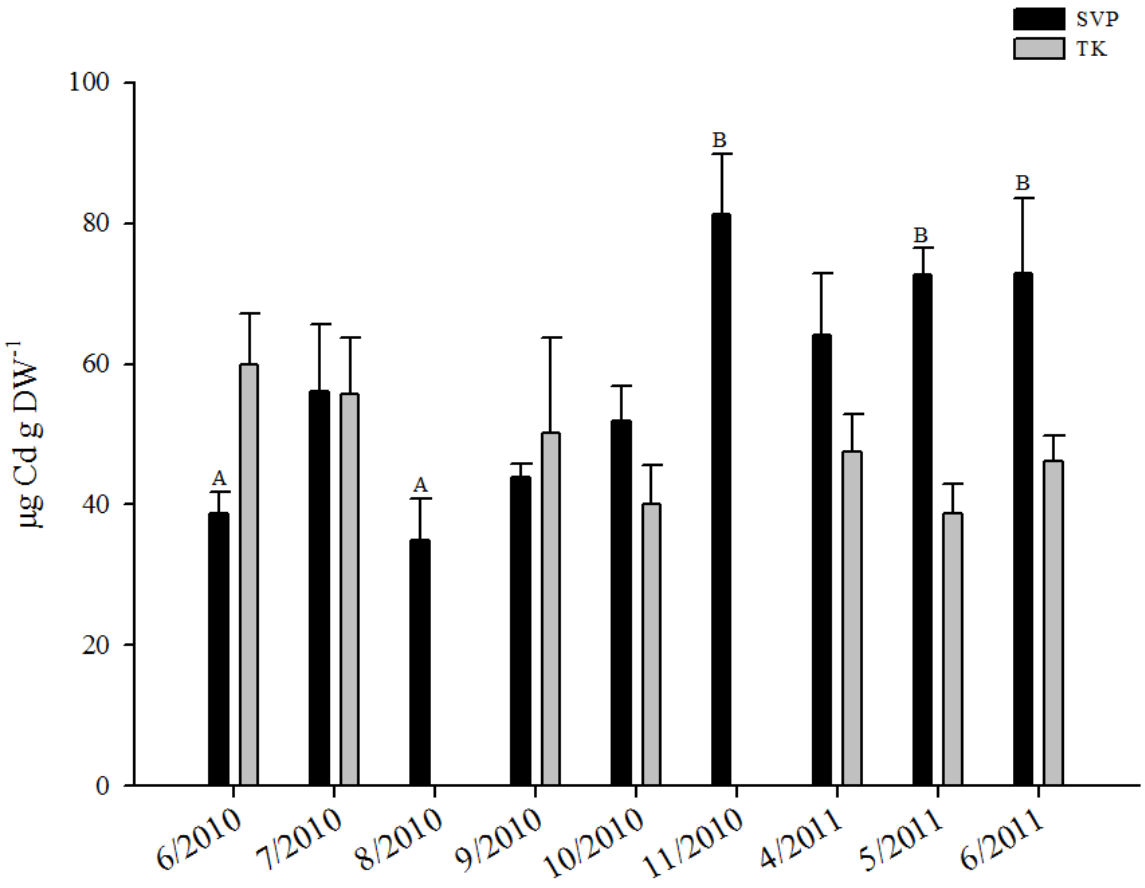

Figure 13: Cd accumulation within digestive gland tissues of adult *C. virginica* placed at SVP or TK from 6/14/10-6/18/11. Black bars denote SVP; grey bars denote TK. Upper case letters indicate significant changes through time at SVP (ANOVA, $p<0.001$, Tukey Post Hoc Test). There was no significant change in Cd accumulation over time at TK (ANOVA, $p>0.05$). NS= no samples (TK; August and November, 2010).

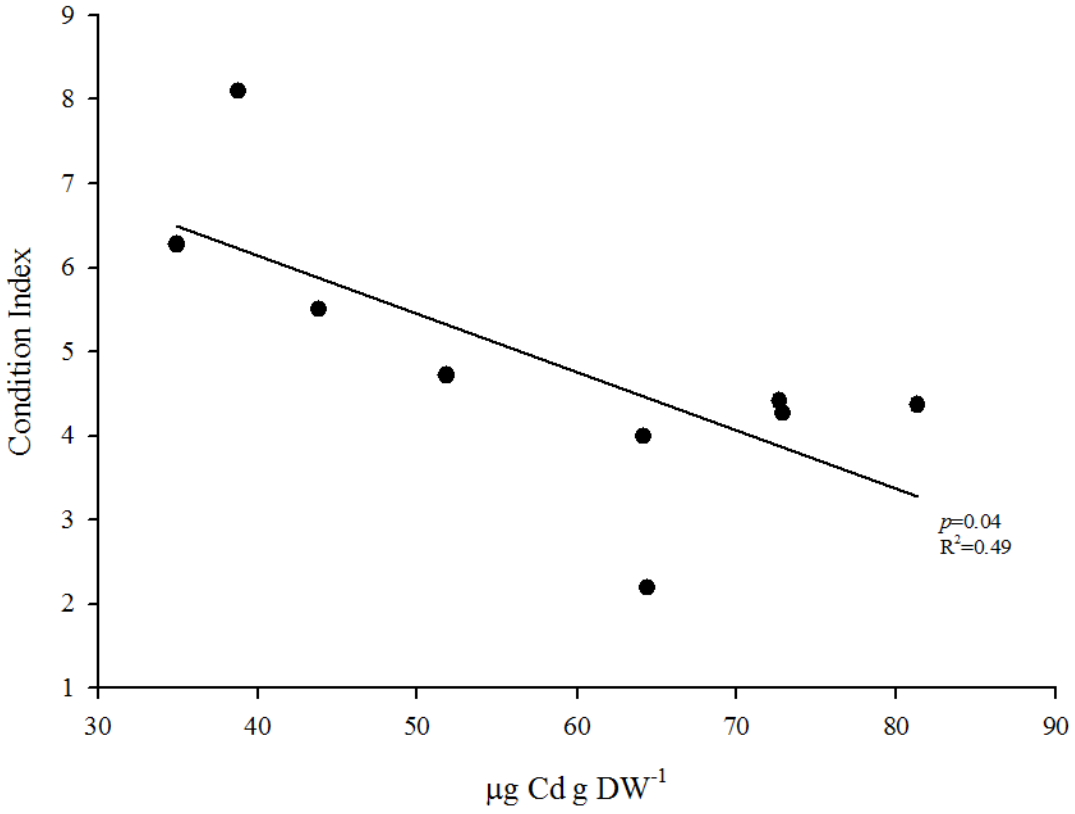

Figure 14: Negative relationship between condition index and Cd burdens in digestive gland tissue of adult *C. virginica* placed at SVP from 6/14/10-6/18/11. Multiple regression, p=0.04, R^2=0.49.

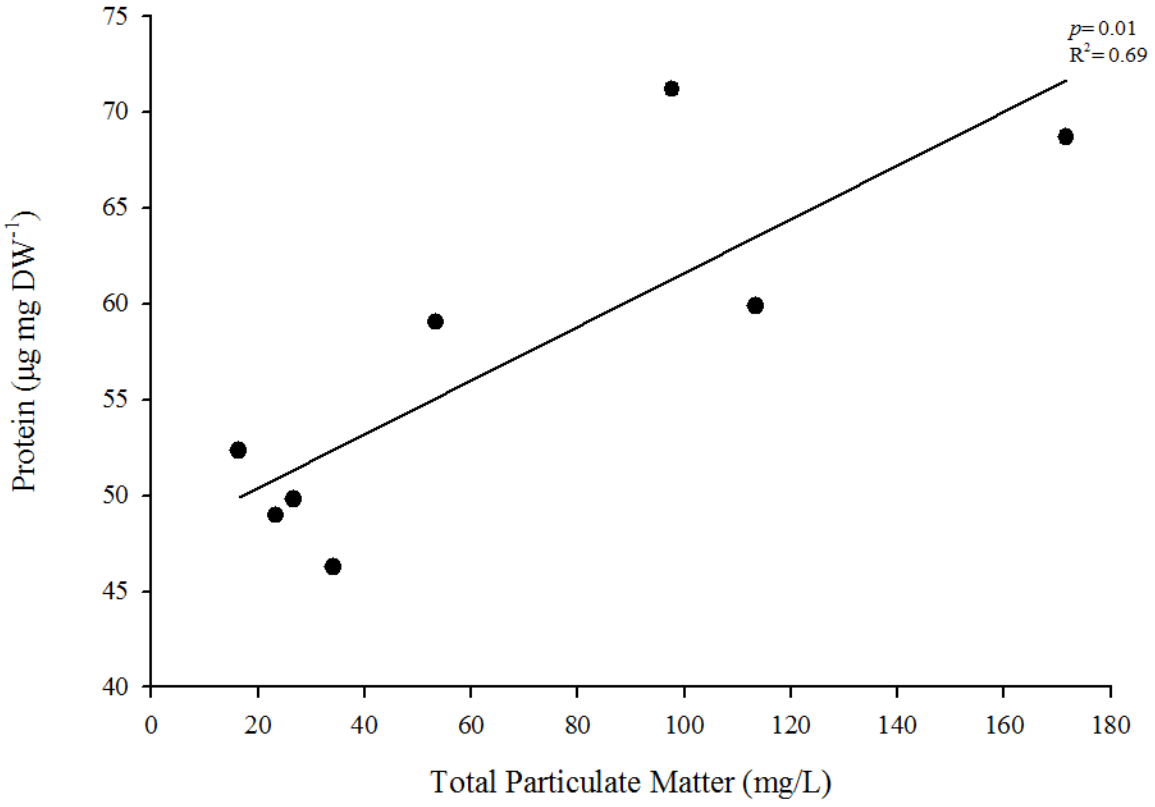

Figure 15: Positive relationship of protein content (in digestive gland) of adult *C. virginica* placed at SVP, to the total particulate matter suspended in the water column (from 6/14/10-6/18/11). Multiple regression, p=0.01, R^2=0.69.

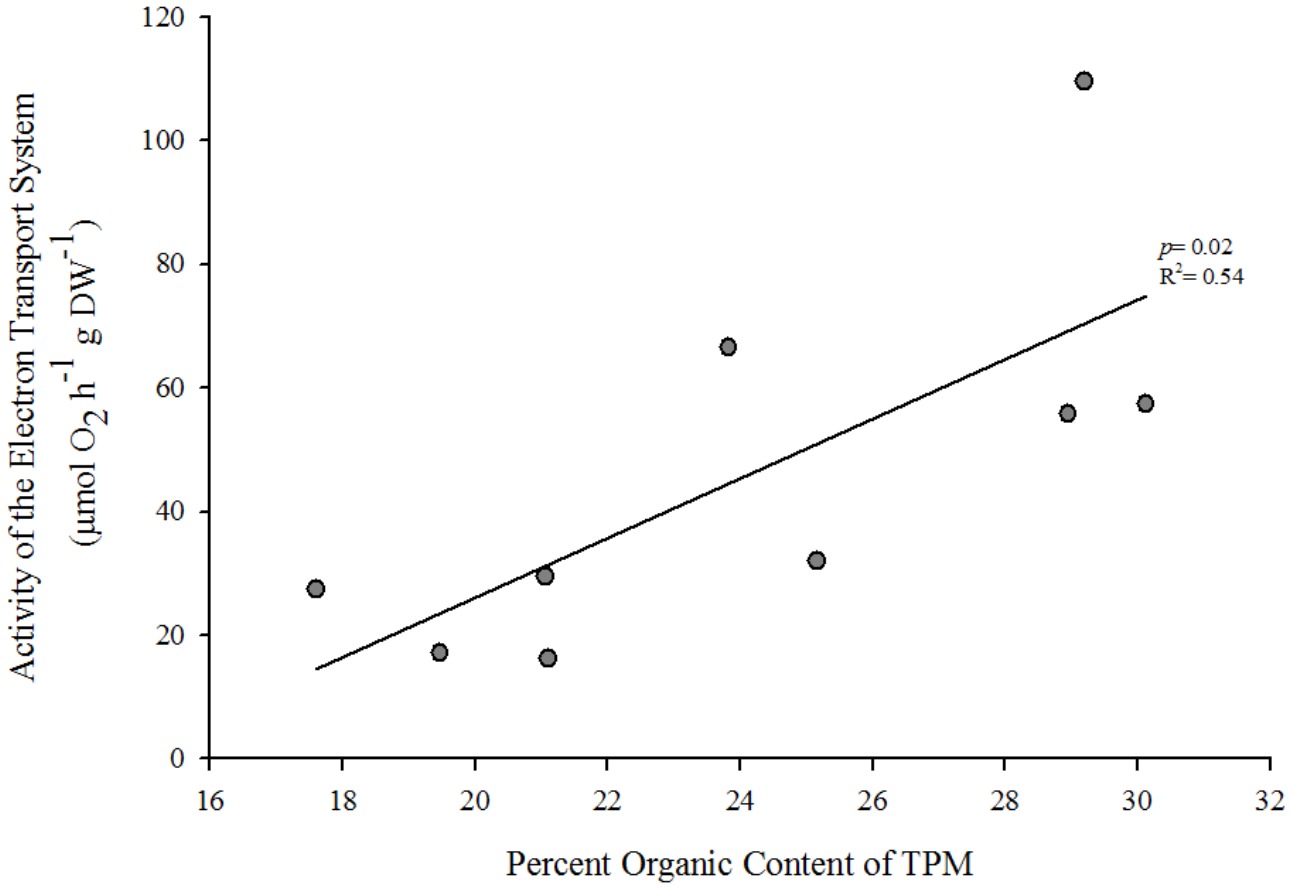

Figure 16: Positive relationship of aerobic potential (ETS activity) of adult *C. virginica* placed at TK, to the organic content found in particulate matter suspended in the water column (from 6/14/10-6/18/11). Multiple regression, p=0.02, R^2=0.54.

