ABSTRACT

Title of Thesis: SURVIVAL AND BIOCHEMICAL HEALTH INDICATORS OF *ELLIPTIO COMPLANATA* DEPLOYED IN ANACOSTIA RIVER TRIBUTARIES FOR MONITORING OF PERSISTENT ORGANIC CONTAMINANTS

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The Anacostia River is one of three regions-of-concern in the Chesapeake Bay Watershed. Persistent organic pollutants (POPs) such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and organochlorine pesticides are known to accumulate in sediment and biota within portions of the Anacostia system, but on-going contaminant sources are poorly understood. The current study investigates relative contaminant burdens in the freshwater mussel *Elliptio complanata* deployed in six non-tidal Anacostia tributaries and an out-of-system reference site. Mussels acquire contaminants during feeding and are a useful tool for monitoring POPs transporting through the system. Mussels were effective at identifying sites with high contaminant loads. The study also investigates the suitability of Anacostia tributaries for reintroduction of *E. complanata* to increase benthic community diversity and potentially improve water quality. Survival and growth during deployment was very good for both

sampling seasons. Biochemical health parameters of deployed mussels suggest that conditions may be suitable for mussel reintroduction.

SURVIVAL AND BIOCHEMICAL HEALTH INDICATORS OF *ELLIPTIO COMPLANATA* DEPLOYED IN ANACOSTIA RIVER TRIBUTARIES FOR MONITORING OF PERSISTENT ORGANIC CONTAMINANTS

by

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Dedication

For my father, Lee Emerson Harrison.

Thank you for all of your love and support
and for passing down your love for the outdoors.

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I would like to thank my lab mate Shannon Edmonds for her assistance in the field and lab, and for her friendship. There were also many technicians and undergraduate students I am so grateful for. I would especially like to thank Natalie Jamison, Matt Mangialardo, Luis Chevez, and Ilana Herold for their many hours of help in the field and lab.

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Chapter 1: Introduction

Overview

The Anacostia Watershed is located within Montgomery and Prince George's Counties, MD, and the District of Columbia (Fig. 1.1). It includes a drainage area of approximately 456 km^2. The tidal portion of the Anacostia River is approximately 14 km, which begins at the confluence of the Northeast Branch and Northwest Branch near Bladensburg in Prince George's County and extends to its confluence with the Potomac River in the District of Columbia. The current average imperviousness of the watershed is 22%, exceeding 50% in many of the small urbanized tributaries (DOEE, 2011). Destruction of wetlands and natural riparian buffer zones greatly decreased the filtration capacity of harmful substances. Thus, the watershed is especially susceptible to contaminants entering the tidal river through nonpoint sources, spills, combined sewer outfalls, and storm drains (Velinsky et al., 2011; DOEE, 2016a).

The Anacostia River is listed as one of three Chesapeake Bay regions of concern identified by the US Environmental Protection Agency (EPA), along with Baltimore Harbor, MD, and the Elizabeth River, VA. The major chemical concerns in the Anacostia River are polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine (OC) pesticides, all of which can persist in aquatic sediments. Fish consumption advisories are in place in both Maryland and the District of Columbia due to levels of PCB concentrations that exceed impairment thresholds (MDE, 2010; MDE, 2018; DOEE, 2016b). Brown bullhead (*Ameiurus nebulosus*) have been used for the last two decades as indicator species of habitat quality in the Chesapeake Bay watershed and

have shown high prevalence of skin and liver tumors in the Anacostia River (Pinkney et al., 2014). Remedial efforts are ongoing to improve impacted areas of the watershed. One goal of these activities is to make the Anacostia River swimmable and fishable by 2025 (USACE, 2010).

The District Department of Energy & Environment (DOEE) is conducting a remedial investigation/feasibility study (RI/FS) aimed at assessing the nature and extent of pollution (RI), and evaluating the feasibility of various remediation options (FS) (DOEE, 2016a). The current status of pollutant loadings into the Anacostia River is unknown. Identifying point and nonpoint sources within the watershed is vital to ensure that remediated sediments are not re-contaminated. The current study was conducted as a two-year sampling plan to compare the bioavailability of organic contaminants among major Anacostia tributaries by deploying caged freshwater mussels for approximately 90 and 150 days each year.

Figure 1.1. Map of the Anacostia River and watershed in Washington, D.C. and Maryland.

Ecological Importance of Freshwater Mussels

Freshwater mussel diversity has declined precipitously due to habitat degradation or destruction. Of the 243 species in North America, 139 are listed as endangered, vulnerable, or of special concern (see the *IUCN Red List*: iucnredlist.org). This is largely attributed to the ecological sensitivity of mussels and their unique life cycles being interrupted by anthropogenic activity. Among the largest ecosystem threats to mussels are water pollution, loss of habitat, and the construction of dams and other fish barriers to the movement of host fish which restricts population expansion (Neves et al., 1997).

Diverse mussel populations are a sign of ecosystem health (Martel et al., 2003). In addition to supporting biodiversity of other benthic organisms (Vaughn and Spooner, 2006), they are a food source for animal species such as mink, otters, and herons. Efforts to conserve mussel species are crucial to prevent further population decline and extinction. The National Strategy for the Conservation of Native Freshwater Mussels was prepared by the National Native Mussel Conservation Committee (NNMCC), collaboration of numerous state and federal entities to address national concerns for mussels by increasing the information exchange between states (NNMCC, 1998). This was one of the first steps toward creating common goals for protecting species and restoring mussel habitat (Haag and Williams, 2014). The strategy was updated in 2016 by the Freshwater Mollusk Conservation Society (FMCS) to include the advancements and successes in the last two decades as well as to prioritize critical issues that remain to be addressed and to recommend new strategies for conservation (FMCS, 2016). According to Haag and Williams (2014), the reintroduction of mussels to areas they once inhabited may accelerate recovery of previously degraded habitats. This approach has been applied

in the Delaware River watershed; one goal of the Partnership for the Delaware Estuary (PDE) is to reintroduce freshwater mussels to improve water quality and biodiversity (PDE, 2013).

Contaminant Uptake

Due to the limited chemical metabolism of persistent organic pollutants (POPs), concentrations taken up by freshwater mussels serve as a good indicator of the bioavailable concentrations in the system. The present study analyzed organic contaminants taken up by freshwater mussels across the major Anacostia River tributaries over a prescribed deployment period. Similar studies have been completed in the Great Lakes region and support the idea that bivalves are effective biomonitoring tools to determine contamination in aquatic systems (Drouillard et al., 2013; Gewurtz et al., 2003; Richman et al., 2011). Mussels have been used successfully as bioindicators of environmental contamination in many studies since the 1970's, although many were focused primarily on metals rather than organic contaminants (Newton and Cope, 2007). The US National Oceanic and Atmospheric Administration (NOAA) National Status and Trends (NS&T) Mussel Watch program started in 1986. Mussel Watch is the longest running continuous contaminant monitoring program in the United States monitoring more than 140 contaminants of concern (Kimbrough et al., 2008). The program extended its monitoring efforts to the Great Lakes in 1992 to monitor contamination in Areas of Concern.

Biochemical and Mussel Health Endpoints

Carbohydrate content is the main energy storage component in mussels and is expected to vary seasonally, increasing during the fall for overwintering (Gray and Kreeger, 2013). Glycogen is the primary storage form of carbohydrates in mussels; thus, reductions in glycogen stores could reduce the ability of mussels to cope with natural and anthropogenic stressors in the environment (Patterson et al., 1999). According to Naimo and Monroe (1999), specific tissues such as mantle could be more sensitive to stressors than whole-body homogenates and therefore more representative of energy stores.

Protein is also essential for maintaining mussel condition. According to Gray and Kreeger (2014), seasonal variability in energy stores such as protein and possibly lipid content are necessary to adapt to changing conditions. Protein stores, especially those of vitellin-like proteins, have the potential to increase over time when caged mussels are exposed to municipal effluents (Gagné et al., 2004), suggesting that elevated protein content over time is an indicator of stress in mussels.

Lipid is another important energy store used for growth and reproductive needs (Pernet et al., 2007; Gray and Kreeger, 2014). Lipid content has been used along with carbohydrate and protein to assess mussel health, but little is known about the specific energy requirements and how they vary seasonally and with reproductive energy needs.

The biochemical composition of bivalves has been used in various studies to determine the overall health of the organisms over time. Understanding seasonal patterns may be useful in predicting response to environmental changes (Baker and Hornbach, 2001) and how the energy constituents change related to gametogenesis. In a study by Zandee et al. (1980), *Mytilus edulis* exhibited increases glycogen, protein, and lipid

content from spring to autumn when there was sufficient food available. While lipid remained constant over the seasonal changes until the beginning of the spawning period, glycogen and protein were lower in the winter and spring possibly due to less food availability and the onset of gametogenesis (Zandee et al., 1980).

The utilization of energy stores when food is limited is an important consideration for long-term survival of mussels. This is a complex process that is not fully understood. Patterson et al. (1999) reports a decline in glycogen stores of food-limited threeridge mussels (*Amblema plicata*) compared to adequately fed individuals. According to Roznere (2016) and McCue (2010), metabolism of energy stores during starvation may begin with the breakdown of glycogen to synthesize glucose; in some cases, a temporary spike in glucose occurs to "overcompensate" for the lack of food before slowly declining again. In the case of *A. plicata*, after using glycogen stores, lipids begin to be utilized for energy if food limitation continues (Roznere, 2016). More prolonged starvation leads to loss of protein content; this loss of protein reserves was observed from autumn through winter in *Mytilus edulis* (Zandee et al., 1980). Studying the way that energy use changes in response to seasonal and food availability changes is beneficial to better understand fitness of mussels throughout seasonal changes and gametogenesis.

Activity of glutathione S-transferase (GST), glutathione reductase (GR), and catalase (CAT) in mussel digestive gland tissue were analyzed in this study as possible indicators of an oxidative stress response, following exposure to organic contaminants. According to Doyotte et al. (1997), these antioxidant defense mechanisms can be useful in the biomonitoring of environmental contaminants in combination with animal condition and chemical analysis of contaminant body burdens. Glutathione S-transferase

enzymes serve as Phase II antioxidant defense mechanisms against many xenobiotics. These enzymes are responsible for defending against oxidative stress-associated DNA damage and lipid peroxidation as a consequence of contaminant exposure (Newton and Cope, 2007). Gowland et al. (2002) found a correlation between the induction of GST and exposure to lipophilic 5- and 6- ring PAHs in blue mussels (*Mytilus edulis*). Glutathione reductase is an antioxidant enzyme integral for catalyzing the reduction of glutathione disulfide to reduced glutathione (GSH) which is critical in resisting oxidative stress (Hellou et al., 2012). Catalase plays an integral role in converting hydrogen peroxide to oxygen and water, assisting in the prevention of free radicals containing reactive oxygen species. It is beneficial to study multiple biomarker responses as there are many antioxidant defense activities which may overlap in the presence of stressors (Borković et al., 2005).

Study Objectives

This thesis was part of an effort to investigate loadings and concentrations of organic contaminants in Anacostia River tributaries. There were two main project goals: 1) measure the bioaccumulation of select persistent organic contaminants in freshwater mussels and passive samplers deployed at various tributary locations; and 2) monitor the survival and health indicators of mussels following the period of deployment. Results of this study are meant to assist in determining a strategy to enhance mussel populations in the watershed. The bioaccumulation data will be published by Dr. Upal Ghosh of the University of Maryland Baltimore County (UMBC). Currently, one year of contaminant data is available (Ghosh et al., 2018), which is presented in the discussion portion of this thesis to aid in interpretation of the survival and health data.

The first objective (addressed by Dr. Ghosh) was to assess the accumulation of contaminants in Anacostia tributaries by mussels and passive samplers. Concentrations of PAHs, PCBs, and OC pesticides in mussel tissue (determined by collaborators at UMBC). Contaminant concentrations were compared between tributaries and used to estimate ongoing fluxes of pollutants into and out of various compartments of the Anacostia River (sediment, porewater, surface water, and atmosphere). Our hypothesis was that the body burdens of contaminants in mussels would differ significantly across study locations. Results of this objective were meant to assist in identifying areas of primary concern.

The second objective (addressed specifically in this thesis) was to evaluate survival and indicators of health of mussels after approximately 90 and 150 days of deployment within the primary tributaries of the Anacostia River. Survival of mussels during the deployment period was expected to be good, as a hardy species was chosen for the study. Our hypothesis was that indicators of health in deployed mussels would differ significantly across study locations including the reference location. Results of this objective were meant to aid in identifying tributaries suitable for reintroduction of mussel populations.

Chapter 2: Materials and Methods

Experimental Design and Study Locations

To address the goals of measuring bioaccumulation and documenting survival and health of mussels, the experimental design consisted of the collection of adult *Elliptio complanata* from a reference location (Zekiah Swamp, La Plata, MD) and deployment at the head of tide in six tributaries of the Anacostia watershed (Figs. 2.1, 2.2). Thus, mussels were collected at Zekiah Swamp, a location selected based on its abundant *E. complanata* population and a reconnaissance that indicated extremely low concentrations of organic contaminants. *E. complanata* was selected based on the hardiness of the species and on its previous use in the Great Lakes (Raeside et al., 2009; Gewurtz et al., 2011; Richman et al., 2011). It is the most common freshwater mussel in Maryland and was recently detected in low numbers in the tidal portion of the Anacostia River (Matthew Ashton, Maryland DNR, pers. comm.). The current and historical abundance in nontidal areas is largely unknown. The Standard Guide for Conducting In-situ Field Bioassays With Caged Bivalves (ASTM, 2002) provides a basic methodology for controlled field exposures using bivalves.

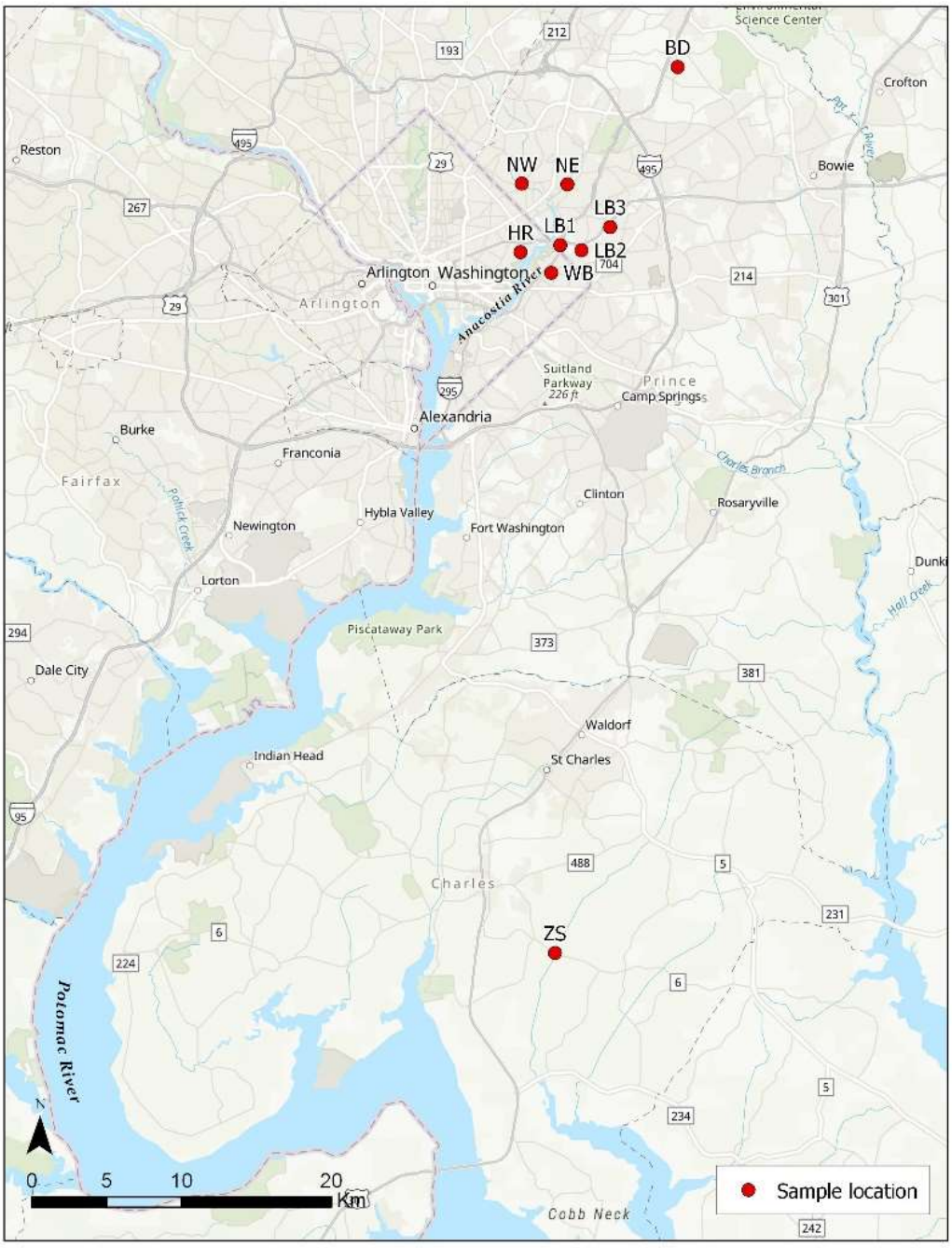

Figure 2.1. Map of all mussel deployment locations for 2016 and 2017 in Maryland and the District of Columbia, including the out-of-system reference. NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB1 = Lower Beaverdam 1; LB2 = Lower Beaverdam 2; LB3 = Lower Beaverdam 3; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp. (LB1 and LB3 were only sampled during 2017.)

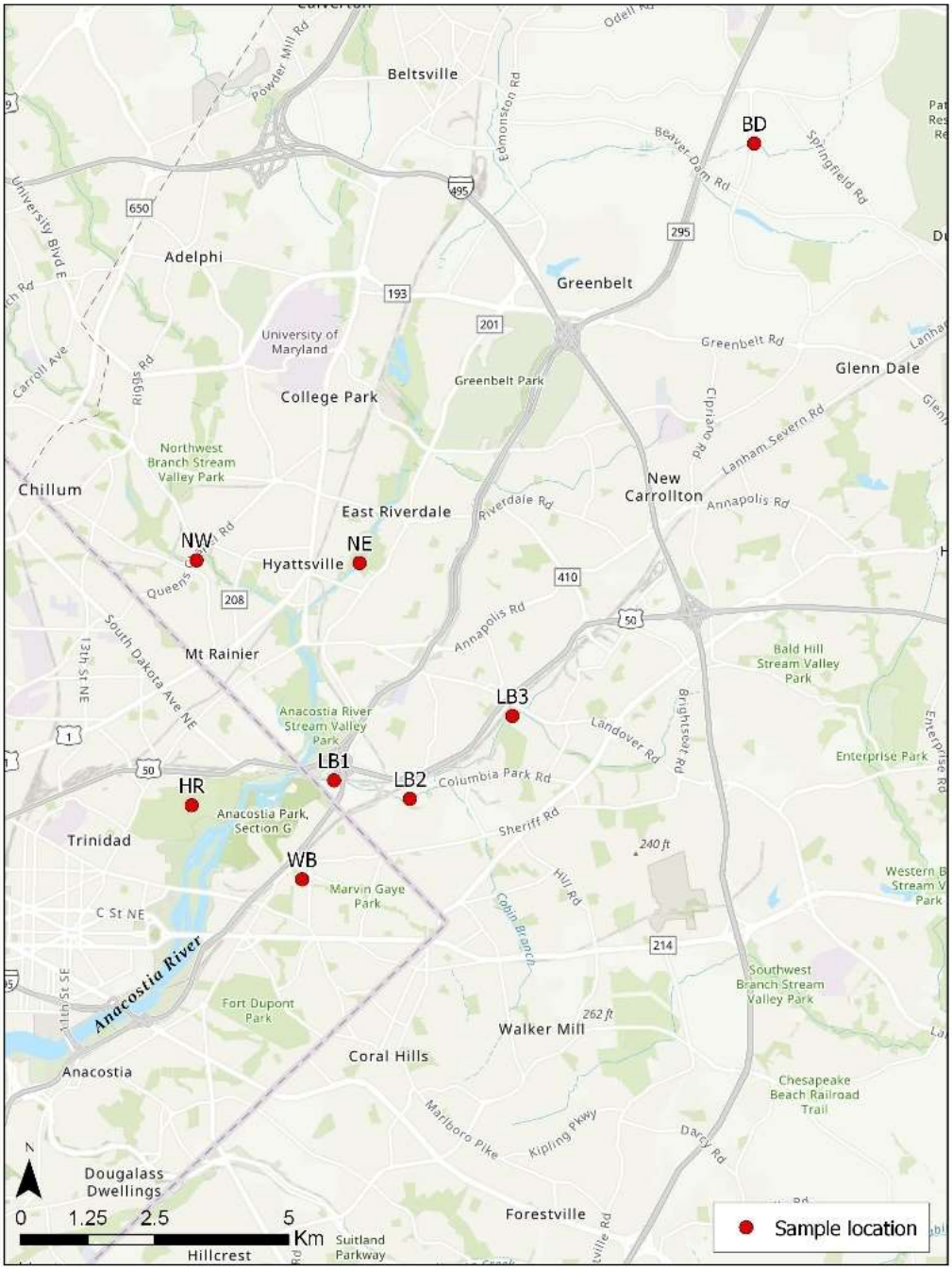

Figure 2.2. Map of Anacostia watershed mussel deployment locations for 2016 and 2017 in Maryland and the District of Columbia. NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB1 = Lower Beaverdam 1; LB2 = Lower Beaverdam 2; LB3 = Lower Beaverdam 3; WB = Watts Branch; BD = Beaverdam Creek. (LB1 and LB3 were only sampled during 2017.)

Mussels collected from Zekiah Swamp in early June 2016 and 2017 were tagged, and length and weight of each mussel were recorded before and after deployment. Some mussels were shucked the day after collection to be used for pre-deployment analyses; the rest were deployed at Zekiah Swamp and at the chosen Anacostia watershed locations. Where possible, deployment locations were selected based on proximity to US Geological Survey (USGS) stream gages so that the flow regime and quantity of water passing over the mussels would be known. Deployment near gages was possible at Northeast Branch, Northwest Branch, Hickey Run, Lower Beaverdam 1 (2017), and Watts Branch. At each location, 6 cages (5 in 2017) were deployed with 8 mussels per cage. Six of the 8 mussels were retrieved from each cage at 91 days (2016 and 2017) and the remainder were retrieved at 146 days (2016) or 154 days (2017). Mussels from the first retrieval were used for both contaminant bioaccumulation analysis and mussel health indicators; those from the second retrieval were used only for the mussel health indicators of total protein and total carbohydrate content. For 2017 mussels deployed for 91 days, the content of glycogen in mantle tissue was analyzed as an additional measure of carbohydrate storage. For the same mussels, the digestive gland activity of regulating enzymes such as glutathione and catalase were analyzed as oxidative stress endpoints.