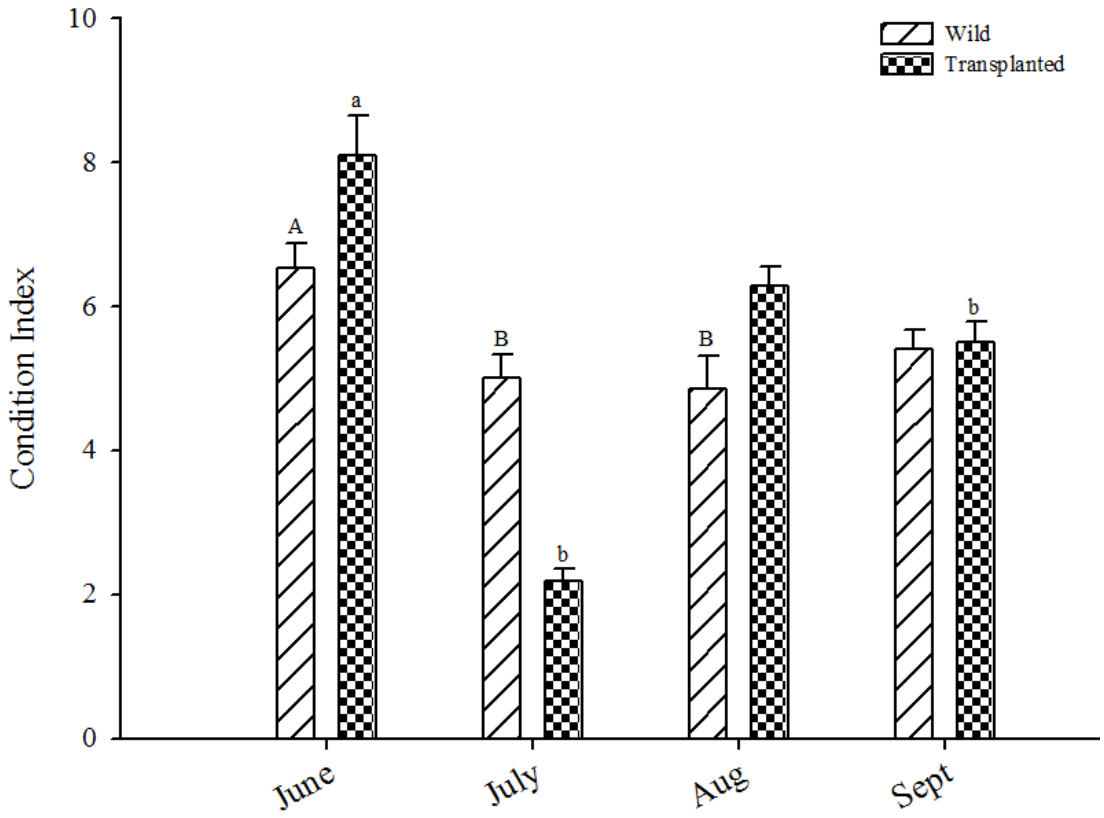

Figure 17: Condition index of wild *C. virginica* collected at SVP and hatchery-reared *C. virginica* transplanted to SVP. Hatched bars denote wild oysters; checkered bars denote transplanted (hatchery) oysters. Upper case letters indicate significant differences between dates of wild oysters (ANOVA, $p<0.01$, Tukey's Post Hoc Test). Lower case letters indicate a significant difference between dates of transplanted oysters (ANOVA $p<0.01$, Tukey's Post Hoc Test). There were no differences between wild and transplanted oysters at any date (ANOVA, $p>0.05$).

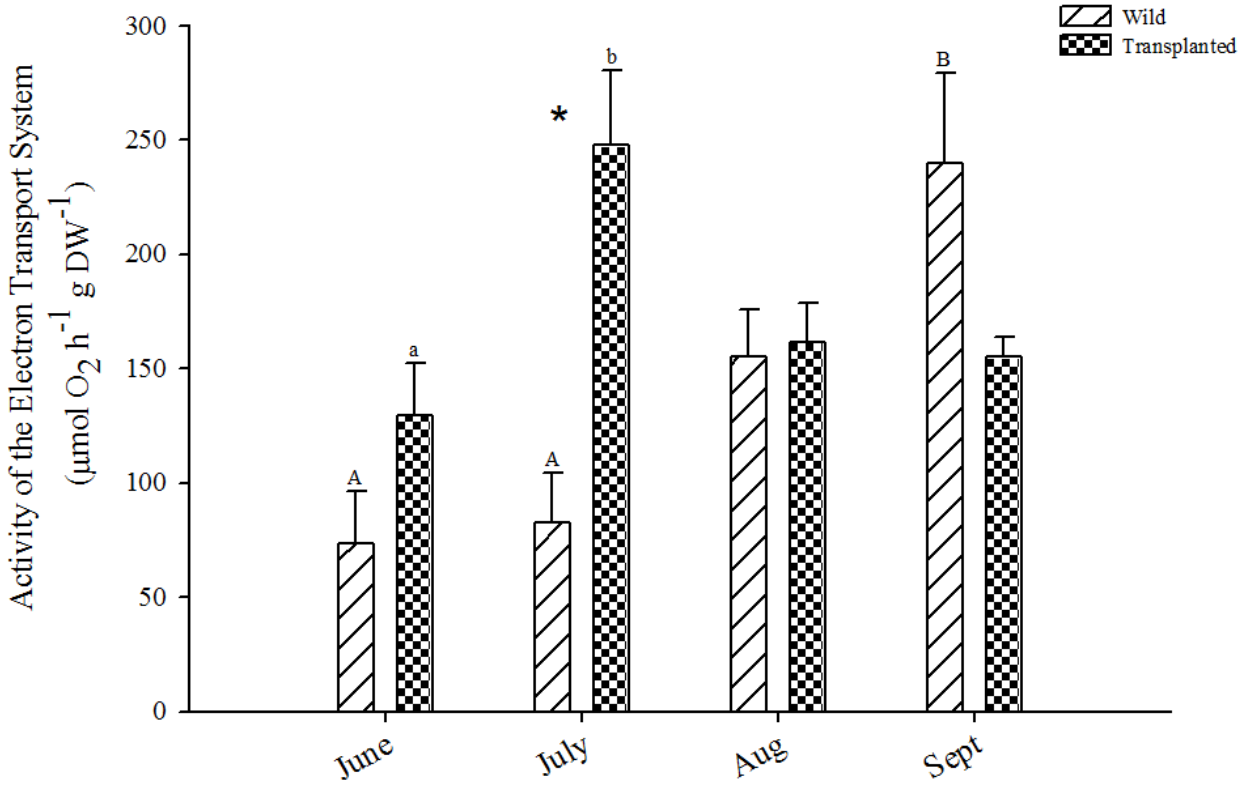

Figure 18: Aerobic potential of wild *C. virginica* collected at SVP and hatchery-reared *C. virginica* transplanted to SVP, as measured by the electron transport system assay (µmol O_2 h^{-1} g DW^{-1}). Hatched bars denote wild oysters; checkered bars denote transplanted (hatchery) oysters. Upper case letters indicate significant differences between oxygen usage of wild oysters (ANOVA, $p<0.0001$, Tukey Post Hoc Test). Lower case letters indicate significant differences between oxygen usage of transplanted oysters (ANOVA, $p<0.02$, Tukey's Post Hoc Test). Asterisk (*) indicates a significant difference between wild and transplanted oysters at this date (ANOVA, $p<0.0001$).

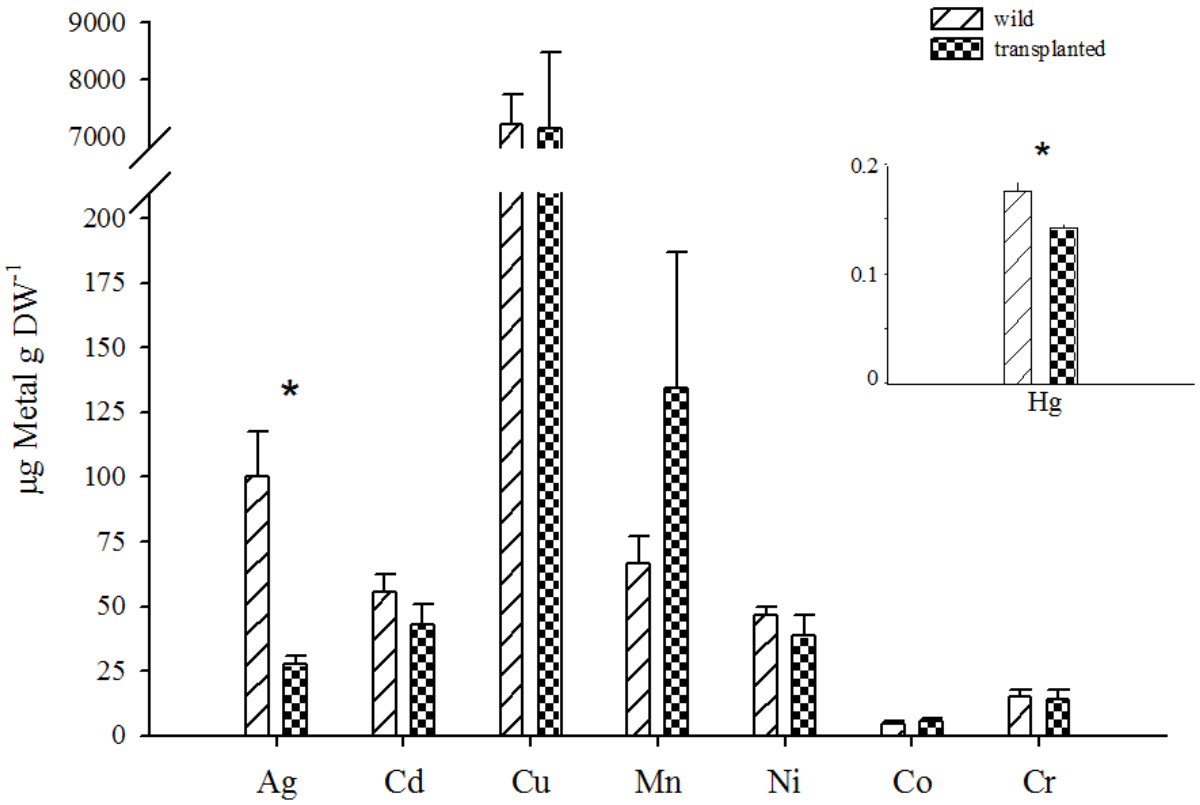

Figure 19: Metal accumulations (µg metal per gram tissue dry weight) of wild C. virginica collected at SVP and hatchery-reared *C. virginica* transplanted to SVP. Hatched bars denote wild oysters; checkered bars denote transplanted (hatchery) oysters. Asterisk (*) indicates a significant difference between wild and transplanted oysters for this metal (Student's *t*-test, $p<0.05$). Inset graph of Hg is included as the y-axis scale is lower (less than 0.5 µg). Y-axis shows a break between 200-7000 µg.

CHAPTER 4

EXAMINING THE RELATIONSHIP BETWEEN METAL EXPOSURE (Cd, Hg), SUBCELLULAR METAL ACCUMULATION, AND PHYSIOLOGY OF JUVENILE *CRASSOSTREA VIRGINICA*

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 (Lannig et al., 2006; Oliver et al., 2003). Impacts on suspension feeding bivalves (such as the eastern oyster, *Crassostrea virginica*) are of particular concern, as they 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. Through feeding and biodeposition (feces and pseudofeces production), oysters' link together dissolved and particulate metal sources, effectively integrating bioavailable metals in the water column and surface sediments (Guo et al., 2002; Ringwood et al., 1999; Shulkin et al., 2003). Dense populations of oysters (such as those that historically occurred in the HRE) have the ability to control phytoplankton assemblages, nutrients, and suspended matter within an estuary (zu Ermgassen et al., 2013).

In past centuries, *C. virginica* was a key species in the HRE abundant throughout lower New York Harbor, Jamaica Bay, Staten Island, and Raritan Bay (Franz, 1982), but urbanization of the area via contaminant inputs (e.g., metals, organics) and habitat destruction (e.g., filling, bulkheading, dredging) has led to the loss of this ecological engineer. Studies of the potential restoration of *C. virginica* have been underway at various sites within the HRE, and have shown varying degrees of survival and growth, indicating that survivorship is site-specific within the HRE (Levinton et al., 2011; Starke et al., 2011; Zarnoch and Schreibman, 2012). Contaminant

inputs into the lower HRE, along with previous habitat destruction, may increase the difficulty of reestablishing a self-sustaining population of oysters. Significant sewage effluents add nutrients to the waters, increasing phytoplankton whose subsequent senescence and decomposition decrease the amount of dissolved oxygen available in the benthos (Brosnan et al., 2006). 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 (Gèret et al., 2002; Volety, 2008). The HRE is a contaminated body of water with elevated levels of many essential and non-essential metals (Balcom et al., 2008; Feng et al., 1998; Kimbrough et al., 2010). Both cadmium (Cd) and mercury (Hg), non-essential trace metals present in the HRE, can lead to toxicity within oysters and other bivalves (Engel, 1999; Gagnaire et al., 2004; Sokolova et al., 2005). Accumulation of these metals has been shown to affect metabolic activity, reproduction, growth and survival. In addition they may alter in cellular responses (i.e., lysosomal destabilization) in oysters (Cherkasov et al., 2007; Ringwood et al., 1998; Roesijadi, 1996).

Oysters have the ability to bioconcentrate metals up to several orders of magnitude higher than ambient concentrations, prior to experiencing any toxicity affects (Mouneyrac et al., 1998; O'Connor, 2002). Accumulated metals may be sequestered in a variety of operationally defined subcellular fractions, including insoluble metal-rich granules (INS), heat-stable proteins (HSP) such as metallothioneins, cellular organelles (ORG), heat-denatured proteins (HDP) such as enzymes, and cell membranes and other cell debris (CD) (Wallace et al., 2003). The metal is either stored and detoxified within the fraction, or possibly passed on to a predator once the

oyster is consumed (Wallace et al., 2003). These fractions can be combined into subcellular compartments to further examine the role of subcellular metal accumulation in physiology, as biologically-detoxified metals (BDM), which are stored in INS and HSP; or adhered to the metal-sensitive fraction (MSF), which consists of ORG and HDP (Wallace et al., 2003). At low concentrations, these metals may be stored or eliminated; at higher concentrations the metals may be bound to metallothionein-like proteins or granular hemocytes thus rendering the metal biologically inactive. For example, several metals (including Cd and Hg) have been shown to bind to metallothioneins and other related proteins (HSP) causing an increase of metal bound within the BDM compartment of bivalves *Macoma balthica* and *Potamocorbula amurensis*, without toxicity. When toxicity has been observed, it corresponded with an increase of Cd bound to the MSF compartment (organelles and enzymes; Wallace et al., 2003). Once these detoxification mechanisms are overtaxed, the excess metal will bind to biologically active areas (i.e., organelles) and cause toxic effects. Using a subcellular compartmentalization approach to analyzing metal accumulation provides insight into how subcellular binding influences physiology, and how changes in subcellular binding may lead to toxicity or detoxification (Goto and Wallace, 2007).

Cadmium has been shown to alter growth rates, reproductive output, enzyme activity (related to cellular respiration), and mitochondrial energetics within oyster tissues (Macey et al., 2010; Ringwood et al., 2004; Sanni et al., 2008; Sokolova et al., 2005; Volety, 2008). Additionally, exposure to elevated levels of Cd may cause increased lysosomal destabilization and lipid peroxidation within tissues of the gills and digestive gland, and decreased levels of glutathione, which all may lead to toxicity in oysters (Gèret et al., 2002; Ringwood et al., 1999; Roesijadi and

Klerks, 1989). Preferential binding of Cd to subcellular components in oyster tissue may lead to alterations in metabolism and manifest as physiological changes (i.e., reduced reproductive output, lower overall condition, changes in filtration rates; Cherkasov et al., 2009; Cooper et al., 2010; Ivanina et al., 2010; Mass Fitzgerald, 2013). The amount of energy reserves within the oyster, reproductive condition, and environmental parameters (e.g., food, temperature, salinity) can affect the amount of metallothioneins present within the oyster, and thus the amount of metal that can be successfully sequestered (Amaird et al., 2006; Erk et al., 2008).