Mussels were deployed alongside passive samplers for comparison of contaminant uptake in mussels deployed for 91 days in 2016 and 2017. Contaminant bioaccumulation was the foundation of the experimental design for this study. This objective was the driving influence in choosing the mussel species, deployment durations, and caging design. Data from contaminant analyses are not reported in the

results for this study but will be part of the discussion as the results in Ghosh et al. (2018) relate to the findings of the current study.

Characteristics of Deployment Locations

Land use data for the study locations (Table 2.1) were obtained from the Anacostia Watershed Restoration Project (AWRP, 2009), USGS stream gage data, and from the Maryland Biological Stream Survey (MBSS 2001). Zekiah Swamp land use information is from the 2011 National Land Cover Database (NLCD) (Homer et al., 2015). Locations were chosen based on accessibility from the road or stream bank, and by water depth. A depth range of 0.3-1m was selected to ensure the cages remained to be submerged throughout dry periods but still allowed us to reach the cages when the water was high.

Table 2.1. Land use and characteristics of each subwatershed for the tributaries chosen for deployment. These reflect the overall catchment and are not reflective of specific deployment locations. *LB1 and LB3 were added for 2017 and are within the general Lower Beaverdam catchment.

Site	Latitude	Longitude	Area (mi^2)	Impervious (%)	Wetlands (%)	Forest Cover (%)	Agriculture (%)	Urban (%)
NE	38.95290	-76.92990	7.2	37	0.3	40	19.4	37.2
NW	38.95276	-76.96500	41.7	19	1.3	28	4.1	61.6
HR	38.91162	-76.96490	1.7	41	1.1	14.4	no data	53
LB1	38.91631	76.93438						
LB	38.91350	-76.91800	14.9	32	0.8	25	12	72
LB3	38.92776	76.89632						
WB	38.89960	-76.94080	3.8	31	0.001	22	no data	63.1
BD	39.02470	-76.84680	13.8	6	7.5	60.7	17	no data
ZS	38.49150	-76.92700	82.7	4	12.1	59.3	17	19

Lower Beaverdam Creek is mostly contained within Prince George's County, with 0.2% of this subwatershed located in the District of Columbia. Impairments in Lower Beaverdam Creek include high volumes of runoff and pollutant and trash loadings (AWRP, 2009). In some major tributaries of Lower Beaverdam Creek, concrete lined channels are in place, including the area behind Smith & Sons scrap metal processing facility near deployment location LB1. These channels were built in the 1950's and 1960's to straighten portions of the tributaries and prevent flooding. The channels, along with other barriers such as culverts and pipes, present significant restoration challenges for the area. Lower Beaverdam Creek contains the greatest proportion of industrial land use of all study locations (17%). Other major land uses are residential (44%) and forest cover (25%).

In 2016, only one deployment location was selected in this tributary, Lower Beaverdam 2, in a residential area near the North Englewood Playground in Cheverly, Maryland (Fig. 2.1). The deployment location is not channelized and includes forested areas on both sides, although banks are incised and degraded.

For the 2017 deployment, two additional sites were chosen along Lower Beaverdam Creek to further assess contaminant bioaccumulation and begin to identify sources of contamination. Lower Beaverdam 1 (LB1) is located near Cheverly, Maryland, approximately 100 m downstream of Joseph Smith & Sons, Inc. and adjacent to a former wastewater treatment plant. This deployment site was tidal (the only one of the study) with water depth changing substantially depending on tidal stage. For this reason, gage height was checked before visiting the deployment site to ensure access. Behind the upstream recycling facility this tributary is a concrete-lined channel which flows out into

an area with degraded stream banks. Lower Beaverdam 3 (LB3) is located in a residential area downstream of the Landover metro station. Due to steeply-incised banks, a rope was anchored above this deployment location for access to perform weekly monitoring.

The Northeast Branch (NE) subwatershed, located entirely within Prince George's County, MD, is formed by the convergence of Indian Creek and Paint Branch. Land use within the subwatershed includes residential (52%), forest (40%), and commercial (10%). Mussels were deployed at a site adjacent to Tanglewood Drive near Fletcher's Field (Fig. 2.1). The Northeast Branch and Northwest Branch converge to form the Anacostia River. The Northwest Branch (NW) is mostly within Montgomery County, with 26% of the subwatershed in Prince George's County and Washington, DC. At the deployment location, the Northwest Branch is wide and varies in depth, with mostly silts and sands. Land use within the subwatershed includes residential (52%), forest cover (28%), agricultural use (4%) and parkland (7%) (AWRP, 2009).

Hickey Run (HR), located entirely within the District of Columbia, has the most impervious surface of the subwatersheds chosen for this study (Table 2.1). The deployment location is in the United States National Arboretum near Beechwood Road. North of Route 50, the main stem (~823 m) and all tributaries of Hickey Run are piped (AWRP, 2009). Remains of an abandoned brick factory now owned by the arboretum are apparent in the streambed, which is comprised of whole bricks and fragments along with mixed sediments as described above. The area is very shallow (~30 cm) with scoured banks. Sewer and oil leaks, as well as urban runoff, have contributed to poor water quality and low biodiversity (AWRP, 2009). Land use in the Hickey Run subwatershed is predominantly parkland (34%), industrial (30%), and residential (29%) (AWRP, 2009).

Watts Branch (WB) flows through the District of Columbia and Prince George's County. In 2016, this deployment location was situated approximately 100 m upstream of the USGS gage. In 2017, the deployment location was moved approximately 200 m upstream due to restoration efforts taking place at the 2016 deployment location. Land uses are residential (70%), forest cover (22%), and parkland (8%) (AWRP, 2009).

Beaverdam Creek (BD) is a tributary of the Northeast Branch. This subwatershed is entirely within Prince George's County, with the majority within the US Department of Agriculture (USDA) Beltsville Agricultural Research Center (BARC). The deployment location for this study was accessed from Soil Conservation Road. This location was selected based on adequate water quality and benthic macroinvertebrate data that suggested mussels could survive in this tributary. Land use is predominantly forest cover (61%) and agricultural (17%) (AWRP, 2009).

Zekiah Swamp Run (ZS) was chosen as an out-of-system reference location for the study, based on suitable habitat, water quality, and an abundant population of freshwater mussels. Land uses in the Zekiah Swamp watershed are forest cover (59%), urban (19%), agricultural (17%), and wetlands (12%) (AWRP, 2009).

Field Methods

Cage System Design and Deployment

Cages to hold mussels during deployment were constructed from polyethylene containers (406.4 mm x 304.8 mm x 114.3 mm; Schaefer Systems, 52356, US Plastic Corporation, Lima, Ohio). Plastic mesh (6.34 mm) was secured to the bottom, sides, and lid on the inside to ensure that water could flow through the cage while containing the

mussels inside the cage. On May 12, 2016, six high-density polyethylene cages (Fig. 2.3) were placed at each of six location within the Anacostia River watershed as well as the reference location at Zekiah Swamp, for a total of 42 cages and 48 mussels per location. Cages were labeled according to site and replicate letter (A-F for 2016; A-E for 2017). Cages were filled halfway with gravel and substrate from the deployment site. Rebar (35 cm) and metal fence posts were anchored in place to secure each cage to the streambed. Stainless steel cable (3.175 mm) was threaded through two sides of each cage and through each piece of rebar/fencepost, then swaged into place using a crimping tool. Cages were left in the streambed for approximately one month before mussels were added, to assess the rate of sediment deposition and ensure that cages would remain anchored to the streambed following high flow or storm events.

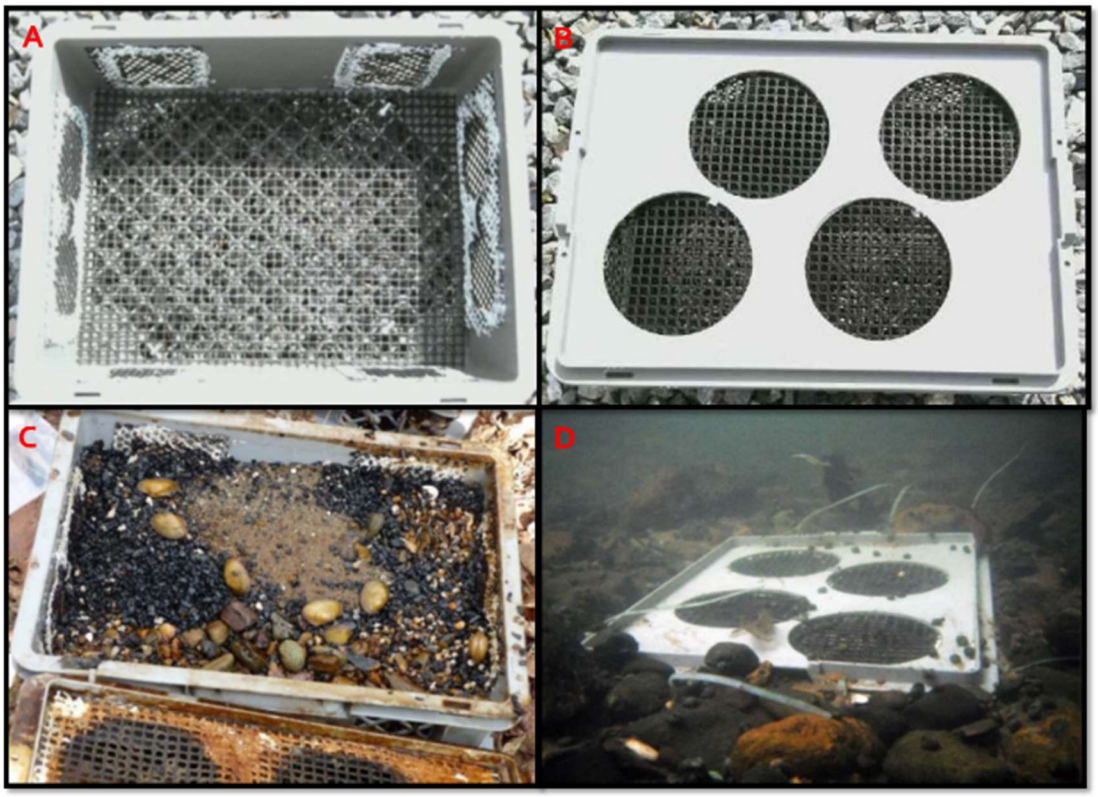

Figure 2.3. Cages constructed from polyethylene containers. (A, B) Cage and lid lined with mesh; (C) Cage filled halfway with sediment from deployment location; (D) Cage submerged and anchored.

Mussel Collection and Deployment

On June 7, 2016, a total of 408 adult *E. complanata* (60-80 mm in length) were collected from Zekiah Swamp. Mussels were collected via mask and snorkel and tactile methods. Mussels were placed in coolers for transport back to University of Maryland College Park (UMCP). All mussels were measured to the nearest 0.1mm with calipers, weighed to the nearest 0.1 g, and tagged using 2 yellow Hallprint tags (one tag per valve). Tags were adhered using Loctite Gel Control® glue. Twenty-four mussels were shucked,

placed in amber vials for pre-deployment analysis, and stored at -40°C. Mussels for deployment were sorted into batches of eight, placed in pre-labeled mesh bags and aerated overnight in coolers with water from the collection location. The following day, mussels were transported to the reference location and to the six Anacostia watershed locations. Eight mussels were placed into each of the six cages at each site for 336 total mussels deployed. Location of each mussel (site and cage) was recorded.

For the 2017 deployment, mussels were collected from Zekiah Swamp on June 5, 2017. Ten mussels were shucked for pre-deployment protein and carbohydrate analysis at UMCP. Twenty-four additional mussels were shucked for pre-deployment contaminant and lipid analyses at UMBC. Mussels were placed in 40 mL glass vials (Thermo Scientific # GVB100C) and stored at -40°C. Remaining mussels were labeled and held overnight as before. The following day, mussels were transported to the nine total deployment sites including the Zekiah Swamp reference location. Eight mussels were placed into each of the five cages for 360 total mussels deployed.

During the 2016 and 2017 deployment, mussel sites and cages were monitored once per week on average to remove any accumulated debris and ensure that all cages were still intact. On several occasions, cages were moved to a deeper part of the stream as water levels had decreased, exposing the tops of some of the cages. The water quality parameters of temperature, conductivity, pH, and dissolved oxygen were measured using a YSI 556 (Yellow Springs Instruments, OH) and recorded each time sites were visited.

Retrieval

At the first retrieval period (91 days in both 2016 and 2017), 6 of the 8 mussels from each cage (36 mussels/site, 252 total mussels in 2016; 30 mussels/site, 270 total

mussels in 2017) were collected and placed into mesh bags labeled according to site and cage. Bags were placed into 20 L buckets labeled according to site, with 4 L of spring water in each bucket. Water quality was also recorded at each site. On arrival at UMCP, an additional 4 L of spring water was added to each bucket and mussels were left to depurate overnight with gentle aeration.

At the second retrieval period (146 days in 2016; 154 days in 2017), the remaining two mussels were removed from each cage and transported to UMCP using the same methods as the first retrieval. At this time, all mussel cages were removed from each site and transported back to UMCP. Water quality was also recorded at each site.

Laboratory Methods

Tissue Preparation and Processing

The day after retrieval, mussels were weighed to the nearest 0.1 g and measured to the nearest 0.1 mm using calipers. Mussels were shucked with spatulas and scalpels over pre-weighed aluminum weigh boats, and wet tissue weight was recorded to the nearest 0.1 g. In 2016, mussel liquor was included in wet tissue weight; in 2017, the liquor was separated before recording wet tissue weight and then returned. Mussels were grossly sectioned with a single-edge razor blade and placed into 40mL vials pre-labeled with mussel tag numbers. Between each mussel, all tools were rinsed with DI water and ethanol. Between each site, all razor blades, water, and ethanol were replaced. Vials containing mussels were stored at -40C in a pharmacy-grade chest freezer. All shells were stored in closure bags labeled for each site for subsequent weighing. In 2017, the digestive gland and two pieces of mantle tissue (approximately 40-60 mg each) were

taken from two of the six mussels collected from each cage at the 91-day retrieval. These samples were stored in cryovials at -20°C; digestive gland tissue was used for glutathione and catalase analysis, and mantle tissue was used for glycogen analysis. These data were used as additional endpoints to determine if there is agreement with contaminant burdens of the mussels deployed for 91 days.

Mussels from the first retrieval were subsequently thawed and homogenized within vials using a Polytron PT 1035®. Samples held for pre-deployment analysis in 2016 were transferred from amber vials to 40mL vials and homogenized. (For 2017 we did not use amber vials, so no transfer was necessary.) Individual mussels were homogenized using two thirty second pulses, stopping the machine to remove any tissue from the blades between pulses. The homogenizer was rinsed with DI water between each mussel and DI water and methanol were used to clean the machine between each cage of mussels. Using pre-labeled, pre-weighed 40 mL vials, approximately 10 g from each of 3 mussels was weighed in an aluminum weigh boat and combined into a vial. These pooled samples were transported to UMBC for the analysis of PAHs, PCBs, organochlorine pesticides, and lipid content (Ghosh et al., 2018). Mussels were pooled to ensure that there was sufficient sample to run the analyses. The remaining homogenized tissue from each individual mussel was left in the original vials and stored at -40°C at UMCP for biochemistry analysis.

Homogenized mussels were lyophilized using a SP Scientific VirTis AdVantage 2.0 for 72 hours. This step was performed on 2 mussels per cage from the first and second deployment periods as well as all pre-deployment individuals for consistent

sample sizes during analysis. The lyophilized samples were pulverized with mortar and pestle, transferred to pre-labeled 20 mL glass vials, and stored at -20°C.

Biochemical Analyses

Mussels were analyzed for protein and carbohydrate content using pre-deployment, 91-day, and 146-day (2016) or 154-day (2017) freeze dried whole-body homogenates. These analyses were performed at UMCP using methods from Gray and Kreeger (2014) modified for use with 96-well microplates. Analysis at each time point allowed differences in concentration to be assessed across the seven deployment locations as well as changes in concentration at each location over the two deployment intervals. In 2017, mantle tissue and digestive gland from pre-deployment and 91-day mussels were used for glycogen (mantle) and glutathione and catalase analyses (digestive gland), respectively. Pre-deployment and 91-day mussels (2016 and 2017) were pooled in groups of 3 and analyzed for lipid content at UMBC using methods modified from Smedes (1999) (Ghosh et al., 2018).

Carbohydrate and Glycogen Analyses

Total carbohydrate was measured following methods from Gray and Kreeger (2014) which were modified from Dubois et al. (1956). Batches of 26 freeze dried samples of whole-body homogenates were prepared at a time. Samples of 2 mg were weighed to the nearest 0.01 mg and added to a 20 ml borosilicate test tube. Lab pure water (1.0 mL), 5% phenol (1.0 mL), and concentrated sulfuric acid (5.0 mL) were carefully added to each sample, vortexing between each addition. Samples were allowed to cool at room temperature for 25 minutes. Six standards were prepared using a solution

of soluble starch and water. One mL of each standard was added to each of 6 test tubes. Phenol and sulfuric acid were added to standards in the same way as they were added to samples. Triplicates of 200 µL aliquots of samples and standards were added to microplate rows. The samples were analyzed in the plate reader (Molecular Devices SpectraMax M2e) at 490 nm.

Mantle glycogen analysis was performed on wet tissue of 2017 mussels deployed for 91 days following methods based on Carr and Neff (1984) and protocols in the Glucose GO Assay Kit (Sigma GAG020). Each microplate was run with 12 samples. All buffers were prepared in advance. Between 30 and 40 mg of mantle tissue was weighed and recorded to the nearest 0.01 mg. Samples were placed in 1.5-mL microcentrifuge tubes. Sodium citrate buffer (100 mM; 600 µL) was added to each tube and homogenized using a polypropylene pestle. Samples were added to a boiling water bath and left for 5 minutes, cooled to room temperature, and centrifuged at 10,000g for 5 minutes. Supernatant from each sample (200 µL) was added in two rows of a 96-well microplate. The first row was treated with 10 µL of 1% amyloglucosidase. Sodium citrate buffer (10 µL) was added to the second row as an untreated control for the enzyme reaction. The microplate was incubated overnight at 25°C. The next day, in a new microplate, 2 µL of both treated and untreated samples were added to 198 µL of nanopure water in duplicate. Five standards were also added to the microplate in duplicate. The final microplate was prepared by adding 30 µL of samples and standards and 60 µL of G.O. reagent, prepared according to instructions in the Glucose GO Assay Kit. The final plate was incubated for 30 minutes at 37°C. The reaction was stopped with 60 µL of 12N sulfuric acid and read with a microplate reader (Molecular Devices SpectraMax M2e) at 540 nm.

Protein Analysis

Total protein was measured using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific; Cat. # 23225). Twenty-four samples of 10 mg each were prepared for each plate. Sample was weighed to the nearest 0.1 mg and added to a 20 ml borosilicate test tube. Sodium hydroxide (0.1 N NaOH) was added to the test tubes (4 mL). Test tubes were homogenized with a Polytron PT 1035 for ten seconds and sonicated for eight bursts of ten seconds each. An additional 4 mL NaOH was added to each tube. Samples were mixed with a vortex and decanted into loosely capped borosilicate test tubes. Samples were incubated at 60°C for 45 minutes. After incubation, samples were vortexed and centrifuged at maximum speed for 10 minutes. Eight standards and a working reagent were prepared during this time according to the BCA Protein Assay Kit protocol and 25 µL of each standard was added in triplicate to a 96-well flat bottom microplate (Thermo Scientific #15041). After samples were centrifuged, 25 µL of supernatant was added in triplicate to the microplate, along with 200 µL of working reagent. Samples were incubated at 37°C for 30 minutes and analyzed in a plate reader (Molecular Devices SpectraMax M2e) at 562 nm.

Glutathione and Catalase Analyses (2017)

Glutathione S-transferase (GST) and glutathione reductase (GR) were analyzed for 2017 mussels deployed for 91 days using wet weight samples of digestive gland tissue. All analyses were run using 10 samples at a time. Digestive gland tissue (40-60 mg) was weighed to the nearest 0.1 mg and placed into a 1.5 mL microcentrifuge tube. GST extraction buffer (600µL) was added to each tube. Samples were centrifuged at 10,000 g for 30 minutes. Activity of GST was determined in 10 µL supernatant using

methods by Habig et al. (1974) modified for a 96-well microplate as detailed by Frasco and Guilhermino (2002). Activity of GR was measured in a 20 µL supernatant by monitoring reduction of 5 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB; 30 µl) following the production of GSH by GR in the presence of 0.1 mM NADPH (30 µl) and 0.1 mM GSSG (90 µl) in 100mM potassium phosphate buffer pH 7.5 with 1 mM EDTA (30 µl). Increase in absorbance was monitored by reading the microplate at 412 nm for 5 minutes.

The activity of catalase was determined using a colorimetric assay. The reaction mixture consisted of 2 µl supernatant, 73 µl 50 mM potassium phosphate buffer pH 7.0, and 25 µl 200 mM H2O2. Samples were incubated for 5 minutes. The reaction was stopped with 900 µl 15 mM sodium azide. A 2 µl aliquot was transferred in duplicate to a 96-well microplate and 250 µl of a solution containing 0.25 mM 4-aminoantipyrine, 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and 0.2 units/ml horseradish peroxidase in 150 mM potassium phosphate buffer pH 7.0 was added. Absorbance was read on a microplate reader (Molecular Devices SpectraMax M2e) at 520 nm after 30 min and catalase activity was quantified using a H_20_2 standard curve.