Research on the effects of Hg exposure on oysters and other bivalves is limited. It is known that oysters are able to accumulate Hg within tissues (Engel, 1999; Kawaguchi et al., 1999), but studies on physiological effects of Hg accumulation are scarce. Gagnaire et al. (2004) removed hemocytes from adult *C. gigas* and exposed the cellular suspensions to varying concentrations of Hg, which led to mortality of hemocyte cells and alterations of cellular function (i.e., enzymatic immunological functions), indicating that immunological mechanisms may be at risk in polluted estuaries. Exposure to elevated concentrations of Hg causes inhibition of several enzymes in *Scobicularia plana*, an infaunal clam (Mazorra et al., 2002). Mercury has also been shown to reduce concentrations of glutathione (which aids in the detoxification process) and GPx (glutathione peroxidase) activity within mitochondria in the scallop *Chlamys farreri* (Zhang et al., 2010). Phagocytic activity in hemocytes of *Mya arenaria* was shown to decrease over time when exposed to high concentrations of Hg (Fournier et al., 2001). Filtration rate of the mussel *Perna perna* was found to be inhibited by Hg exposure, due to breakdown of the front cilia bands of the gills (Anandraj et al., 2002; Gregory et al., 2002).

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 the environmental variables controlling oyster physiological responses. We measured condition index, energy storage (amount of proteins), and energy usage [via the electron transport system (ETS) assay] in relation to the total body burden and subcellular partitioning of Cd or Hg. It was hypothesized that oysters exposed to elevated ranges of Cd or Hg may exhibit impaired health as determined by lowered overall condition indices and protein storage, and increases in metabolic activity. By using this information, in conjunction with concurrent field exposures (Mass Fitzgerald, 2013), it will be possible to inform future restoration efforts within the urbanized HRE.

METHODS

Laboratory Exposures

Juvenile eastern oysters, *C. virginica,* were obtained from Aeros Cultured Oyster Company in Southold, NY. Oysters used for Cd exposures were obtained in June 2011 (22.9 ± 3mm shell length); a second group used for Hg exposures was obtained in July 2011 (40.6 ± 7.5mm shell length). Oysters were transported on ice back to the laboratory, placed into tanks with a salinity of 25 and temperature of 25^{o}C. Seawater was prepared with artificial sea salts (Reef Crystals®)

and deionized water. Oysters were acclimated to this temperature and salinity for 72 hours prior to being transferred into treatment containers.

For each metal (Cd and Hg), three treatment regimes were created (Control, Low, and High) in triplicate using 19L containers. The Low exposures were designed to mimic 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 container which was also supplied with gentle aeration. The nominal concentrations of Cd used were 0.6μM (Low-Cd treatment), and 2.4μM (High-Cd treatment). These concentrations are above the EPA guideline for maximum concentration Cd in marine systems (0.36μM; US EPA 1999, 2001). Nominal Hg concentrations used were 0.014μM (Low-Hg treatment) and 0.056μM (High-Hg treatment); the EPA guideline for maximum concentration of Hg in marine systems is 0.009μM (US EPA 1999, 2001). Preliminary experiments revealed that all concentrations used were sublethal and would not cause significant mortality within the exposure period.

Control treatments (Con-Cd and Con-Hg) contained no metal. For Low-Cd treatments, 1.08mL of 1000ppm $CdCl_2$ (in HNO_3) was added to each container, resulting in 0.6μM Cd concentration. For High-Cd treatments, 4.32mL of 1000ppm $CdCl_2$ (in HNO_3) was added to each container, resulting in 2.4μM Cd concentration. For Low-Hg treatments, 45μl of 1000ppm $HgCl_2$ (in HNO_3) was added to each container, resulting in 0.014μM concentration. For High-Hg treatments, 180μl of 1000ppm $HgCl_2$ (in HNO_3) was added to each container, resulting in 0.056μM concentration.

Every 48 hours, containers were emptied, refilled with fresh artificial seawater, and respiked with the appropriate concentration of metal. Oysters were fed daily with Instant Algae® commercial algal blend (2mL per container, moderately mixed in), added after the water was changed and metals added. The water was tested regularly to ensure that the metal remained within the desired range of concentrations during the 48 hours.

Each laboratory exposure was performed for four 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 container, and enumerated to determine mortality. Mortality was minimal in each container (< 10%). Subsamples of oysters were frozen on dry ice and stored for future analysis (-80^{o}C). Twenty oysters were sampled per container (n=3 per treatment) to determine condition index and protein content of tissue, ten oysters per container were sampled to determine the activity of the electron transport system; and four oysters were sampled per container for metal analysis.

Physiological Endpoints

Condition index was determined using the methods of Crosby and Gale (1990), using the total, dry shell and dry tissue weights (g). The percentage of protein in dried tissues 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 modified methods of Madon et al. (1998). Briefly, oysters 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

10,000*g* for 20 minutes 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: ETS μmol O_2 h^{-1} g DW^{-1}= $\{(E_{corr} * V_{hom} * 60/t * 1 * V_{rxn}) / (V_{inc} * DW * 31.8)\}$ 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 were shucked, weighed for wet weight (g), and homogenized with TRIS buffer (pH 7.6). A subsample ($1/6^{th}$ the total volume) was then removed to determine total body burden (TOT). Sequential centrifugation and heat treatment steps were used to separate the oyster tissue into operationally-defined fractions (INS, HSP, ORG, HDP, CD; Wallace et al., 2003; Figure 1). When determining Cd concentrations, all fractions (including TOT) were placed in a 60°C drying oven for a minimum of three days, digested with trace-metal grade nitric acid, resuspended in 2% nitric acid, and analyzed using Graphite-Furnace Atomic Absorption Spectrophotometry to estimate the amount of Cd present (Brown and Luoma, 1995). To determine Hg concentrations, all fractions were dried at 60°C for a minimum of three 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; Klajokic-Gaspic et al., 2006).

Statistics

Data were analyzed to determine the relationships between metal exposure, differential subcellular binding, and various physiological endpoints (overall condition, energy storage and usage). For all physiological parameters and metal concentrations are presented as treatment mean ± standard error. Where appropriate, data was transformed (either log-10 or arcsine transformations; Zar, 1999) for meeting assumptions of Nested ANOVA. Bonferroni Post-Hoc tests were used to identify differences between treatments. 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) were found to have any metal detectable. All analyses were completed on Statistica 7.1 (StatSoft Inc ®) and Sigma Plot 10 (Systat Software Inc ®).

RESULTS

Cadmium exposures

Physiological endpoints

Condition index of oysters prior to the exposures was 8.43 ± 0.23. After the four week exposure period, the condition index for the High-Cd oysters was significantly lower than the Low-Cd and Con-Cd treatments (ANOVA, $p<0.05$) (Figure 2). The condition index for oysters in the Con-Cd treatment was highest, at 6.17 ± 0.10. Oysters that were exposed to 0.6μM Cd for four weeks showed a slight decrease in overall condition (5.83 ± 0.17), while those exposed to 2.4μM Cd were lower with a condition index of 4.99 ± 0.30 (ANOVA, $p< 0.0001$). The Low-Cd treatment was 19.1% lower than the Con-Cd treatment; the High-Cd treatment was 26.8% lower than the Con-Cd treatment.

A significant difference between the protein content (μg mg DW^{-1}) of High-Cd oysters and those in the Low-Cd and Con-Cd treatments was observed after four weeks exposure (Kruskal-Wallis, $p< 0.01$). The average amount of protein in Con-Cd oysters was 511.92 ± 4.05 μg mg DW^{-1}, which decreased to 493.59 ± 7.42 μg mg DW^{-1} when oysters were exposed to 0.6μM Cd for four weeks. Oysters exposed to 2.4μM Cd had 461.15 ± 11.06 μg mg DW^{-1}, which was a decrease of 9.92% from Con-Cd oysters (Figure 3).

Oysters in the High-Cd treatment had a significant decrease in aerobic potential compared to the Con-Cd and Low-Cd treatments (Kruskal-Wallis, $p<0.05$). High-Cd oysters produced 103.11 ± 16.74 μmol O_2 h^{-1} g DW^{-1} compared to the Con-Cd treatments (201.16 ± 7.49 μmol O_2 h^{-1} g DW^{-}

^{1}), which is a 49% decrease from the control. Oysters exposed to 0.6μM Cd had slightly lowered energy usage from the control oysters (202.24 ± 54.84 μmol O_2 h^{-1} g DW^{-1}) (Figure 4).

Metal accumulation and subcellular fractionation

Measurements of total body burden of oysters exposed to 0.6μM Cd or 2.4μ M Cd for four weeks showed accumulated Cd within the cells, but the oysters did not accumulate Cd at a linear rate. The dose of Cd given to the High-Cd treatment was 4-fold higher than the Low-Cd treatment, but the total body burdens of Cd were only approximately two times higher in High-Cd oysters than Low-Cd oysters (29.54 ± 2.42 μg Cd g DW^{-1} versus 70.89 ± 6.87 μg Cd g DW^{-1}). There was no Cd detected in the Con-Cd treatments (Figure 5).

Significant differences between Low-Cd treatments and the High-Cd treatments in all fractions were observed after four weeks of exposure (Student's *t*-test; $p<0.05$) (Figure 5). When the body burdens of Cd within specific fractions of the cell were examined, the same trend was found. Approximately 2 times more Cd was found in the High-Cd treatment than the Low-Cd treatment, in the INS, ORG, HDP, and HSP fractions. Estimated subcellular burdens (μg Hg g DW^{-1}) for each fraction are listed in Table 1.

When these values were translated into percentages of the total body burden, the amount of Cd associated with the Cell Debris (CD) fraction, or "lost" during the sample processing could be estimated (Table 2). In opposition to the estimated body burdens, there were no significant differences between the percentage of total accumulated metal associated with any fraction in the Low-Cd and High-Cd treatments (Student's *t*-test, $p> 0.05$). In the Low-Cd treatment,

approximately 2.58 ± 0.33% Cd was associated with INS, 16.41 ± 2.36% Cd associated with HSP, 10.59 ± 1.19 % with HDP, and 10.5 ± 1 % with ORG. It was estimated that 63.12 ± 3.49% Cd was associated with the CD fraction or not recovered ("lost"). In the High-Cd treatment, 2.61 ± 0.33 % of Cd was associated with INS, 20.31 ± 0.95% Cd was associated with HSP, 8.36 ± 0.43% Cd was associated with HDP, and 10.71 ± 0.65% Cd was associated with ORG. This indicates that 60.83 ± 2.4% was associated with CD or lost.

The percentage of metal associated with MSF or BDM was calculated in each treatment following Wallace et al. (2003). There were no significant differences between the percentage of total Cd within each subcellular compartment (Student's *t*-test, $p>0.05$). In the Low-Cd treatment, 20.13 ± 2% of Cd was bound to MSF; in the High-Cd treatment, 18.37 ± 0.91% was bound to MSF. The Low-Cd treatment had 18.43 ± 2.49% of Cd associated with BDM, while the High-Cd treatment had 22.69 ± 0.87% (Table 2). However, there was a significant difference between the accumulations of Cd (μg) within each compartment (MSF/BDM) (Student's *t*-test, $p< 0.01$). There was a higher amount of Cd accumulated in MSF (12.53 ± 2 μg Cd g DW^{-1}) and BDM (14.24 ± 1.85 μg Cd g DW^{-1}) in High-Cd treatment than in Low-Cd treatments, where MSF was 5.65 ± 0.77 μg Cd g DW^{-1} and BDM was 6.30 ± 1.5 μg Cd g DW^{-1} (Table 1).

Mercury exposures

Physiological endpoints

The final condition indices for Con-Hg, Low-Hg, and High-Hg treatments were significantly lower than the initial condition index value (ANOVA; $p< 0.001$). The initial condition index for Hg oysters was 6.75 ± 0.34, and decrease of 31.4% was observed from the initial sample to the

final sample in the Con-Hg treatment. After four weeks in the respective Hg treatments, there was no difference in the condition indices between the Con, Low and High groups (ANOVA, $p > 0.05$). The mean condition index for all treatments was 5.14 ± 0.12 (Figure 6).

Oyster protein content was similar among treatments (ANOVA, $p > 0.05$). Con-Hg oysters had 484.02 ± 5.38 μg mg DW^{-1}. Oysters exposed to 0.014μM Hg had protein concentrations of 484.72 ± 4.69 μg mg DW^{-1}, while those exposed to 0.056μM Hg had 476.98 ± 9.93 μg mg DW^{-1} (Figure 7).

There were no significant differences in aerobic potential for oysters exposed to Con-Hg, Low-Hg or High-Hg treatments for four weeks (ANOVA, $p > 0.05$). The average metabolic rate of Con-Hg oysters was 85.6 ± 39.5, the Low-Hg treatment was 92.57 ± 18.3 μmol O_2 h^{-1} g DW^{-1}, and the High-Hg treatment was 106.59 ± 24.7 μmol O_2 h^{-1} g DW^{-1} (Figure 8).

Metal accumulation and subcellular fractionation

The High-Hg treatment oysters accumulated significantly more Hg than those in the Low-Hg treatment (Student's *t*-test, $p < 0.01$). There was no Hg detected in the Con-Hg treatments. The High-Hg exposure (0.056μM Hg) was 4-fold greater than the Low-Hg exposure (0.014μM Hg), but the total body burdens of oysters in High-Hg treatment were approximately nine times higher (14.75 ± 1.33 μg Hg g DW^{-1} for Low-Hg and 131.75 ± 27.94 μg Hg g DW^{-1} for High-Hg) suggesting they accumulated Hg at a rate above linear (Figure 9).

Accumulations within specific subcellular compartments varied between 1.5-5 times higher in High-Hg oysters than Low-Hg oysters. There was a significant difference between the Low-Hg treatment and the High-Hg treatment with respect to total body burdens (TOT), INS, ORG, and HDP (Student's *t*-test; $p < 0.0001$) (Figure 9). There was no significant difference with respect to HSP subcellular binding; however, many of the oysters had HSP levels below the detection limit. Estimated subcellular burdens (μg Hg g DW^{-1}) for each fraction are listed in Table 1.

Subcellular burdens were used to estimate the relative percentage of the total accumulated Hg bound to different compartments (Table 2). As with the estimated subcellular body burdens, there were significant differences between the percentages of Hg bound to INS, ORG, HDP and CD/loss fractions in the Low-Hg treatment and the High-Hg treatments (Student's *t*-test; $p < 0.01$). In the Low-Hg treatment, 3.73 ± 0.38% Hg was associated with INS, 17.71 ± 0.91 % Hg associated with ORG, 14 ± 1.87% Hg associated with HDP, and 2.01 ± 0.6% Hg associated with HSP. Therefore, 64.48 ± 2.64% Hg was either associated with the CD fraction or lost during the fractionation process. Within the High-Hg treatment, 1.90 ± 0.23% Hg was bound to INS, 0.36 ± 0.03% Hg was associated with HSP, 10.02 ± 1.02% Hg was bound to ORG, and 7.39 ± 1.19% Hg was associated with HDP. The remaining 80.39 ± 1.6% of Hg was bound to the CD fraction or lost.

The percentage of Hg sequestered within the MSF compartment was significantly higher in the Low-Hg treatment (31.71 ± 2.26%) than the High-Hg treatment (17.41 ± 1.46%; Student's *t*-test, $p<0.0001$). Additionally, there was significantly more Hg bound within the BDM compartment in the Low-Hg oysters (4.27 ± 0.62 %) than the High-Hg oysters (2.2 ± 0.23%; Student's *t*-test,

$p<0.01$) (Table 2). Using these percentages to calculate the estimated subcellular burdens (μg Hg g DW^{-1}) within each subcellular compartment, it was shown that significantly more Hg accumulated in the High-Hg oysters than the Low-Hg oysters (Student's *t*-test, $p<0.0001$). The Low-Hg treatment had 4.71 ± 0.57 μg Hg g DW^{-1} bound to MSF, and 0.66 ± 0.09 μg Hg g DW^{-1} bound to BDM. The High-Hg treatment had 19.61 ± 2.48 μg Hg g DW^{-1} bound to MSF, and 2.38 ± 0.26 μg Hg g DW^{-1} bound to BDM (Table 1).