Data Analysis

Analysis was performed using SigmaStat version 12.0 (SysStat Software, Inc., San Jose, CA USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey all-pairwise multiple comparison procedure. Datasets not passing assumptions of normality and homogeneity of variance were analyzed using a Kruskal-Wallis test by ranks, followed by a Dunn's all pairwise multiple comparison procedure. Measured starting lengths of mussels and lengths of the same mussels measured again

after deployment were compared using a one-way ANOVA. Significant differences in lengths between mussels at day 0 vs day 91, and at day 0 vs day 146 (or day 154 in 2017) were evaluated using a paired t-test. For the analysis of carbohydrate, glycogen, protein, lipid, and glutathione, ANOVA was performed on proportion data based on site to compare biochemical data between sites and with the reference location. Significantly different means or medians were determined at a level of $p < 0.05$. For protein and carbohydrate data, a one-way ANOVA was performed on proportion data for each location based on deployment time (day 0, day 91, and day 146 (154 for 2017)) to detect any significant variability over the deployment period. Datasets not passing assumptions of normality and homogeneity of variance were analyzed using a Kruskal-Wallis test by ranks, followed by a Dunn's all pairwise multiple comparison procedure. Comparisons were also made between the first and second deployment periods for each site using an unpaired t-test.

Chapter 3: Results

Survival and Growth

In 2016, survival was > 99.7%, with only one dead mussel of the 336 deployed. In 2017, there were two dead mussels of the 360 deployed. In addition, three cages were lost in 2017 due to storm events or theft before the 91-day retrieval; two at Northwest Branch and one at the Zekiah Swamp reference location. Two mussels were unaccounted for at Lower Beaverdam 2 on the 146-day retrieval. Aside from missing animals and cages, both deployment years had very high survival rates.

In 2016, no significant differences were found in lengths between locations at day 0 (ANOVA, $p = 0.303$), day 90 ($p = 0.029$) or day 146 ($p = 0.907$). The mean lengths of mussels from all 7 deployment locations increased over time from day 0 to day 91 (paired t-test; $p < 0.001$ for all locations, Table 3.1). Increase in shell length ranged from mean ± one standard deviation of 0.4 ± 0.7 mm (Hickey Run) to 1.6 ± 0.65 mm (Lower Beaverdam Creek). The mean length of mussels from all locations except Hickey Run increased from day 0 to day 146 (paired t-test; $p \leq 0.004$ for locations excluding Hickey Run). Increase in shell length ranged from 0.2 mm (Hickey Run; $p = 0.052$) to 2.1 mm (Northwest Branch). Apart from Hickey Run, all locations saw an increase in shell length equal to or greater than that of the mussels deployed at Zekiah Swamp (0.6 mm).

Table 3.1. Statistical comparisons of the initial and final lengths of 2016 mussels deployed for 91 days and 146 days (mean ± 1 standard deviation). Statistically significant comparisons ($p < 0.05$) indicated in bold.

	91-Day Mussels			146-Day Mussels		
	Initial Length (mm) n = 36	Day 91 Length (mm) n = 36	Paired t-test	Initial Length (mm)	Day 146 Length (mm)	Paired t-test
NE	70.0 ± 3.6	71.1 ± 3.5	**p < 0.001**	68.8 ± 3.1 n = 11	70.2 ± 3.4 n = 11	**p = 0.004**
NW	69.9 ± 4.3	71.0 ± 4.2	**p < 0.001**	68.0 ± 4.6 n = 12	70.1 ± 4.0 n = 12	**p < 0.001**
HR	68.1 ± 4.2	68.4 ± 4.2	**p < 0.001**	69.8 ± 4.7 n = 12	70.1 ± 3.1 n = 12	p = 0.052
LB	69.4 ± 4.1	71.0 ± 4.0	**p < 0.001**	69.8 ± 3.4 n = 12	71.1 ± 3.1 n = 12	**p < 0.001**
WB	68.9 ± 3.4	69.9 ± 3.5	**p < 0.001**	67.5 ± 4.5 n = 12	69.2 ± 4.6 n = 12	**p = 0.001**
BD	68.9 ± 4.3	69.5 ± 4.2	**p < 0.001**	69.0 ± 3.9 n = 12	69.8 ± 3.8 n = 12	**p = 0.001**
ZS	70.1 ± 4.0	70.8 ± 4.1	**p < 0.001**	70.6 ± 5.2 n = 12	71.2 ± 5.4 n = 12	**p < 0.001**

NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

In 2017, no significant differences were found in lengths among locations at day 0 (K-W, $p = 0.136$), day 90 (K-W, $p = 0.567$) or day 154 (ANOVA, $p = 0.201$). The mean lengths of mussels from all 9 deployment locations increased significantly from day 0 to day 91 (paired t-test; $p \leq 0.018$ for all locations, Table 3.2). Increase in shell length ranged from mean ± one standard deviation of 1.3 ± 1.9 mm (Hickey Run) to 2.3 ± 3.1 mm (Northwest Branch). The mean length of mussels from all locations except Watts Branch and Zekiah Swamp increased from day 0 to day 154 (paired t test; $p \leq 0.025$ for all locations excluding Watts Branch and Zekiah Swamp). Increase in shell length ranged from 0.4 ± 0.7 mm (Watts Branch) to 3.4 ± 3.1 mm (Lower Beaverdam 3).

Table 3.2. Statistical comparisons of the initial and final lengths of 2017 mussels deployed for 91 days (median with minimum and maximum values) and 154 days (mean ± 1 standard deviation). Statistically significant comparisons ($p < 0.05$) are indicated in bold.

	91-Day Mussels			154-Day Mussels		
	Initial Length (mm)	Day 91 Length (mm)	Paired t-test	Initial Length (mm)	Day 154 Length (mm)	Paired t-test
NE	67.1 (60.2, 75.6) n = 29	68.4 (61.5, 76.5) n = 29	**p < 0.001**	68.7 ± 5.8 n = 9	70.8 ± 5.1 n = 9	**p = 0.002**
NW	68.3 (62.2, 73.6) n = 18	71.0 (63.8, 78.0) n = 18	**p < 0.001**	71.4 ± 4.8 n = 6	73.0 ± 4.7 n = 6	**p = 0.025**
HR	69.3 (61, 75.4) n = 30	70.5 (62.5, 79.5) n = 30	**p < 0.001**	69.1 ± 5.6 n = 10	70.0 ± 5.1 n = 10	**p = 0.016**
LB1	70.3 (59.7, 75.8) n = 30	70.7 (61.2, 78.8) n = 30	**p < 0.001**	68.7 ± 4.9 n = 10	70.1 ± 5.0 n = 10	**p = 0.002**
LB2	68.0 (60.2, 74.3) n = 30	69.4 (62.1, 75.4) n = 30	**p < 0.001**	67.0 ± 5.4 n = 8	68.6 ± 4.8 n = 8	**p = 0.006**
LB3	68.0 (61.0, 76.0) n = 30	69.8 (62.8, 76.7) n = 30	**p < 0.001**	64.8 ± 4.2 n = 10	68.1 ± 5.9 n = 10	**p = 0.002**
WB	68.2 (62.9, 76.8) n = 30	68.5 (62.7, 76.8) n = 30	**p = 0.017**	66.1 ± 3.7 n = 10	66.5 ± 3.6 n = 10	p = 0.088
BD	68.7 (62.4, 76.6) n = 30	69.3 (61.6, 77.2) n = 30	**p = 0.018**	67.0 ± 3.9 n = 10	67.7 ± 3.6 n = 10	**p = 0.004**
ZS	68.5 (61.2, 75.4) n = 24	70.4 (61.7, 76.2) n = 24	**p < 0.001**	67.0 ± 3.9 n = 8	68.1 ± 4.4 n = 8	p = 0.110

NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB1 = Lower Beaverdam 1; LB2 = Lower Beaverdam 2; LB3 = Lower Beaverdam 3; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

Biochemical Results 2016

Carbohydrate

Only one statistically significant difference was found in carbohydrate concentrations between deployment locations at 91 days based on one-way ANOVA ($p = 0.002$). Specifically, Lower Beaverdam Creek had significantly higher carbohydrate content at this deployment interval than at Beaverdam Creek and Zekiah Swamp based on Tukey's multiple comparison test (Fig. 3.1). This difference did not persist in mussels deployed for 146 days (ANOVA, $p = 0.084$). Carbohydrate content was significantly higher in 146-day mussels than in 91-day mussels at three locations based on t-tests (Northeast Branch, Northwest Branch, and Beaverdam Creek; Table 3.4a). Variations in carbohydrate content for day 0, day 91, and day 146 mussels are shown in Fig. 3.4b. Carbohydrate content fluctuated at all locations over time excluding Lower Beaverdam and Watts Branch (Table 3.4b). At Northwest Branch, carbohydrate stores in 91-day mussels were lower than pre-deployment mussels, and moderately elevated in 146-day mussels. At all times, mean % carbohydrate on a dry weight basis fell between 32.8 % and 47.9 %.

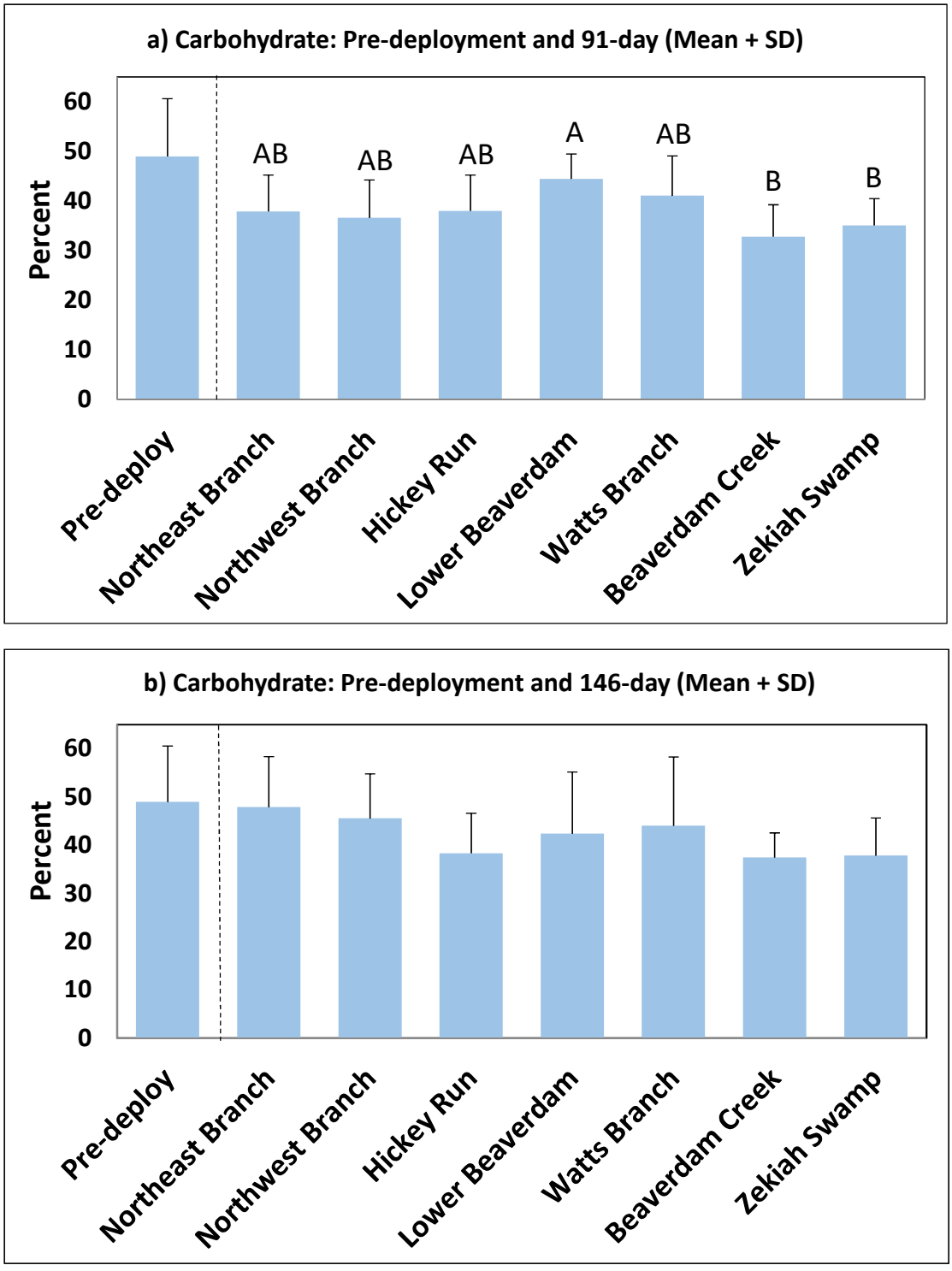

Figure 3.1. Carbohydrate content (% dry wt) in mussel whole-body homogenates (mean + SD): a) pre-deployment and 91-day mussels; b) pre-deployment and 146-day mussels. Columns with different letters are significantly different. Pre-deployment mussels are not compared statistically with post-deployment mussels in this analysis.