DISCUSSION

Cadmium exposures

Exposure of juvenile *C. virginica* to elevated, but sub-lethal, concentrations of Cd (2.4μM) led to changes in physiology at different levels of biological organization, including whole body and subcellular. Oyster physiological condition significantly decreased in all treatments after four weeks of exposure, with a similar decline observed in subcellular physiology. However, oysters in the High-Cd treatment had significantly lower protein content and ETS activity than the control and the Low-Cd treatment suggesting that sublethal exposures of Cd can further decrease overall health status. Protein content was approximately 50.8 μg g DW^{-1} less in oysters exposed to 2.4μM Cd (a significant decrease of 9.9%) as compared to the control. A significant (49%) decrease in aerobic potential was also observed with oysters in High-Cd treatment relative to the Con-Cd oysters. Similar results were observed when Gėret et al. (2002) exposed *C. gigas* to 1.8μM for 21 days, and found significant accumulation within gills and digestive glands while exhibiting lowered protein content. Additionally, Sokolova (2004) saw reductions in

mitochondrial activity (increases in mitochondrial uncoupling and proton leak, inhibition of oxidative respiration) when exposing oysters to low levels (1μM) of Cd, and Ivanina et al. (2010) found significantly lower levels of important molecules (i.e., ATP) which aid in responses to oxidative stressors.

Accumulation of Cd by juvenile oysters was less than linear. The concentration difference between the Low-Cd and High-Cd treatments was 4-fold (0.6μM versus 2.4μM), the total body burdens of oysters in High-Cd treatments was 2-fold higher than the total body burdens of oysters in Low-Cd treatments (Figure 5). Several other studies of field and laboratory bivalves have indicated a linear relationship between Cd accumulation and exposure regimes (Markich et al., 2001; Perceval et al., 2006; Serra et al. 1995). Therefore, the accumulation rates below linear that were found in the present study could be a result of the stressors of the laboratory experiment on the oysters. The decline in condition index in the control treatment (Figure 2), suggests that the experimental setup and handling could have contributed to reduced feeding and subsequently less accumulation of metals. Alternately, the saturation of certain subcellular compartments and subcellular binding mechanisms (i.e., metallothionein-like proteins) can lead to a decline in the rate of uptake (Serra et al., 1995). While the concentrations chosen did not lead to significant mortality in the experiments, the sublethal concentration may have been high enough to lead to subcellular toxicity, resulting in a slower uptake rate of Cd. Declines in the amount of stored protein, and the observed energy expended by the oysters (ETS assay) indicate that the oysters were filtering below the optimal rate when exposed to 2.4μM Cd for four weeks (Figures 3, 4).

When examining the subcellular partitioning of Cd within oyster cells, it was observed that Cd did not partition differently in oysters exposed to High-Cd or Low-Cd treatments. In each treatment, the percentages bound to the MSF and BDM compartments did not significantly differ, nor did the percentage of Cd estimated in CD, or lost during processing. Oysters exposed to 2.4mM Cd accumulated approximately 18.4% in MSF and 22.7% in BDM, which leaves 58.9% that was either bound to CD or lost. Wallace et al. (2003) exposed *Potamocorbula amurensis* (similar size to the *C. virginica* used in the current study) to 0.032μM Cd for 14 days, and found 41.4% of Cd bound to the MSF fraction, 20.7% bound to BDM, and 37.9% bound to CD (or lost). The increase in the percentage of Cd bound to MSF was likely due to species' differences in physiological rates and tolerance to metal toxicity (Lee et al., 1998; Wallace et al., 2003). To our knowledge, this is the first study of juvenile *C. virginica* and subcellular fractionation of non-essential trace metals; however, when adult *C. virginica* were exposed to sublethal Cd concentrations (0.22μM), 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 hepatopancreas tissues (Sokolova et al., 2005). These results also suggest that Cd accumulation will strongly affect energy budgets (storage and usage).

Cadmium is known to partition primarily to the BDM subcellular compartment (HSP and INS) as exposure to Cd has caused the induction of metallothioneins in several species of bivalves (Wallace et al., 2003). 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 within cells (Cooper et al., 2010; Roesijadi & Robinson, 1994).

The four week exposure used in the current study may have been long enough to induce metallothionein production, as 20.9% of accumulated metal was partitioned to the HSP fraction (more than any other fraction).

The estimate of metal in CD gives us the percentage of Cd bound to both the debris fraction, which consists of tissue fragments, cell membranes, and other parts that do not fall into the other operationally-defined categories (Wallace et al., 2003). It also provides an estimate of the percentage of Cd that may have been lost during the processing. Loss typically accounts for 10-15% of metal (Wallace et al., 1998), but it cannot be calculated directly using the methods of the present study (due to the addition of NaOH). While cell membranes are not the primary binding site for Cd, accumulation in membranes can lead to changes in structure and function of phospholipids and other associated molecules (i.e., cholesterol; Zakhartsev et al., 2000).

Mercury exposures

Even though Hg exposure regimes were 8-fold higher than the EPA guidelines (0.009μM; US EPA 1999, 2001), there were no significant changes in physiology at either the whole-body or subcellular levels (Figures 6-8). A preliminary experiment to determine the exposure regimes used 0.056 μM as the highest concentration; no mortality was found during the preliminary three week exposure, and this was then chosen as the High-Hg exposure. While a higher concentration could have been used and may have lead to significant changes in physiology, it would have been environmentally unrealistic. Sediment concentrations of Hg within the HRE have been reported between 0.0025-0.01 μmol g DW^{-1}; the majority of the Hg is found within

the upper 25cm of sediment, making it easily resuspended and available to bivalves (Heyes et al., 2004).

In contrast to Cd, Hg accumulated in cells at a rate greater than linear. While the High-Hg exposure was four-fold higher than the Low-Hg exposure, the total body burden of oysters from High-Hg was approximately nine times higher than those in the Low-Hg treatment (Figure 9). To our knowledge this is the first study examining the effects of Hg exposure on juvenile *C. virginica*; however, previous studies using other marine bivalves have shown increased accumulation of Hg in tissues. *Perna perna* mussels exposed to 0.25μM Hg for 24 days were found to accumulate 87μg Hg g DW^{-1} in soft tissues, increasing 600-fold from the control sample (Anandraj et al., 2002). This was a 4.5-fold increase over the High-Hg exposure in the current study, and yet still did not lead to significant changes in physiology (filtration rates; Anandraj et al., 2002). Exposure of the Japanese scallop *Chlamys farreri* to very low concentrations of Hg ($2.4x\ 10^{-4}$ μM) did not elicit significant 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 in the current study caused impaired enzymatic pathways that were not examined during the current study.

Within subcellular compartments, Hg accumulation did change between those oysters exposed to 0.014μM and those exposed to 0.056μM. When oysters were exposed to the lower Hg concentrations, there was a greater percentage of Hg bound to the MSF and BDM fractions;

however, when oysters were exposed to the higher Hg concentrations, significantly more metal was found in the CD fraction (or lost). It can be assumed that the same amount of loss (approximately 10-15%) would be found in both fractions, since the same protocol for digestion and analysis of Hg was followed. This suggests more of the Hg was bound to cell debris, which can include cell membranes and other cell parts that are high in lipids. Mercury will readily bind to the thiol group in the lipids and membrane fragments that constitute the CD subcellular fraction (Dang and Wang, 2010). Bivalves collected at Hong Kong sites with elevated Hg were subjected to subcellular fractionation for total and methyl Hg, and it was found that highest percentage of methyl Hg was stored in the HDP and CD fractions (Pan and Wang, 2011). When examining total accumulation of Hg, Dang and Wang (2010) exposed bivalves to low concentrations of Hg (0.001μM), and discovered that 49-57% of the total Hg was in the CD fraction (similar to what was found in the present study). When looking at the methyl portion of Hg (MeHg), the authors found that 43-73% of the MeHg was bound to the HSP fraction (Dang and Wang, 2010). In the current study, only 0.36-2.01% of Hg was found to be accumulated within the HSP fraction, indicating that most of the metal accumulated by juvenile *C. virginica* was likely not methyl Hg. Huang et al. (2008) found similar results when exposing the abalone *Haliotis diversicolor* to Hg through dissolved and particulate uptake. Abalone exposed to Hg had approximately 50% of their metal burdens bound to CD, and very low percentages of Hg bound to HSP.

CONCLUSION

Exposure of juvenile *C. virginica* to elevated, yet sublethal, concentrations of Cd (2.4μM) for four weeks resulted in significant changes in physiology (i.e., lowered physiological condition, decreases in protein content, and changes in aerobic potential). The majority of Cd was found to accumulate in the subcellular fractions HSP, ORG, and HDP. The accumulation of Cd onto the HDP fraction likely impacted enzymatic activity in various areas of the oysters' 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 will lead to alterations in protein storage as metallothioneins accumulate Cd.

In contrast, there were no discernible changes in physiology when juvenile *C. virginica* were exposed to elevated, sublethal concentrations of Hg (0.056μM) for four weeks. Hg accumulated most heavily in the ORG and HDP fractions. Additionally, a large percentage of Hg was found to be associated with the CD fraction, although the exact amount is unknown (and a percentage of this is likely "lost" during processing as well; Wallace et al., 2003). While no physiological endpoints were altered during exposures, it is likely that subcellular changes may have occurred that were not examined during this study.

Oysters were observed to accumulate Cd approximately 2.4x higher when exposed to the High-Cd treatment versus the Low-Cd treatment. However, oysters in the High-Hg treatment

accumulated metal approximately 9x higher than the Low-Hg treatment. In each case, the High treatments were 4x higher than the Low treatments. This discrepancy in metal uptake rates can be due to many factors, including biological rates and metal dynamics. Oysters (*Saccostrea cucullata*) have been shown to uptake Hg slower than other bivalves, but also have a slower loss rate (Pan and Wang, 2011). If oysters are able to accumulate mercury it will likely persist in cells for a longer period, leading to toxicity. Oysters were shown to accumulate Hg linearly with increasing concentrations when Hg was spiked into tanks with sediment and food (Pan and Wang, 2011); in the current lab study, oysters accumulated Hg at rates above linear indicating that the exposure was more efficient when sediment was not available to bind the Hg or that interspecfic differences in uptake constants are present within oysters (between *Saccostrea cucullata* and *Crassostrea virginica*). Another reason for differences in uptake rates is the biological half-life of both Cd and Hg. Though it was not estimated during this experiment, loss rates of Cd and Hg vary within species and exposures. The half-life of Cd in bivalves is approximately 500 days (Baudrimont et al., 2003) while the half-life of Hg is only 12-33 days (Pan and Wang, 2011). If uptake rates of Cd were higher than loss rates, then it is possible that Cd body burdens after 30 days would have been at least 4x higher than the Low-Cd treatments (linear accumulation). With Hg, the elevated accumulation rate (9x higher) suggests that the uptake rate is higher than the loss rate, thus accounting for a higher accumulation. Both exposures were for the same amount of time (30days) and the procedure was the same during both exposures. One difference was the size of oysters; as the Hg exposure took place six weeks later than the Cd exposure, oysters were larger at the hatchery and therefore significantly larger in length (Student's *t*-test, $p<0.0001$) but had a significantly lower condition index (Student's *t*-

test, $p<0.0001$). These differences in size may account for differences in physiological rates, which would lead to differences in uptake rates.

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

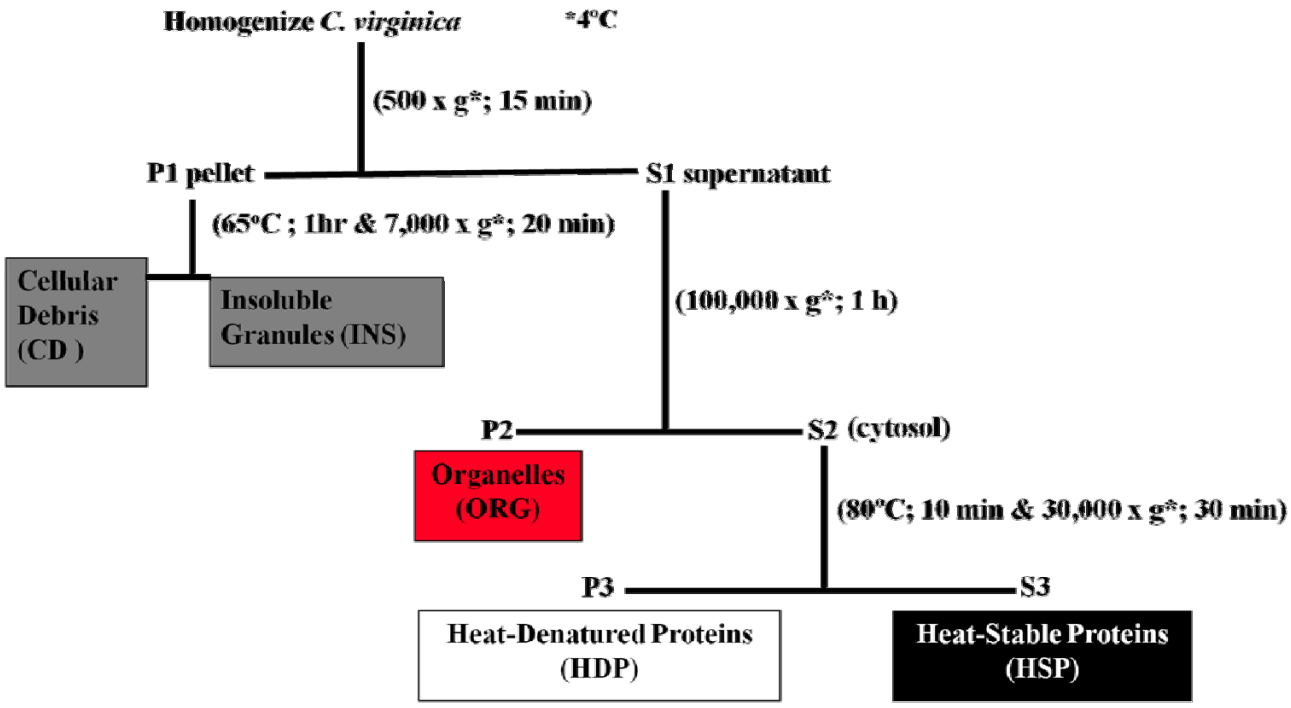

Figure 1: Diagram of subcellular fractionation procedure (after Wallace et al., 2003). CD= cell debris; INS= Insoluble Granules (i.e., metal rich granules); ORG= Organelles; HDP= Heat-Denatured Proteins (i.e., enzymes); HSP= Heat-Stable Proteins (i.e., Metallothionein-like proteins). Temperature, spin intensity, time indicated for each step, resulting in either pellet (P) or supernatant (S).

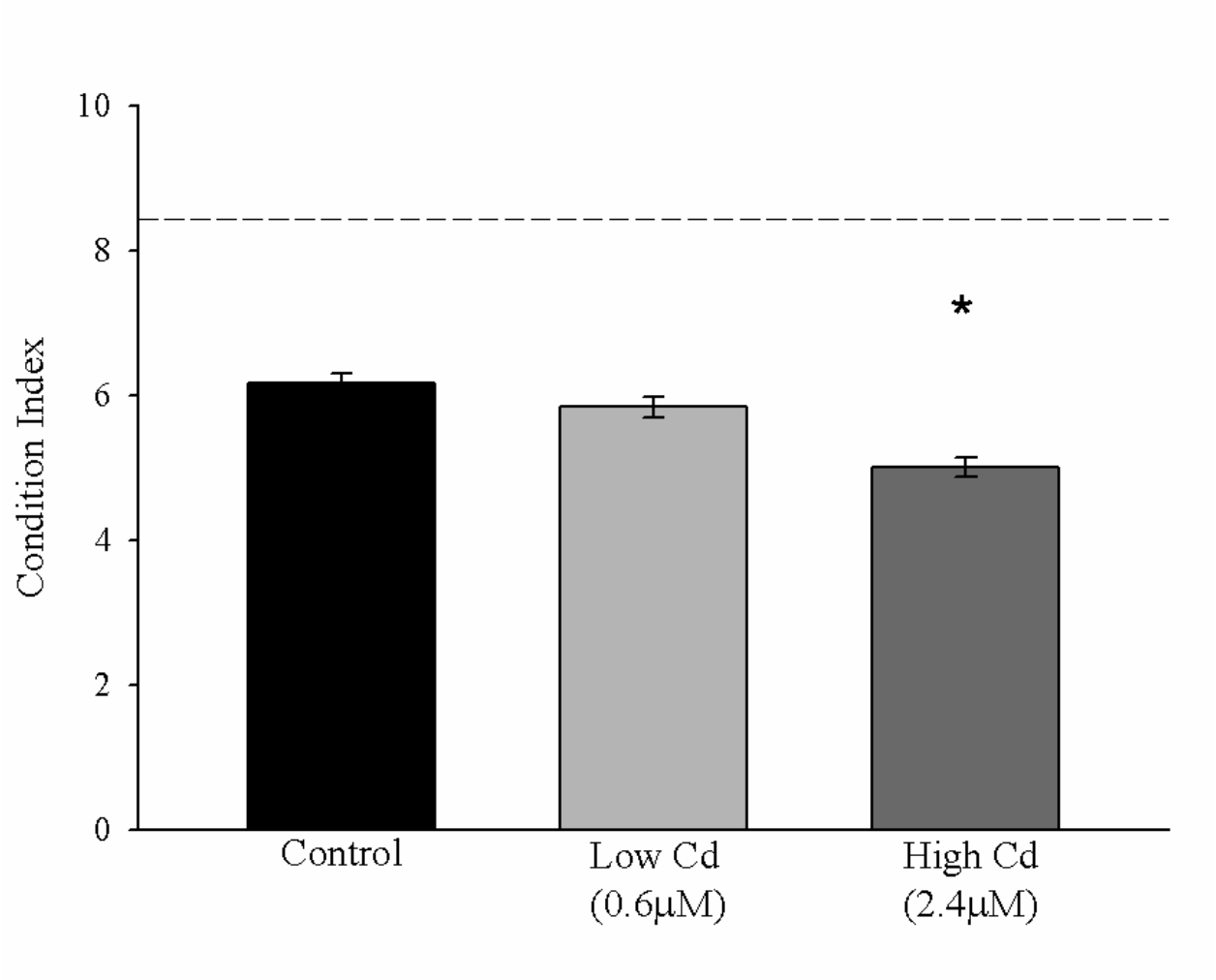

Figure 2: Condition index of oysters exposed to 0.6μM (Low-Cd treatment) or 2.4μM (High-Cd treatment) for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ±1 SE. Asterisk (*) indicates a significant difference between the High-Cd treatment and Con-Cd and Low-Cd treatments (ANOVA, $p<0.0001$). Dashed line represents the average condition index of initial (prior to placement in treatment containers) subsample ($n=20$).

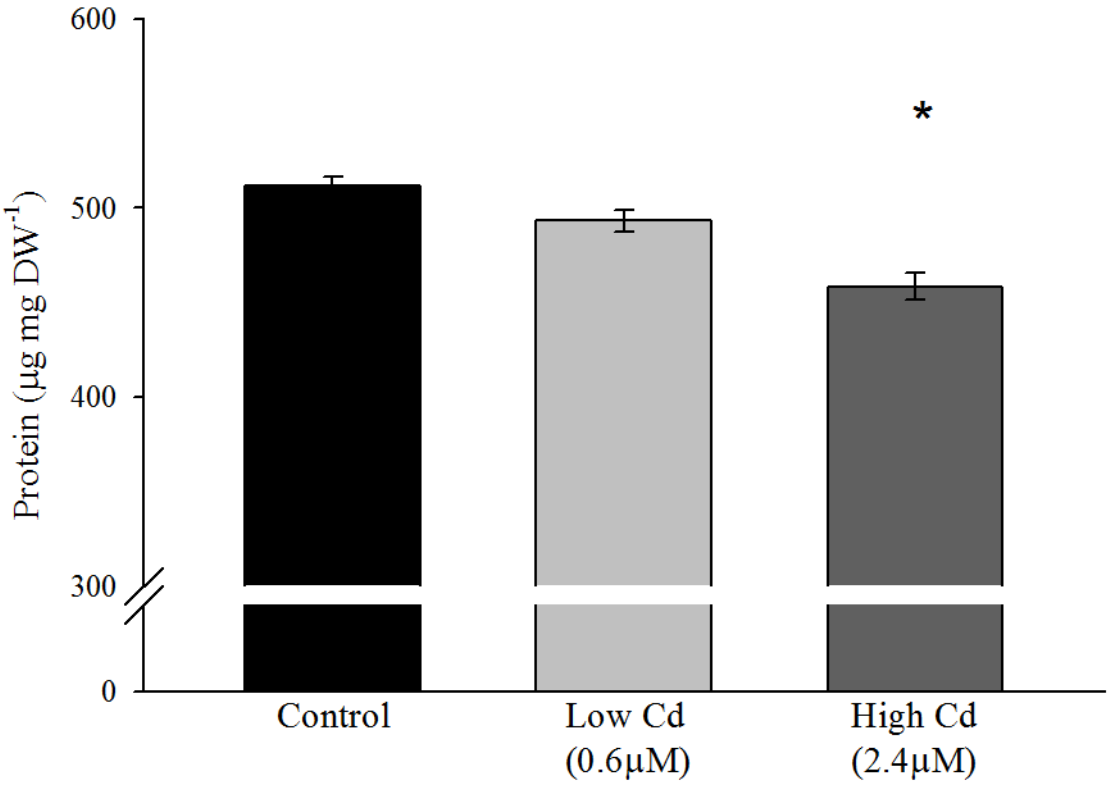

Figure 3: Protein concentration of oysters exposed to 0.6µM (Low-Cd treatment) or 2.4µM (High-Cd treatment) for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ±1 SE. Asterisk (*) indicates a significant difference between the High-Cd treatment and Con-Cd and Low-Cd treatments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

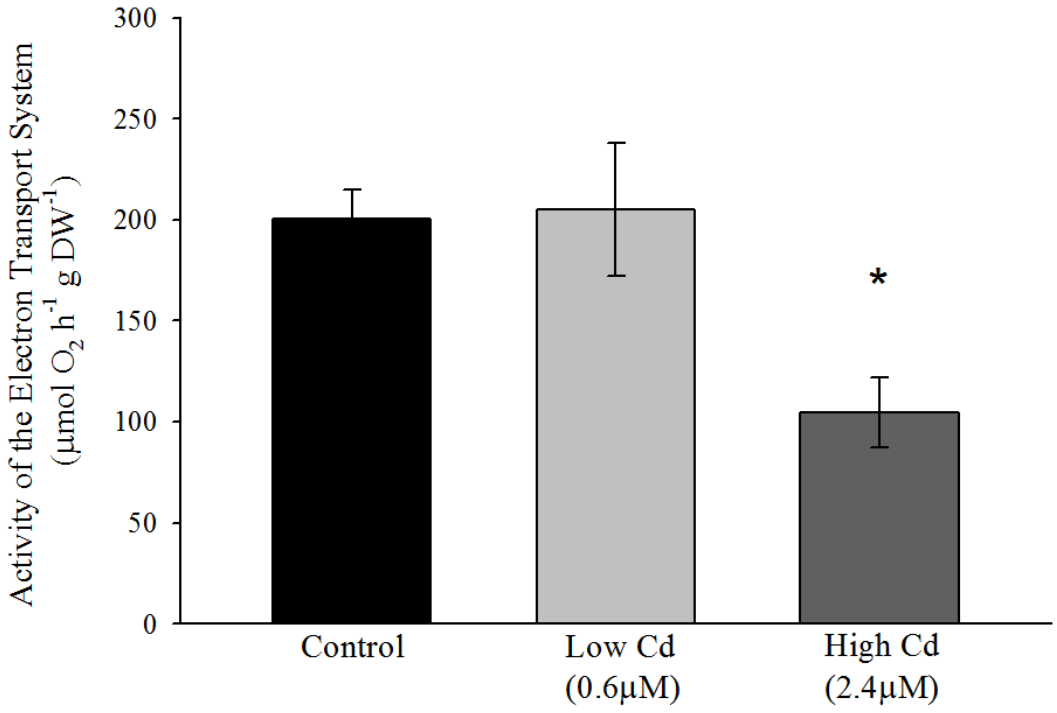

Figure 4: Aerobic potential of oysters exposed to 0.6µM (Low-Cd treatment) or 2.4µM (High-Cd treatment) for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ±1 SE. Asterisk (*) indicates a significant difference between the High-Cd treatment and Con-Cd and Low-Cd treatments (Kruskal-Wallis, $p < 0.05$).

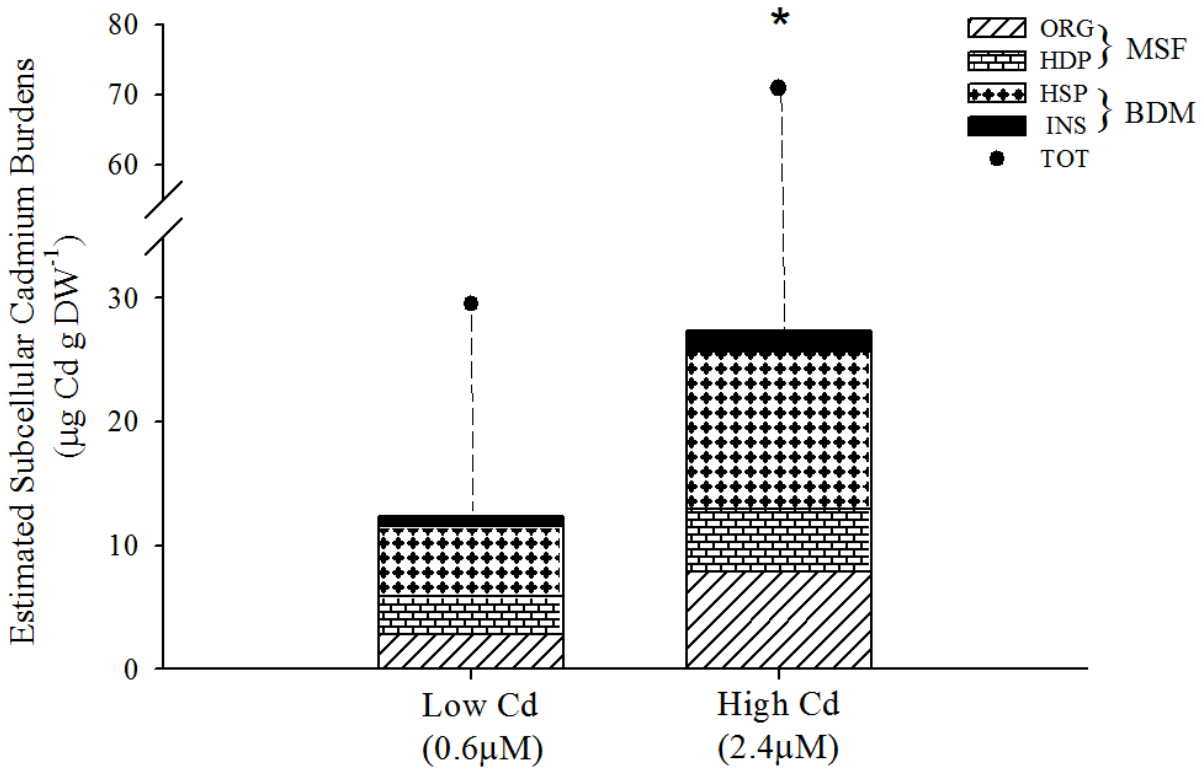

Figure 5: Total body burdens, and estimated subcellular burdens of Cd in oysters exposed to 0.6µM (Low-Cd treatment) or 2.4µM (High-Cd treatment) for 4 weeks. Con-Cd treatment is not shown due to Cd burdens below the detection limit of the machine. Bars represent the mean of all oysters sampled from each treatment ±1 SE. Asterisk (*) indicates a significant difference between all fractions (Student's *t*-test, $p < 0.05$). 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); MSF= Metal Sensitive Fraction; BDM= Biologically Detoxified Metal.

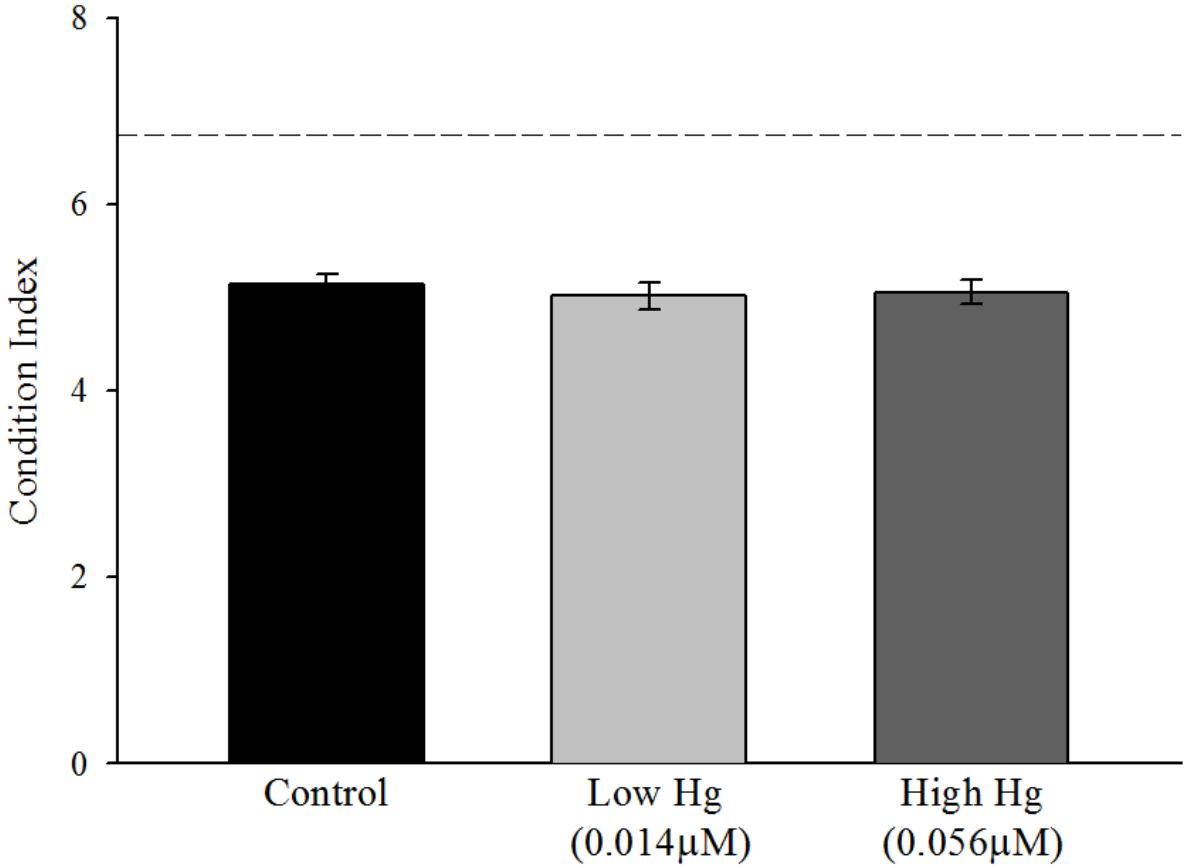

Figure 6**:** Condition index of oysters exposed to 0.014µM (Low-Hg treatment) or 0.056µM (High-Hg treatment) for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ±1 SE. There were no significant differences between Con-Hg, Low-Hg, and High-Hg treatments (ANOVA, $p > 0.05$). Dashed line represents the average condition index of initial (prior to placement in exposure containers) subsample (n=20).