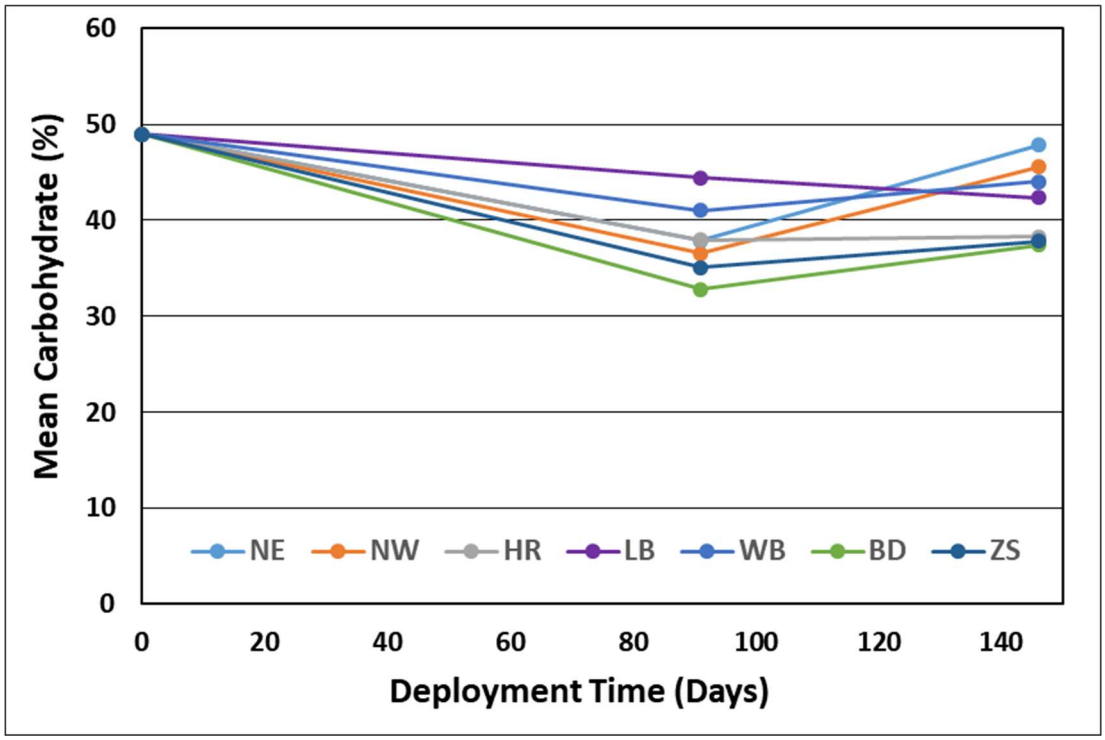

Figure 3.2. Variation of mean carbohydrate content (% dry wt) in mussel whole-body homogenates from pre-deployment, 91-day, and 146-day mussels at each location. Pre-deployment mussels are shown as one point as they were all collected from Zekiah Swamp. (NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.)

Protein

Protein concentrations showed statistically significant differences between 91-day mussels deployed at several locations based on one-way ANOVA ($p < 0.001$). Specifically, protein content in mussels deployed at Zekiah Swamp and Beaverdam Creek was significantly lower than that of several locations and protein content in Lower Beaverdam Creek was significantly higher than at all other deployment locations based on Tukey's multiple comparison test (Table 3.3; Fig. 3.3). The differences were not found to persist in the 146-day mussels. Protein content changed modestly between 91-

day and 146-day deployment; this difference was significant at several locations (Table 3.4a). Specifically, protein content was lower at Lower Beaverdam Creek and higher at Watts Branch and Beaverdam Creek between deployment intervals. Variations in protein content for day 0, day 91, and day 146 mussels are shown in Table 3.4b. Protein content was elevated at each deployment time at several locations, with the highest content in 146-day mussels (Northwest Branch, Lower Beaverdam, Watts Branch, and Beaverdam; Table 3.4b). At all times and locations, mean % protein on a dry weight basis fell between 24.2% and 31.7%.

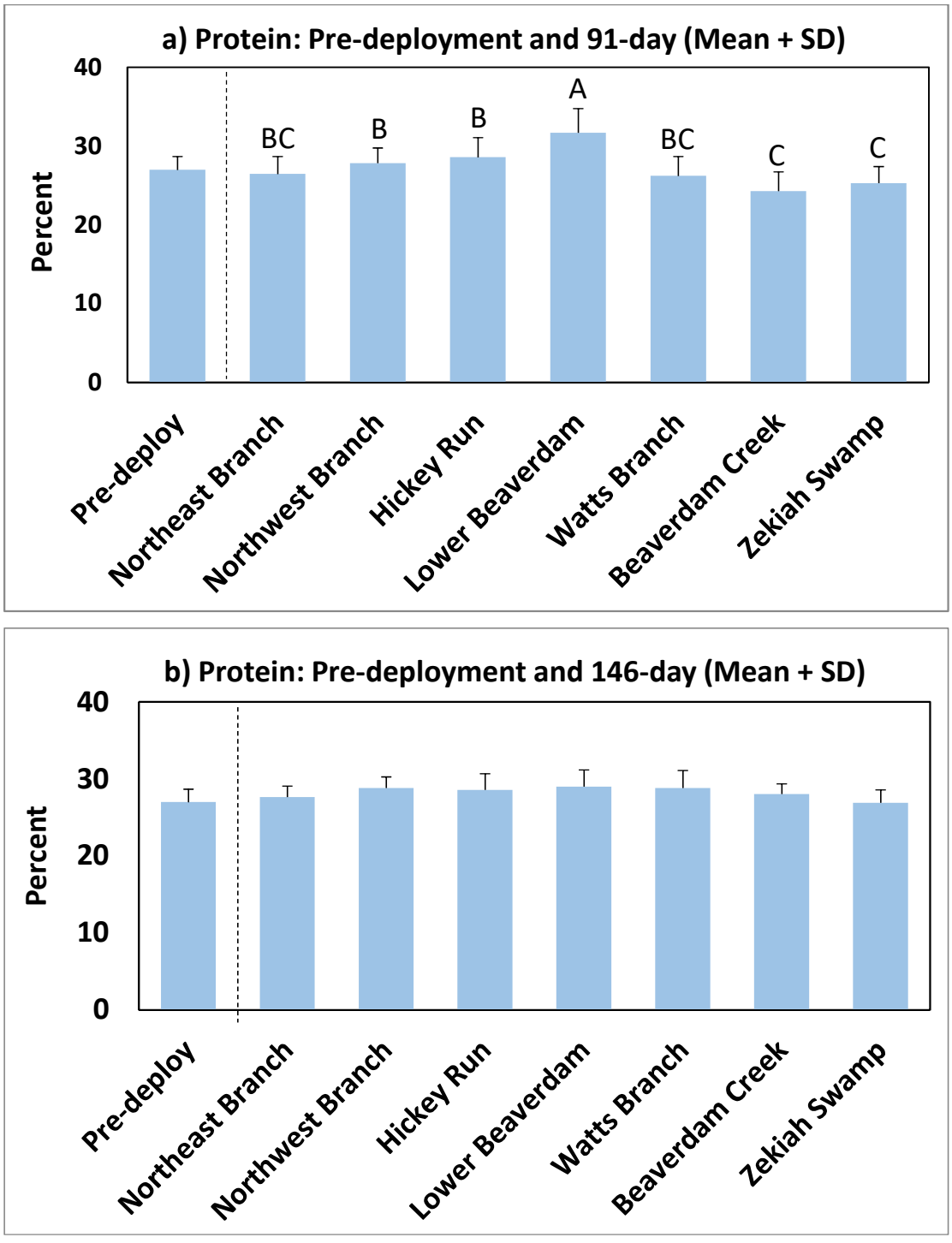

Figure 3.3. Protein content in 2016 mussel whole-body homogenates (% dry wt; mean + SD): a) pre-deployment and 91-day mussels; b) pre-deployment and 146-day mussels. Columns with different letters are significantly different; refer to Table 3.3 for sample size and explanation of statistics. Pre-deployment mussels are not compared statistically with post-deployment mussels in this analysis.

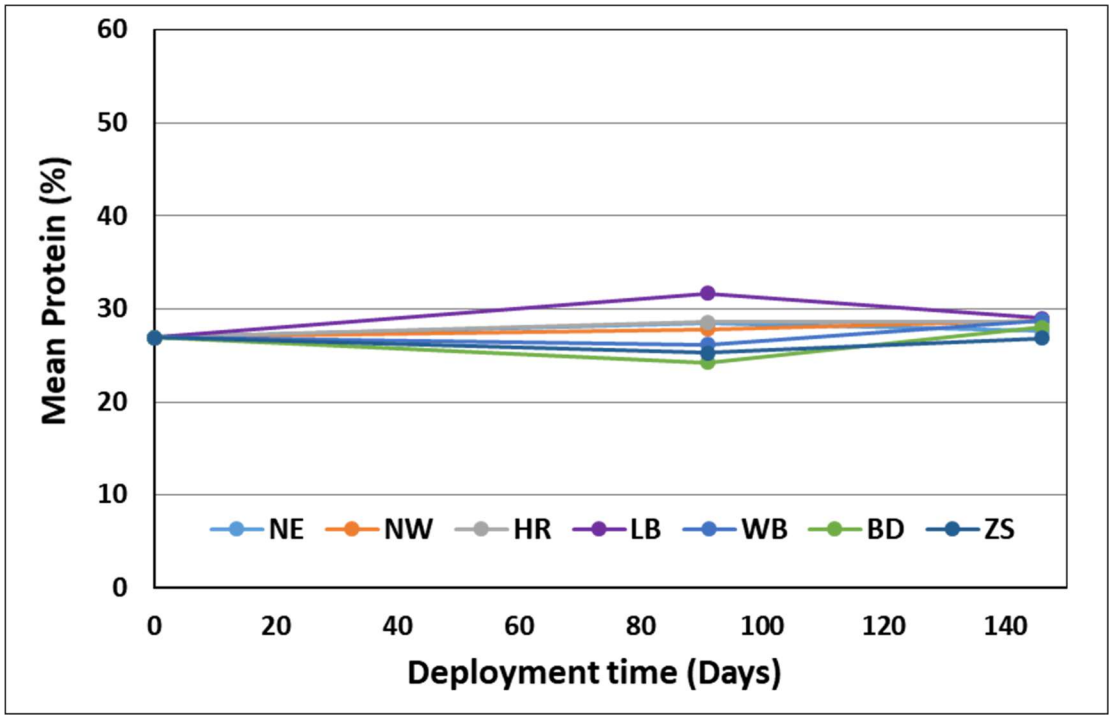

Figure 3.4. Variation of mean protein content (% dry wt) in mussel whole-body homogenates from pre-deployment, 91-day, and 146-day mussels at each location. Pre-deployment mussels are shown as one point as they were all collected from Zekiah Swamp. (NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.)