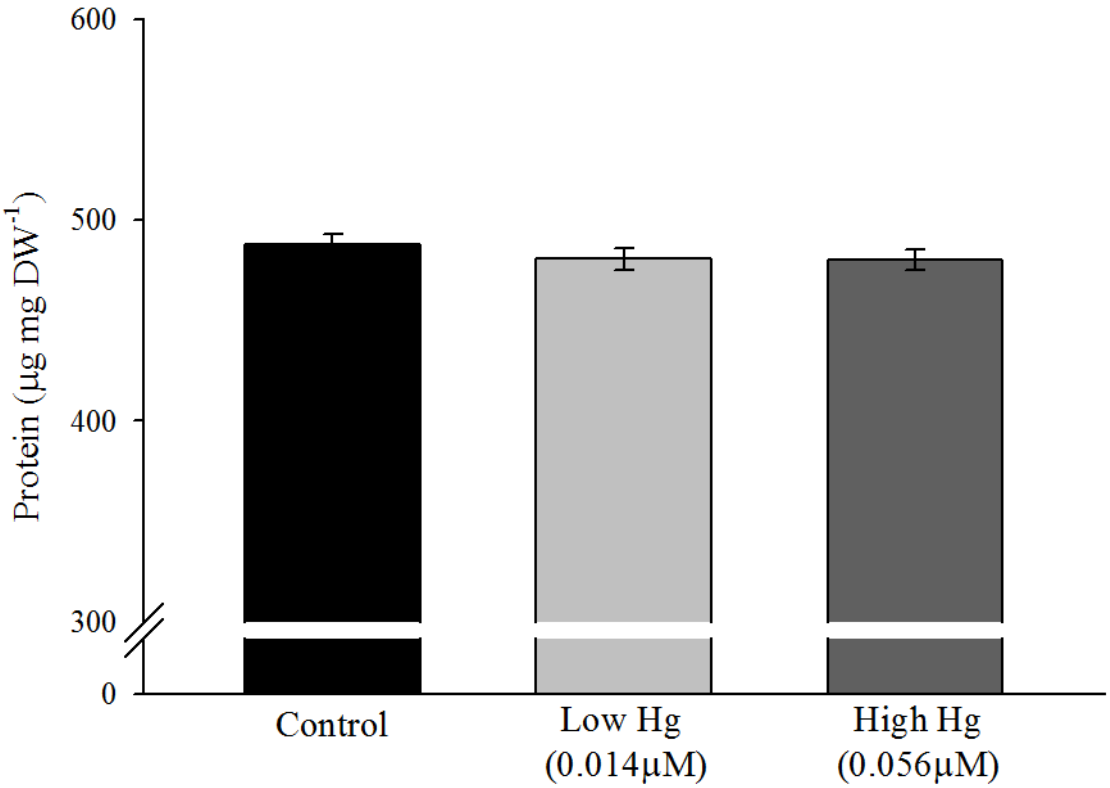

Figure 7: Protein concentration of oysters exposed to 0.014µM (Low-Hg treatment) or 0.056µM (High-Hg treatment) for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ±1 SE. There were no significant differences between Con-Hg, Low-Hg, and High-Hg treatments (ANOVA, $p > 0.05$).

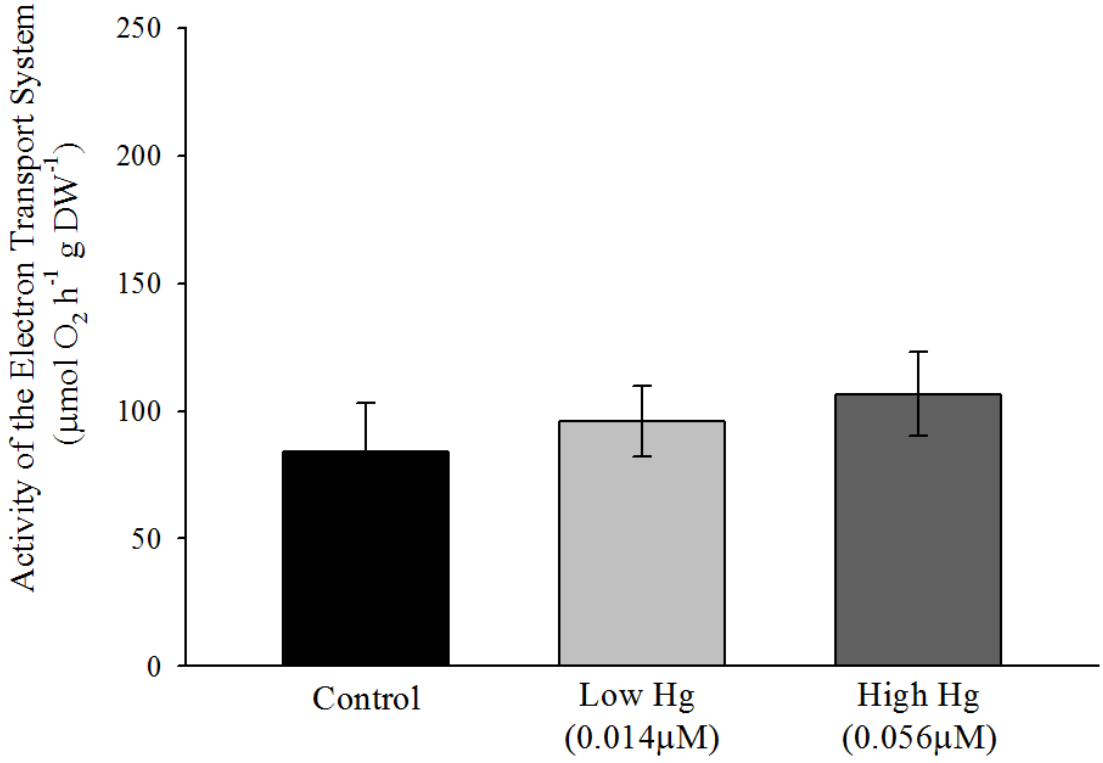

Figure 8**:** Aerobic potential of oysters exposed to 0.014µM (Low-Hg treatment) or 0.056µM (High-Hg treatment) for 4 weeks. Bars represent the mean of all oysters sampled from each treatment ±1 SE. There were no significant differences between Con-Hg, Low-Hg, and High-Hg treatments (ANOVA, $p > 0.05$).

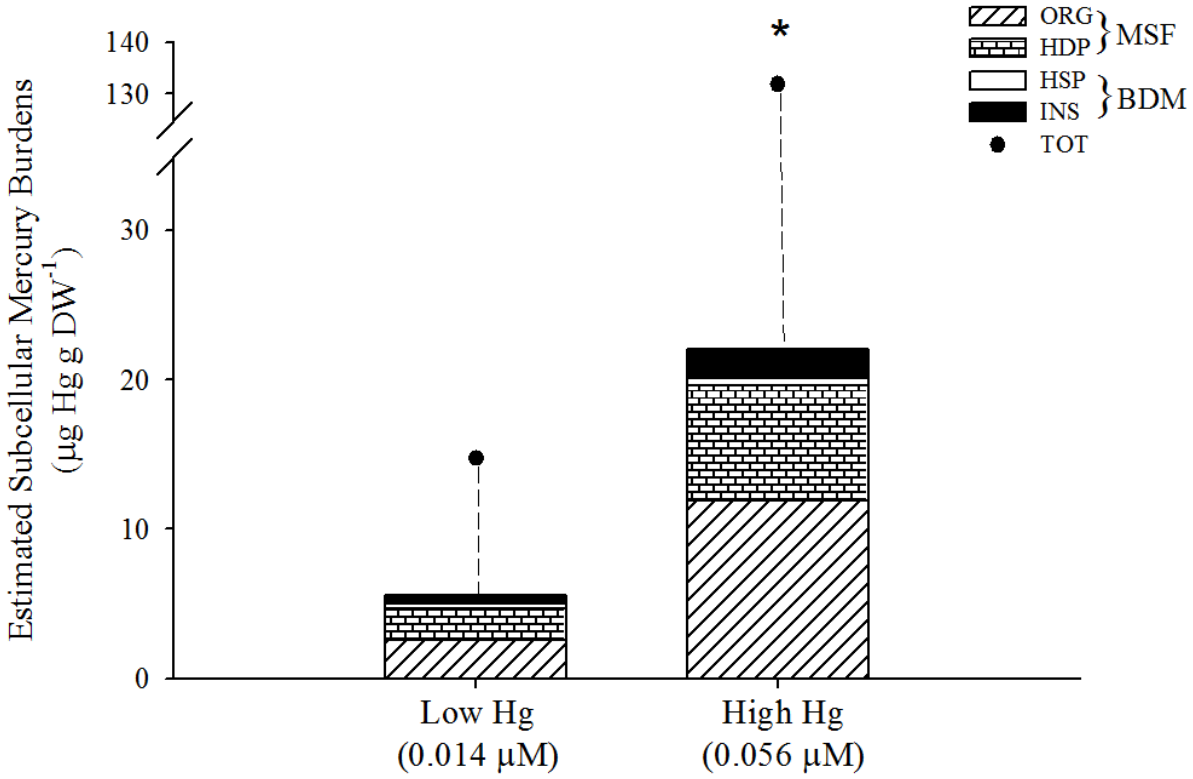

Figure 9: Total body burdens, and estimated subcellular burdens, of Hg of oysters exposed to 0.014µM (Low-Hg) or 0.056µM (High-Hg) for four weeks. Con-Hg treatment is not shown due to Hg burdens below the detection limit of the machine. Bars represent the mean of all oysters sampled from each treatment ±1 SE. Asterisk (*) indicates a significant difference between INS, ORG, HDP, and TOT fractions (Student's *t*-test, $p < 0.0001$). There was no significant difference between the HSP fraction (Student's *t*-test, $p > 0.05$). 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); MSF= Metal Sensitive Fraction; BDM= Biologically Detoxified Metal.

	Estimated quantity (μg g DW^{-1})				
Fraction	**Low-Cd**	**High-Cd**		**Low-Hg**	**High-Hg**
TOT	29.54 ± 5.19	70.89 ± 11.12		14.75 ± 4.61	131.75 ± 96.77
INS	0.79 ± 0.2	1.70 ± 0.21		0.57 ± 0.25	1.99 ± 0.72
HSP	5.64 ± 1.48	12.69 ± 1.84		2.60 ± 0.92	11.92 ± 6.53
HDP	3.12 ± 0.57	5.09 ± 0.53		2.11 ± 1.21	7.69 ± 3.61
ORG	2.79 ± 0.5	7.86 ± 1.77		0.31 ± 0.1	0.46 ± 0.2
BDM	6.30 ± 1.5	14.24 ± 1.85		0.66 ± 0.09	2.38 ± 0.26
MSF	5.65 ± 0.77	12.53 ± 2		4.71 ± 0.57	19.61 ± 2.48

Table 1: Estimated metal accumulations of Cd or Hg in juvenile oysters exposed to Low or High doses of Cd or Hg for four weeks. All values are reported as mean μg metal g DW^{-1} (n=3) ± 1 SE. Exposure concentrations: Low-Cd = 0.6μM, High-Cd = 2.4μM, Low-Hg= 0.014μM, High-Hg= 0.056μM. 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 Metals; MSF= Metal Sensitive Fraction.

	Percentage of total metal				
Fraction	**Low-Cd**	**High-Cd**		**Low-Hg**	**High-Hg**
INS	2.58 ± 0.33%	2.61 ± 0.33%		3.73 ± 0.38%	1.9 ± 0.23%
HSP	16.41 ± 2.36%	20.31 ± 0.95%		2.01 ± 0.6%	0.36 ± 0.03%
HDP	10.59 ± 1.19%	8.36 ± 0.43%		14 ± 1.87%	7.39 ± 1.19%
ORG	10.5 ± 1%	10.71 ± 0.65%		17.71 ± 0.91%	10.02 ± 1.02%
CD	63.12 ± 3.49%	60.83 ± 2.4%		64.48 ± 2.64%	80.39 ± 1.6%
BDM	18.43 ± 2.49%	22.69 ± 0.87%		4.27 ± 0.62%	2.2 ± 0.23%
MSF	20.13 ± 2%	18.37 ± 0.91%		31.71 ± 2.26%	17.41 ± 1.46%

Table 2: Percentage of total metal accumulated in each subcellular fraction and compartment, for juvenile oysters exposed to Low or High doses of Cd or Hg for four weeks. Exposure concentrations: Low-Cd = 0.6μM, High-Cd = 2.4μM, Low-Hg= 0.014μM, High-Hg= 0.056μM. As per Wallace et al. (2003): 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); CD= Cell Debris; BDM= Biologically Detoxified Metals; MSF= Metal Sensitive Fraction.

CHAPTER 5

CONCLUSION

Overall summary

The objective of this study was to gain an understanding of how a highly urbanized environment, such as the HRE, will influence oyster physiology. This information could then be used to influence management decisions and oyster restoration in the lower HRE. By using a field transplant study to determine site-specific effects of the environment on oysters, physiological responses to environmental parameters and anthropogenic inputs (Cd and Hg) were assessed to determine any relevant relationships. Oyster responses are highly site-specific, with multiple abiotic factors influencing physiology. Though metal accumulation was related to overall condition (in both juvenile and adult *C. virginica*), subcellular metal accumulation could not be related to subcellular physiology which suggests that it is likely synergistic effects of abiotic and anthropogenic influences affecting subcellular physiology.

The first part of this study (Chapter 2) noted that environmental parameters influence oyster condition and subcellular physiology (tissue biochemistry, energy usage). Sites with higher TPM, poorer food quality, and increased anthropogenic influences (including sediment metal accumulations) had oysters with decreased physiological condition and increased Cd accumulation. The metal accumulation was primarily in the cell debris (CD) and biologically detoxified metal (BDM) compartments, indicating that oysters exposed to more metal were able to detoxify a higher percentage of accumulated metal after four months. Sites with lower environmental Cd concentrations had oysters with increased Cd accumulation in the metal sensitive fraction (MSF) compartment. Changes in juvenile oyster health and subcellular physiology indicate that site selection is imperative when initiating a new restoration project.

The second part of this study (Chapter 3) noted that site selection is also important when assessing reproductive output and adult oyster health. Adult oysters declined in overall condition when placed at a site with increased TPM and poorer food quality, but differences in subcellular physiology (i.e., proteins and Vtg percentage) were not evident indicating that adult oyster physiology is not as sensitive to abiotic degradation as juvenile physiology. Analysis of wild adult oysters at SVP suggests that oysters naturally occurring in the HRE may have acclimated to the impacted conditions (i.e., increased particulates, lower chlorophyll-*a*) and are able to tolerate increased accumulation of metal contaminants and poor food quality.

The third part of the study specifically examined the role of non-essential metals (Cd and Hg) in juvenile physiology and metal accumulation (Chapter 4). Juvenile oysters were exposed to increasing concentrations of Cd or Hg (above the EPA maximum) for four weeks and physiological endpoints (overall condition, subcellular physiology) and subcellular metal accumulation were analyzed. Exposure to Hg did not lead to changes in subcellular health, but the majority of metal accumulated in the MSF compartment. This suggests that increased metal accumulation, perhaps after a long-term field transplanting, may eventually lead to toxicity as oysters do not sequester the majority of Hg in the BDM compartment. The amount of Hg accumulated during the exposure was between two and ten orders of magnitude higher than field-exposed oysters, suggesting that none of the field sites used had a level of Hg high enough to result in accumulating enough metal to cause toxicity and physiological changes. Exposure to Cd did lead to declines in overall condition and subcellular physiology as exposure concentrations increased. In treatments with significantly impacted physiological condition, accumulated Cd was two times higher than the highest amount of Cd accumulated (with an

increase in accumulation in the BDM compartment) at field sites suggesting that field accumulated Cd was also not high enough to be the sole cause of subcellular toxicity. Therefore, the synergistic effects of habitat degradation (i.e., increased sedimentation, poor food quality, low DO) and Cd accumulation would more likely account for site-specific differences in overall health and subcellular physiology of oysters rather than Cd alone.

Review of previous studies on oysters in the HRE:

Oyster restoration in the HRE has been a subject of discussion and research since the early 2000s. Beginning in 2004 as a series of small transplants to determine survivorship, projects have scaled up in size and complexity, with each adding to the pool of information on oysters in the HRE. Pilot studies by the NYC Parks department and NY/NJ Baykeeper were the first to use artificial reefs to examine habitat impacts on the sediment, survival and growth of spat, and recruitment of natural oysters to the reef (Mass and Ruzicka 2008; NY/NJ Baykeeper, 2005). These studies were small in size (less than ½ acre footprint), and mainly used volunteers and staff for monitoring protocols. Protocols were focused on mortality and size of oysters, and indexing other invertebrates and fish found on the reef. These projects provided a base understanding of oyster survivorship at sites in the HRE, and a working knowledge of rules and regulations associated with performing oyster restoration in both NY and NJ (NYC-DEC, NYS-DEP, NJ-DEP).

Many scientific studies by investigators at local universities also began to increase in frequency in the early 2000s. Site suitability, mortality and growth of juvenile *C. virginica*, interactions of *C. virginica* with the substrate, and reef usage (by fish and invertebrates) have been examined

(Starke et al., 2012; Ravit et al., 2012; Zarnoch and Schreibman 2012; Levinton et al., 2011; Ravit et al., *unpubl.*; Zarnoch et al. *unpubl.*; Hollein et al., *unpubl.*). Research sites from the lower Hudson River (Hastings-on-Hudson, Ossining), Bronx River (Soundview Park), East River (Pier 40), Bay Ridge Flats, Jamaica Bay, Rockaway Inlet, NY Harbor (Liberty Flats) Raritan Bay, and Navesink River have all provided data which adds to the growing knowledge base for the HRE. Additional studies by NY/NJ Baykeeper (Keyport and Navesink Reefs; NY/NJ Baykeeper), Hudson River Foundation (Grizzle et al., 2013), NYC Department of Parks and Recreation (Conrad et al., 2012; Mass and Ruzicka 2008) have solidified relationships between principal investigators, government agencies, and non-profit non-government organizations.

The Hudson Raritan Estuary Comprehensive Restoration Plan (CRP) has a goal of 500 acres of restored oyster habitat by 2015 (Bain et al., 2007). While these small scale projects are important, a larger scale project has to be attempted in order to strive towards the goals of the CRP. The largest pilot study to date in the HRE has been the multiple reef project spearheaded by the Hudson River Foundation and NY/NJ Baykeeper. The Oyster Restoration and Research Project (ORRP) was formed in 2009 as a partnership of over 40 organizations, institutions, and community groups within the NY/NJ area, with the goal of oyster restoration in the HRE. The first phase of this project used five sites over a large spatial scale in the HRE: Hastings-on-Hudson (Hudson River), Bay Ridge Flats (Upper Bay), Great Kills, Staten Island (Raritan Bay), Governor's Island (Upper Bay), and Soundview Park (Bronx River/ East River). After a two-year study, with 0.12 $acre^2$ subtidal artificial reefs installed and monitored at each location, it was determined that the sites with the most potential (i.e., most oyster survival and best reef integrity

after two years) were Soundview and Governor's Island (Grizzle et al., 2013). The second phase of this study is now (2013-2015) building a larger (1 acre) subtidal reef at Soundview Park, to be seeded with live oysters (spat-on-shell) and monitored for recruitment of natural spat as well as growth and survival of aquacultured spat-on-shell (M. Comi, pers. comm.). The second phase will last for two years as well, and provide information on habitat restoration (building subtidal reefs), recruitment of natural oysters (from a wild population at Soundview; Medley, 2010), growth and survival of spat-on-shell, and habitat usage by fish and other invertebrates. Additionally, genetics of wild versus aquacultured spat at Soundview are also being examined; wild adult oysters were removed from Soundview and transferred to an aquaculture facility at The Urban Assembly New York Harbor School (Governor's Island) where they were induced to spawn, and natural broodstock and spat-on-shell are being cultured (P. Malinowski, pers. comm.).

Restoration in the HRE: regulations and policy

A recent review paper (Ravit et al. 2012) highlights the differences between management and restoration of oysters and reef habitat in the HRE versus other Atlantic estuaries. Restoration in neighboring estuaries (Long Island Sound, Delaware Bay) have promoted oyster restoration for both aquaculture (commercial) and habitat (ecological) restoration, but closed waters and health concerns in the HRE render commercial restoration impossible. Ecological restoration of oyster reefs, to promote healthy water quality, fish habitat, and shoreline stabilization, is the goal of restoration within the HRE. Oyster populations in the mid-Atlantic estuaries are estimated to be below 10% of historic levels, and in many areas (including the HRE) are "ecologically extinct" (Beck et al., 2009). One major problem with restoration in the HRE is the dual-state control of

the estuary. Waterways flow between NY and NJ, but restoration and management practices between the governing agencies (NYC-DEP and NYS-DEC, NJ-DEP) are not consistent. Many of the sites chosen during the present study were in NY due to regulations against scientific permits within Raritan Bay (Figure 2; Ravit et al. 2012). An additional problem with NJ policy seems to be the discrepancy between regulations in northern NJ (within the HRE, including Newark and Raritan Bays), central NJ (including Barnegat Bay), and southern NJ (Delaware Bay). The only area that represents a fishery is Delaware Bay, which has seen declines in the oyster industry due to loss of habitat and increases in disease prevalence (Ravit et al. 2012; Ewart and Ford 1993). As of 2012, NJ –DEP considers all areas north of, and including, Raritan Bay to be "closed waters to shellfishing" meaning no scientific research or restoration is allowed in these areas (NJDEP 2012). Waters south of Raritan Bay are under different regulations; Barnegat Bay is permitted to have a large shellfish restoration program even without natural oyster habitat (which HRE does have) and declining water quality (Ravit et al., 2012). These discrepancies in policy and state lines make larger restoration projects in the HRE difficult to implement. The only current restoration project in northern NJ is located within the secure naval base, Naval Weapons Station Earle (NY/NJ Baykeeper) in eastern Raritan Bay, providing a secure location where no oysters can be illegally poached off of the reef (which is a large part of the NJ-DEP ruling to ban all research permits in northern NJ waters; Ravit et al., 2012).

Future directions for research and restoration in the HRE:

The small-scale variation in environmental parameters encountered in the HRE is a difficulty that many projects have to overcome. Within a small spatial area (approximately 8000 km2), the lower HRE has dramatic shifts in salinity, dissolved oxygen, suspended particulates, and

chlorophyll-a concentrations. Additionally, the HRE is one of the most heavily impacted estuaries in the United States, with several toxic inorganic and organic pollutants, in addition to nutrient enrichment (nitrogen and phosphorus) (Bokuniewicz et al., 2006). Areas of contaminant "hot spots" can be found throughout the estuary, and several Superfund sites are also in the HRE (Gowanus Canal, Newtown Creek, and parts of the Passiac River, Hackensack River, Newark Bay, and Raritan Bay). These localized sites can have severe impacts on biota in the area, and guide where future restoration and research can occur.

When considering research and restoration sites within the HRE, survivorship of oysters is used as a precursory screening measure. Oysters are often placed in nets or cages, placed in the subtidal, and monitored (including over winter) for survival and growth. If a site is suitable for oyster survival, the next precursory screening measure is to observe and measure the wave energy and hydrodynamics of the site. Several sites used in preliminary studies (i.e., Bay Ridge Flats, Liberty Flats, Keyport Harbor) were thought to be suitable based on oyster survival. However, after a reef was constructed, it was found to be unsuitable due to high wave energy. The high energy and flow of water causes the shell to be washed off the reef and spread out, which degrades the reef structure and leads to increased oyster mortality (Grizzle et al., 2013; NY/NJ Baykeeper 2005).

Once a suitable site is found, research on the effects of the chosen site on oyster physiology is necessary. Growth and survival are important, but an oyster reef cannot flourish if the spat cannot grow to a consistent size, reach reproductive age, and begin producing more spat. The presence of toxic pollutants, both organic and inorganic, is important to survey at a site prior to

beginning a restoration. Trace metal contamination is elevated throughout the HRE as are organic pollutants (NOAA 1993, 1995). Non-essential trace metals, PCBs, and PAHs can all lead to changes in physiology, declines in reproductive efforts, and mortality in *C. virginica*.

Any physical anomalies (i.e., lesions and other tissue deformities) and diseases (MSX, Dermo) should be assessed, as these will lead to reduced reproductive capacity and growth, and eventually mortality (Wang et al. 2010). Diseases found among *C. virginica* in the HRE include Dermo (*Perkinsus marinus* parasite) and MSX (*Haplosporidium nelson* parasite). These two disease can infect oysters of all ages, though the prevalence of both is generally higher in adult oysters than spat simply due to the fact that the larger volume of water filtered by adults means they will encounter parasitic particles more often (Ford and Tripp, 1996). Dermo disease infects live oyster cells, leading to a reduction in physiological function and eventual death. The level of intensity of infection can vary, and oysters with "light intensity" may not show physiological alterations (Ford and Tripp, 1996). It is possible that inbreeding within the wild populations found in the HRE has lead to a natural "resistance" and therefore lower infection rate; hatchery transplanted oysters would not have this resistance and therefore may experience higher mortality than wild populations. Oysters infected with MSX often lose stores of tissue glycogen, protein, and lipid and appear emaciated and in poor condition before death. MSX also impacts several physiological functions of the oyster, including reproduction. In the HRE, Medley (2010) assayed wild oysters at several sites and found the highest prevalence of Dermo and MSX in the Hackensack River (NJ). Oysters at Soundview Park had a 25% prevalence of Dermo, and 12.5% of MSX, suggesting that this site is more suitable for future restoration due to its low occurrence of disease. Levinton et al. (2013) found infection intensities of transplanted oysters

to Jamaica Bay and Raritan Bay low during the first year, but increasing after a two year exposure at each site. MSX was in approximately 21% of the oysters at Raritan Bay, but only 3% in Jamaica Bay. Dermo was found in low intensities at both Jamaica Bay and Raritan Bay.

Eutrophication can lead to system-wide alterations by initiating changes in trophic webs and affecting numerous biota. The long term effects of eutrophication, including lowered dissolved oxygen and harmful algal blooms, can lead towards system-wide environmental deterioration of a habitat and community. Kirby and Miller (2005) hypothesized that growth rates of subtidal *C. virginica* in Chesapeake Bay were altered by eutrophication and, later, environmental deterioration. However, the authors argue that these two phases are different, and affect the biota accordingly. Eutrophication added nutrients into the water column, and thus lead to increased primary production. This type of system, when the food web is dominated by top-down grazing by infaunal and epifaunal filtering organisms, is conducive to oyster growth and allows for larger, faster growing oysters (Kirby and Miller, 2005). As seen at the sites in Jamaica Bay, oysters with elevated growth rates were found at the same sites that saw periodic elevations in chlorophyll-*a* abundance (Figures 5,8 Chapter 2). These sites are known to have high nutrient inputs as well as periodic algal blooms during summer months (Benotti et al., 2007), which have helped to enhance oyster growth rates, as observed in historical samples from Chesapeake Bay (Kirby and Miller, 2005). After years of eutrophication, negative impacts from consistently elevated algal abundances begin to take place, and the combined impacts of algal blooms and removal of oysters (due to overfishing and habitat removal) begin to switch the system towards a system dominated by bacterial decomposers ('environmental deterioration', Kirby and Miller, 2005). As Jamaica Bay has not had oysters abundant in any location since the early 20th

century, it can be inferred that while growth rates are elevated at these sites (versus other locations in the HRE), this is actually a reduced growth rate compared to the hypothetical maxima that would have occurred before environmental deterioration had begun. The entire system of the HRE has been deteriorated, and numerous shifts in the trophic webs have taken place to compensate for the removal of species and habitat, and the addition of excess nutrients and algal abundances. This will be an important factor to consider when moving forward with oyster restoration, as the system has shifted and the food web may be different than what oysters optimally feed on.

As the current study demonstrated, areas of the HRE that were more eutrophic (Jamaica Bay) had higher growth rates. The tissues of oysters at Jamaica Bay had the highest carbohydrate, lipid, and protein concentrations. In addition, these oysters accumulated very little Cd. These findings all lead to the conclusion that Jamaica Bay would be a suitable area for a restored subtidal reef. However, caution must be taken when extrapolating up to a long-term reef restoration; during the four months this study was conducted, no tissue anomalies or significant metal (Cd or Hg) accumulation occurred. If oysters were left at field sites for a longer period of time (such as two years), adverse abiotic conditions may lead to impaired physiology. Subcellular fractionation analysis revealed that the majority of accumulated Cd was bound to the MSF compartment (36-63%), indicating that sensitive cellular components may be altered due to metal accumulation. Mitochondria are known to be a key subcellular target for Cd, and decreases in metabolism are observed in response to increased Cd accumulation (Cherkasov et al., 2010). The data suggests that oysters are not preferentially detoxifying Cd as only 18-23% of metal was bound to the BDM compartment (Figure 12, Chapter 2). The lack of apparent

toxicity observed during this study may be because (1) the exposure time was too short, or (2) the amount (μg Cd g DW^{-1}) was not high enough to elicit a toxic response. In order to assess the possibility of a long term project at this site, a longer exposure period would be necessary; as exposure time increases, accumulation to the MSF compartment may increase and lead to future toxicity.