Lipid

In 2016, statistically significant differences in lipid concentrations were found between batches of pooled mussels from the 91-day deployment at several locations based on one-way ANOVA ($p < 0.001$). Hickey Run mussels showed significantly lower lipid content than all other Anacostia locations except Beaverdam Creek (Figure 3.5). Conversely, Lower Beaverdam mussels had higher lipid content than Beaverdam and Zekiah Swamp mussels. No comparison was made between 91-day and 146-day lipid

content as lipid was not measured for 146-day mussels. Mean lipid % on a dry weight basis ranged from 2.9% to 3.7% across all deployment locations.

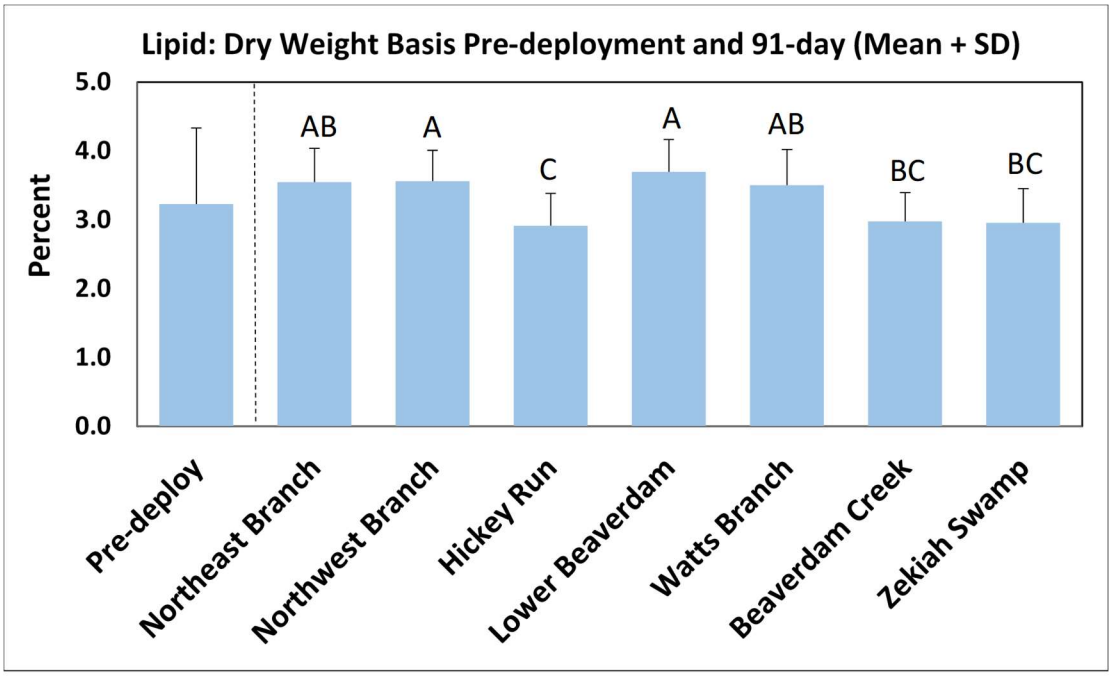

Figure 3.5. Lipid content in 2016 mussel pooled whole-body homogenates comprised of 3 mussels (% dry wt; mean + SD) for mussels prior to deployment (pre-deploy) and following 91-day deployment. Columns with different letters are significantly 1different; refer to Table 3.3 for sample size and explanation of statistics. Pre-deployment mussels are not compared statistically with post-deployment mussels in this analysis.

Table 3.3. Biochemical data (mean + SD) on a percent dry weight basis for 2016 mussels deployed for 91 and 146 days in six Anacostia tributaries and Zekiah Swamp

	NE (n=11)	**NW (n=12)**	**HR (n=12)**	**LB (n=12)**	**WB (n=12)**	**BD (n=12)**	**ZS (n=12)**	**Statistical Analysis[a]**
				0-91 Days				
Carbohydrate	37.9 ± 7.3 AB	36.6 ± 7.6 AB	38.0 ± 7.2 AB	44.4 ± 5.0 A	41.0 ± 8.1 AB	32.8 ± 6.4 B	35.1 ± 5.4 B	$p = 0.002$
Protein	26.4 ± 2.2 BC	27.8 ± 1.9 B	28.6 ± 2.5 B	31.7 ± 3.1 A	26.2 ± 2.5 BC	24.3 ± 2.5 C	25.3 ± 2.1 C	$p < 0.001$
Lipid	3.5 ± 0.5 AB	3.6 ± 0.4 A	2.9 ± 0.5 C	3.7 ± 0.5 A	3.5 ± 0.5 AB	3.0 ± 0.4 BC	3.0 ± 0.5 BC	$p < 0.001$
				0-146 Days				
Carbohydrate	47.9 ± 10.5	45.6 ± 9.2	38.3 ± 8.3	42.4 ± 12.8	44.0 ± 14.3	37.4 ± 5.1	37.8 ± 7.8	$p = 0.084$
Protein	27.6 ± 1.4	28.8 ± 1.4	28.6 ± 2.1	29.0 ± 2.2	28.8 ± 2.3	28.0 ± 1.3	26.9 ± 1.7	$p = 0.061$

NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

[a] Groups with different letters are significantly different at $p < 0.05$ using Tukey's test following ANOVA.

Table 3.4a. Statistical comparisons of 91 vs. 146-day carbohydrate and protein data on a percent dry weight basis for mussels from Anacostia tributaries and Zekiah Swamp. Mean ± 1 standard deviation or median with minimum and maximum. Statistically significant comparisons at $p<0.05$ are in bold. Data failing parametric assumptions were analyzed by Mann-Whitney (M-W) U test.

	CARBOHYDRATE			**PROTEIN**		
	91-day (n=12)	146-day (n=11)	Statistics	91-day (n=12)	146-day (n=11)	Statistics
NE	**39.0 (21.8, 46.3)**	**50.5 (24.2, 61.1)**	**M-W p=0.013**	26.4±2.2	27.6±1.4	p=0.14
NW	**36.6±7.6**	**45.6±9.2**	**p=0.016**[a]	27.8±1.9	28.8±1.4	p=0.17
HR	38.2 (23.4, 49.8)	38.9 (17.8, 52.2)	M-W p=0.800	28.6±2.5	28.6±2.1	p=0.98
LBD	43.9 (36.7, 54.3)	42.0 (14.7, 65.9)	M-W p=0.930	**31.7±3.1**	**29.0±2.2**	**p=0.02**
WB	41.0±8.1	44.0±14.3	p=0.750[a]	**26.2±2.5**	**28.8±2.3**	**p=0.014**
BD	**32.8±6.4**	**37.4±5.1**	**p=0.050**[a]	**24.3±2.5**	**28.0±1.3**	**p<0.001**
ZS	35.1±5.4	37.8±7.8	p=0.343 [a]	25.3±2.1	26.9±1.7	p=0.051

NE = Northwest Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

[a] log-transformed data.

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Table 3.4b. Statistical comparison of 2016 carbohydrate and protein data in pre-deployment, 91-day, and 146-day mussels at each location. Mean ± 1 standard deviation or median with minimum and maximum. Statistically significant comparisons at $p<0.05$ are indicated by different letters. Data failing parametric assumptions were analyzed by the Kruskal-Wallis (K-W) test followed by Dunn's method.

	Carbohydrate				Protein			
	Pre-Deploy	**Day 91**	**Day 146**	**Statistics**	**Pre-Deploy**	**Day 91**	**Day 146**	**Statistics**
NE	49.0 ± 11.6 B	37.9 ± 7.3 A	47.8 ± 10.5 AB	p = 0.020	27.0 ± 1.7	26.4 ± 2.2	27.6 ± 1.4	p = 0.303
NW	49.0 ± 11.6 A	36.6 ± 7.6 B	45.6 ± 9.2 AB	p = 0.011	27.0 ± 1.7 B	27.8 ± 1.9 AB	28.8 ± 1.4 A	p = 0.040
HR	49.0 ± 11.6 A	38.0 ± 7.2 B	38.3 ± 8.3 B	p = 0.011	27.0 ± 1.7	28.6 ± 2.5	28.6 ± 2.1	p = 0.118
LB	49.0 ± 11.6	44.4 ± 5.0	42.4 ± 12.8	p = 0.313	27.0 ± 1.7 B	31.7 ± 3.1 A	29.0 ± 2.2 B	p < 0.001
WB	49.0 ± 11.6	41.0 ± 8.1	44.0 ± 14.3	p = 0.272	27.0 ± 1.7 AB	26.2 ± 2.5 B	28.8 ± 23 A	p = 0.019
BD	49.0 ± 11.6 A	32.8 ± 6.4 B	37.4 ± 5.1 B	p < 0.001	27.0 ± 1.7 A	24.3 ± 2.5 B	28.0 ± 1.3 A	p < 0.001
ZS	44.1 (35.9, 71.9) A	33.9 (28.4, 47.0) B	34.8 (29.2, 54.1) B	p = 0.003	27.0 ± 1.7	25.3 ± 2.1	26.9 ± 1.7	p = 0.054

NE = Northwest Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

[a] Groups with different capital letters are significantly different at $p < 0.05$ using Tukey's test following ANOVA or Dunn's method following Kruskal-Wallis (K-W) test.

Biochemical Results 2017

Carbohydrate and Glycogen

Carbohydrate concentrations showed no significant differences among deployment locations for 91-day mussels based on Kruskal-Wallis test by ranks ($p = 0.068$). For 154-day mussels, there were significant differences between several locations based on one-way ANOVA ($p = 0.010$). Based on Tukey's multiple comparison test, the mean at Lower Beaverdam 3 was significantly higher than that at Watts Branch, with all others intermediate (Fig. 3.6; Table 3.5). Between day 91 and day 154, only two of the nine deployment locations saw a significant change in carbohydrate concentrations. Carbohydrate content was significantly lower in 154-day mussels than in 91-day mussels deployed at Watts Branch (t-test, $p = 0.020$) and Zekiah Swamp (t-test, $p = 0.019$) (Table 3.6a). Variations in carbohydrate content for day 0, day 91, and day 146 mussels are shown in Fig. 3.7. Only two locations showed significant changes in carbohydrate content over time. Watts Branch and Zekiah Swamp both had the lowest mean carbohydrate content in 154-day mussels compared to pre-deployment and 91-day mussels (Table 3.6b). At all times, mean % carbohydrate on a dry weight basis fell between 34.8% and 54.1%.