Another site that had high growth rates and healthy tissue was FBF, located in the Rockaway Inlet. Condition index and shell growth were very high here as was biochemical content of tissue, suggesting that oysters did not have to expend a lot of unnecessary energy to grow (see Figures 5-8 Chapter 2). This site has low TPM and high chlorophyll-*a* concentrations, indicating that food quality is higher than other sites which aids in faster growth. These oysters also developed gonad tissue within their first year (data not shown), indicating that they would mature quickly in this environment. Total accumulation of Cd was low (similar to Jamaica Bay) with little Cd bound to the MSF and BDM compartments. The majority (63%) of metal was associated with CD; this is an estimate as part of this metal may have been lost during processing. The low percentage of metal associated with MSF (30%) suggests that oysters here would likely not experience toxicity. It is still possible that continued exposure (longer than one year) would lead to toxicity as increased metal accumulation taxes detoxification mechanisms, but this site appears to be suitable for a long term study based off the physiological endpoints. One downside to this site is that FBF is located in an active inlet, with high wave energy and boat/barge traffic. This would make it difficult to install a subtidal reef, and therefore would not make for the most suitable location.

Tuckerton, a site outside the HRE and therefore permitted to have oyster restoration projects, was presumed to have the healthiest oysters, with high energy reserves and low metal accumulation. However, condition indices and carbohydrates in oyster tissue were lower than Jamaica Bay oysters (yet more than Soundview). This could be due to the fact that these oysters were hung from a dock into the subtidal zone (nets were exposed from the water at mean low tide). Sediment and fouling organisms (tunicates, sponges) were often found coating the bags and were scrubbed off at every sampling event. The site is located adjacent to an Atlantic Ocean inlet (Little Egg Inlet), resulting in low food availability (chlorophyll-*a*) but also low TPM (see Figure 3-4, Chapter 2). Both juvenile and adult oysters accumulated very little Cd and Hg at this site, and less than 40% of the Cd accumulated was bound to the MSF compartment. This site has high wave action, but oyster restoration efforts in the Mullica-Great Bay estuary have been successful in the past with restoring the fishery beds to a self-sustaining population (NJDEP-FWS, 2007).

The only site used in the current study with oyster restoration underway is Soundview Park. This site was considered one of the most impacted study sites, with high sediment inputs, hard substrate and marsh loss, increased metal and organic contaminants, and other anthropogenic inputs (rusted cars, garbage and other debris litter the site). Water quality at the site is poorer than other sites (hypoxic events were recorded 2009-2011), and chlorophyll-*a* content is lower. However, this site has the only natural population of oysters out of all sites used for the present study; oysters grow on the rip-rap shoreline, submerged tires and debris. Oysters were discovered in the park in 2004 by the Natural Resources Group of NYC Department of Parks and Recreation (Harris and Mass, 2008). The substrate alternates between soft-bottomed mud and

harder substrates (firmer sand, with rocky outcroppings) which provide some subtidal substrate for oysters and other invertebrates. While the natural population is encouraging, increased sedimentation and poor food quality make it difficult for transplanted juveniles, adults, and spat-on-shell to grow to reproductive size (this study; Grizzle et al. 2013; Mass and Ruzicka 2008). The juveniles used in the current study had thin, brittle shells leaving the oysters vulnerable to predators. Additionally, thin shells make it difficult for a reef to accrete and form refugia and microhabitats (Coen and Luckenbach, 2000; Bartol et al., 1999) for settling spat and other invertebrates. Oysters from SVP also displayed linear accumulation of Cd and Hg (see Figure 12-13, Chapter 3). It is possible that the natural population of oysters at SVP is better acclimated to the abiotic conditions of the site, even with increased metal accumulations (see Chapter 3; Ravit et al., *submitted*) these oysters are able to survive. However, growth rates of the natural population are low, shells are thin, and oysters do not occur in multiple size classes (Conrad et al., 2012; Mass and Ruzicka, 2008; pers. obsv.). Since 2004, only one major spat set has been observed (summer 2012), with the majority of oysters representing a similar size class (less than 20mm) at last observation (pers. obsv., K. Kalchmayr, pers. comm.). With low growth and poor tissue quality, and abiotic factors suggesting site degradation, restoration at SVP may be compromised. Though aquacultured spat-on-shell may survive and the substrate may add habitat, poor growth, tissue abnormalities (Ravit et al., *unpubl.*), and accumulation of toxic metals in oyster cells may lead to long term problems and reproductive failure (thus, not a self-sustaining reef).

This study was designed to garner an overall understanding of the potential of long-term oyster restoration in the HRE. Pilot projects (such as this study), with short term exposures and small

spatial scales, are necessary to determine the usefulness and suitability of sites. The responses of juvenile oysters at several different sites (Chapter 2) illustrate the site-specific nature of environmental parameters (i.e., seston characteristics), and thus how site-specific physiological measures will be. While a site may promote faster growth and healthier tissue, long term accumulation of toxins, influence from the environment (i.e., disease, eutrophication) and physical parameters (i.e., wave energy) may reduce the suitability of the site. Examination of adult oysters at a potential restoration site (Chapter 3) is another key component of future research. A reef can only be self-sustaining if adults can reproduce spat and the spat recruit to the reef and begin to grow and reproduce themselves. If reproductive capacity and tissues are impacted by site-specific abiotic factors, a long-term restoration would require constant additions of spat-on-shell. Additionally, reefs in an estuary act like metapopulations, interconnected by the planktonic spat and recruitment patterns; a single reproductive reef would likely not thrive unless other reefs were near (Haase et al. 2012). Finally, the influence of Cd and Hg accumulation on physiology was examined to determine if these non-essential metals (present in the HRE, with several "hot spots" of high concentration; NOAA 1993, 1995) may cause physiological declines that would affect survival and growth of juvenile oysters. It was found that Hg did not lead to any physiological changes, even at levels 4-fold higher than the EPA maximum; however, high levels of Cd exposure (2.4μM; also 4-fold higher than the EPA maximum) did cause changes in subcellular physiology (Chapter 4). As sites are selected for potential restoration, it is imperative that non-essential trace metals are examined as high sediment concentrations of Cd, and possibly other metals and organic contaminants (not examined here), will lead to subcellular physiological changes, reproductive decline, and mortality.

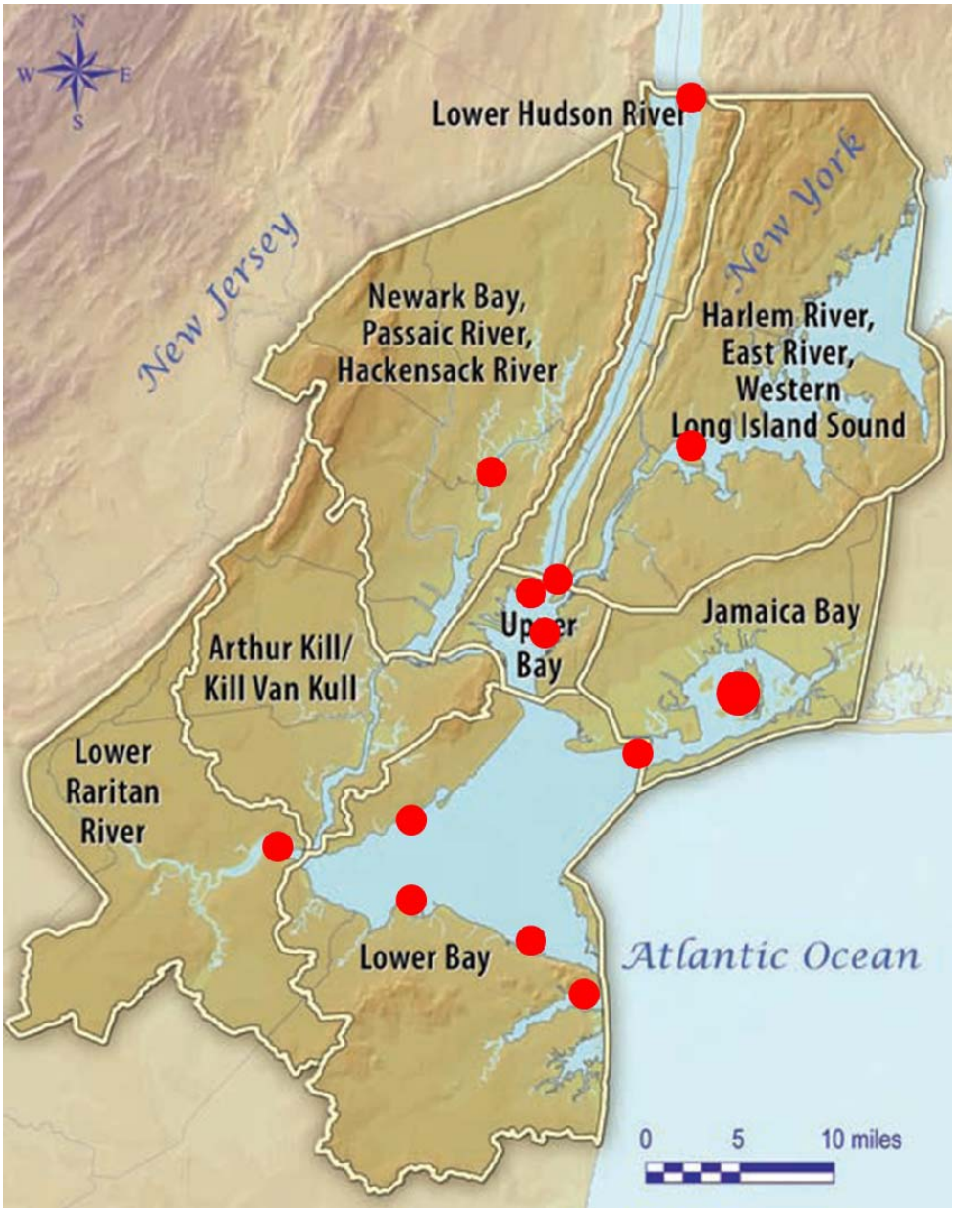

Figure 1: Map of the lower Hudson Raritan Estuary (reprinted from Hudson Raritan Estuary Comprehensive Restoration Plan, 2009). Circles represent significant oyster restoration projects since 2004. Sites include (clockwise from north) Hastings-on-Hudson (ORRP), Soundview Park, Bronx (NYC Parks, ORRP, this study), Jamaica Bay (reef site at Dubois Pt- NYCDEP, several sites- this study), Rockaway Inlet (this study), Navesink River/ Sandy Hook (NY/NJ Baykeeper), NWS Earle (NY/NJ Baykeeper), Keyport (NY/NJ Baykeeper), Western Raritan Bay (NY/NJ Baykeeper), Staten Island/ Raritan Bay (ORRP), Bay Ridge Flats (ORRP), Liberty Island (NY/NJ Baykeeper), Governor's Island (ORRP), and Hackensack River (NY/NJ Baykeeper).

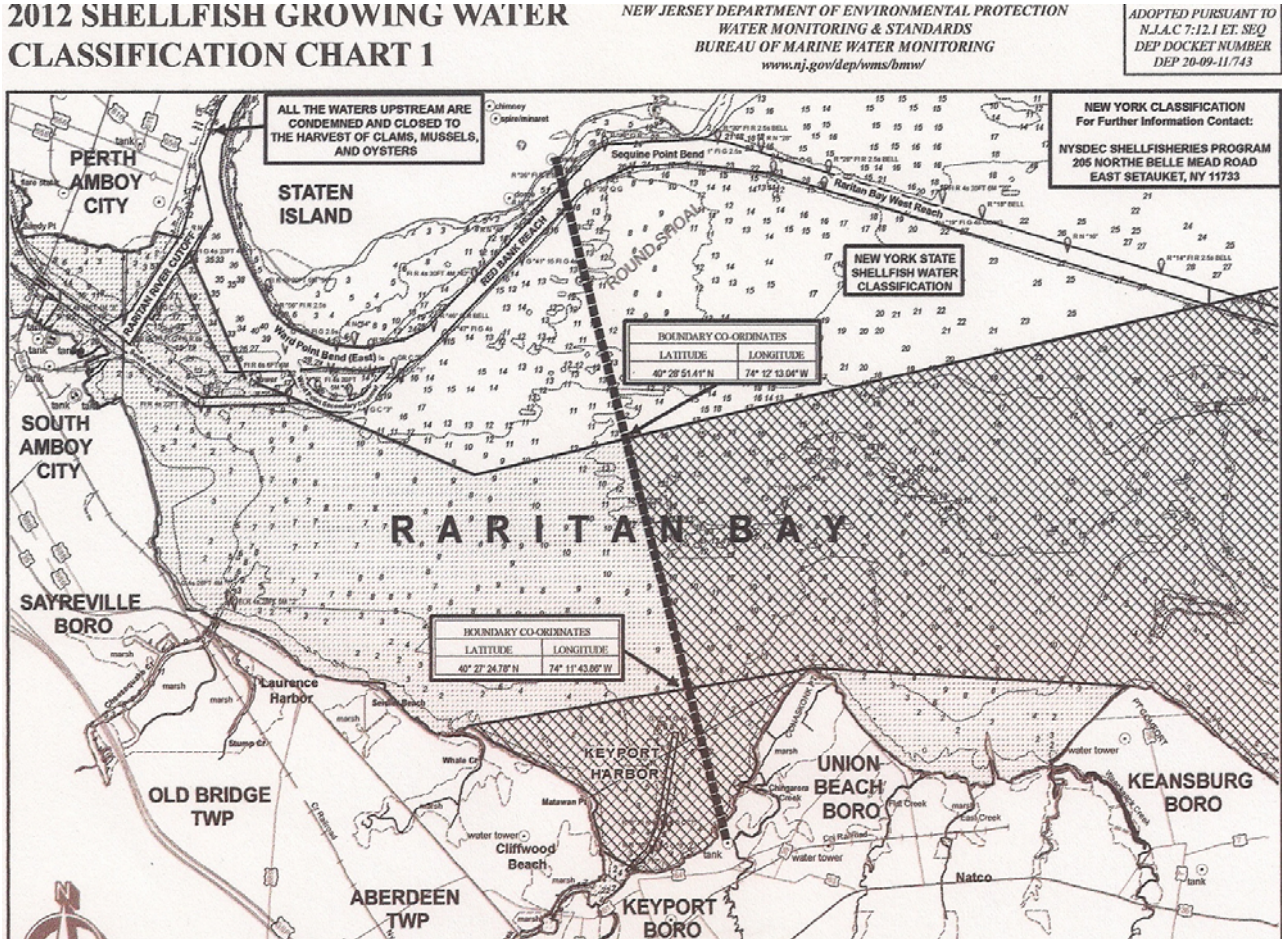

Figure 2: Raritan Bay Shellfish classifications (as per NJ-DEP, 2012). Areas shaded (light and dark) are closed to shellfishing. All waters in the Arthur Kill (north of Staten Island/ Perth Amboy) are closed as well. Areas unshaded are deemed NY-DEP property and are subject to NY regulation. All areas under NJ-DEP regulations are shaded; light shading means "prohibited area", dark shading deems areas "special restricted area: water condemned for the harvest of oysters, clams, and mussels except harvesting for further processing may be done under special permit from NJ DEP" (NJ-DEP, 2012). This includes Keyport Harbor (where the previous NY/NJ Baykeeper reef was located) and over ½ of Raritan Bay.

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