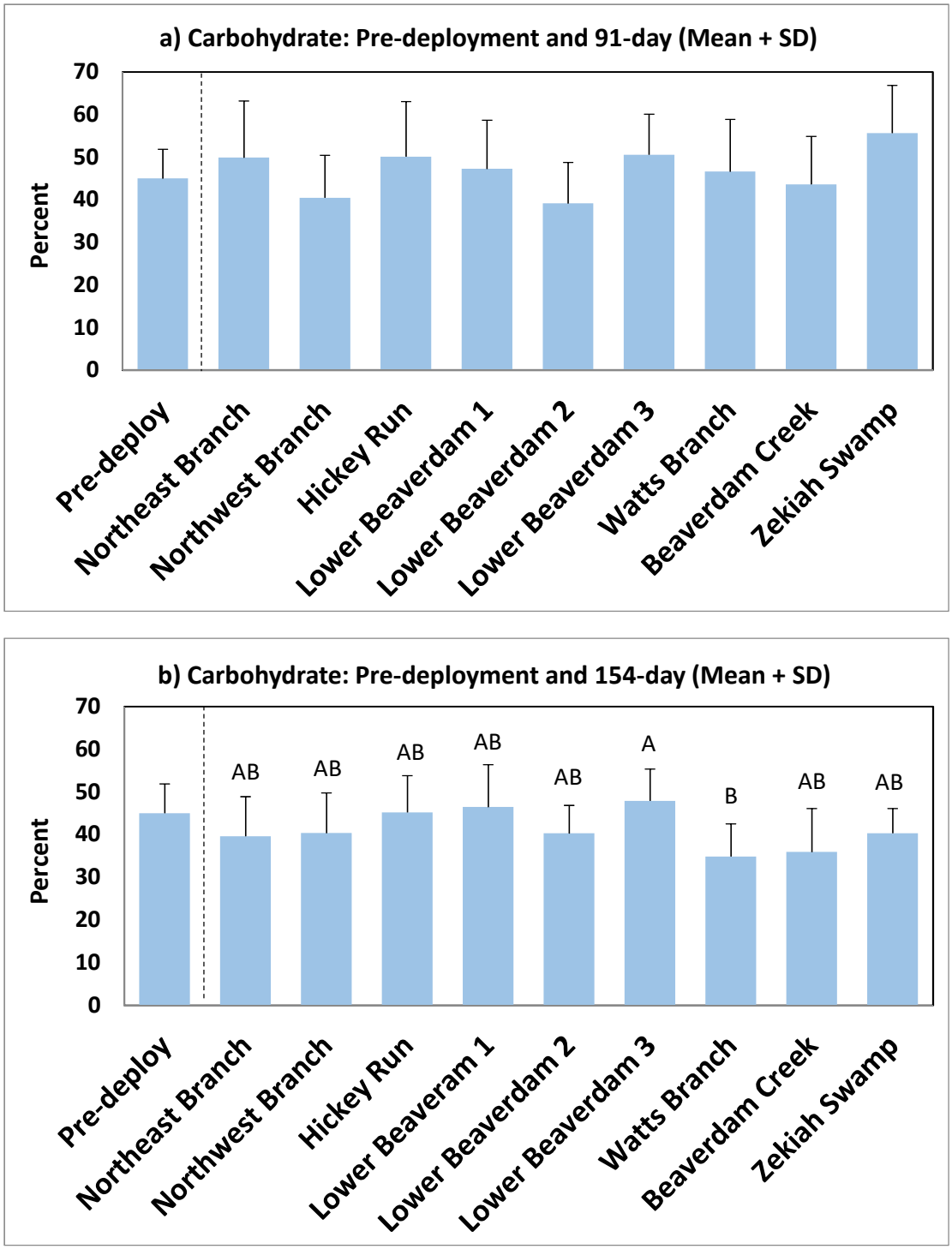

Figure 3.6. Carbohydrate content (% dry wt) in mussel whole-body homogenates (mean + SD): a) pre-deployment and 91-day mussels; b) pre-deployment and 154-day mussels. Columns with different letters are significantly different; refer to Tables 3.5 and 3.6 for sample size and explanation of statistics. Pre-deployment mussels are not compared statistically with post-deployment mussels in this analysis.

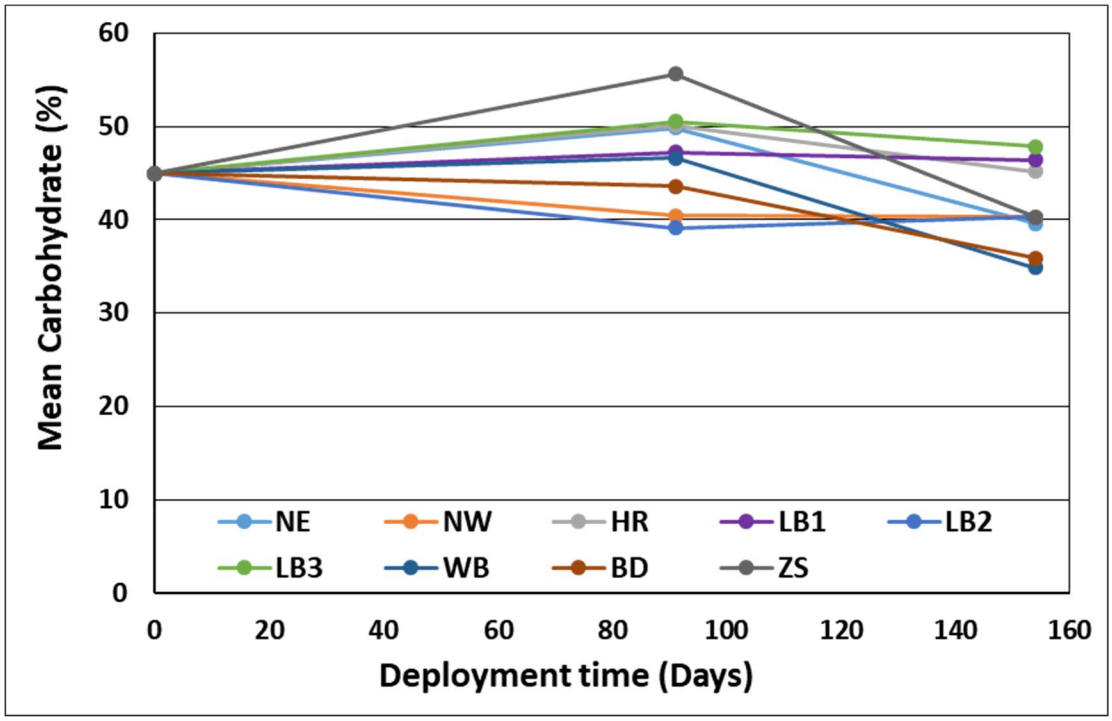

Figure 3.7. Variation of mean carbohydrate content (% dry wt) in mussel whole-body homogenates for 2017 from pre-deployment, 91-day, and 154-day mussels at each location. Pre-deployment mussels are shown as one point as they were all collected from Zekiah Swamp. (NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek (1, 2, and 3); WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.)

Glycogen content in mantle tissue showed significant differences between several locations in 91-day mussels from the 2017 deployment (Figure 3.8). The mean glycogen content on a wet mantle tissue weight basis ranged from 23 mg/g (Northwest Branch) to 68 mg/g (Zekiah Swamp) (2.3% to 6.8%).

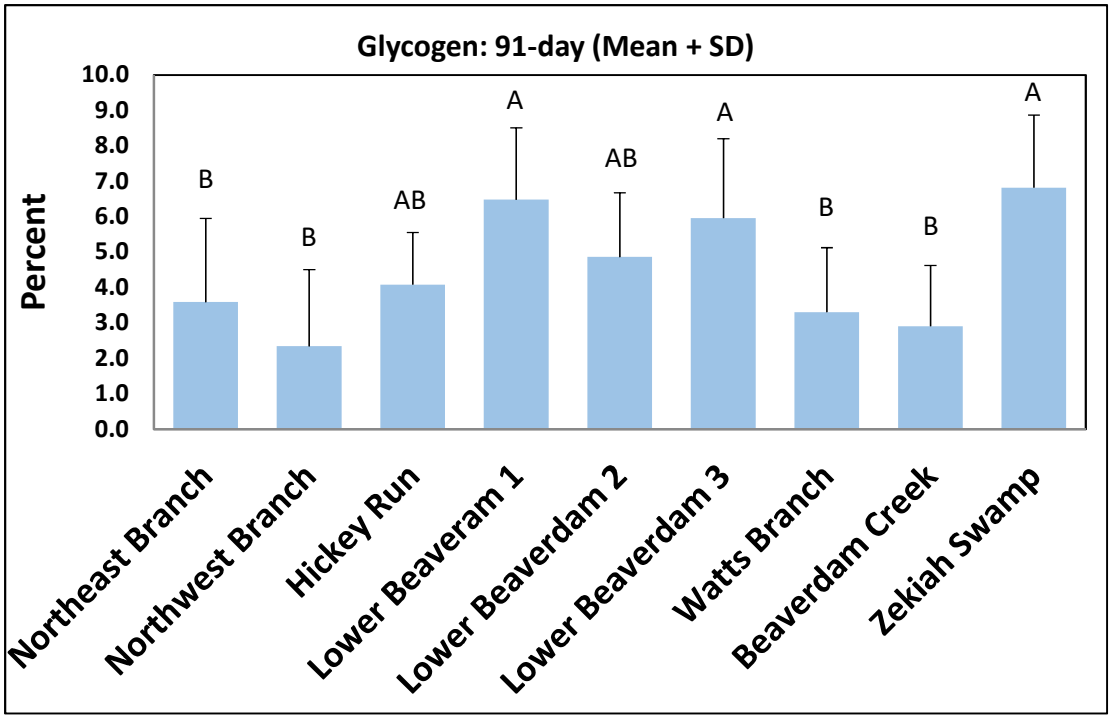

Figure 3.8. Glycogen content (% wet weight) in mussel mantle tissue (mean + SD) in 2017 mussels deployed for 91 days. Columns with different letters are significantly different.

Protein

Protein concentrations showed statistically significant differences between 91-day mussels deployed at several locations based on one-way ANOVA ($p = 0.008$). Specifically, protein content in mussels deployed at Zekiah Swamp was significantly lower than that of several locations, and protein content in two Lower Beaverdam Creek sites (LB2 and LB3) was significantly higher than at all other deployment locations (Table 3.5; Fig. 3.9). These differences were not found to persist in the 154-day mussels. There was a significant increase in protein content between 91-day and 154-day mussels (t-test, $p < 0.05$ for all locations except Northwest Branch and Lower Beaverdam 3; Fig. 3.10). Northwest Branch and Lower Beaverdam 3 saw modest increases in protein

between mussels from the two deployment intervals (Table 3.6a). Variations in protein content for day 0, day 91, and day 154 mussels are shown in Fig. 3.10. All locations showed increased protein content in 154-day mussels compared with pre-deployment and 91-day mussels (Table 3.6b). At all times and locations, mean % protein on a dry weight basis fell between 25.9% and 33.6%.

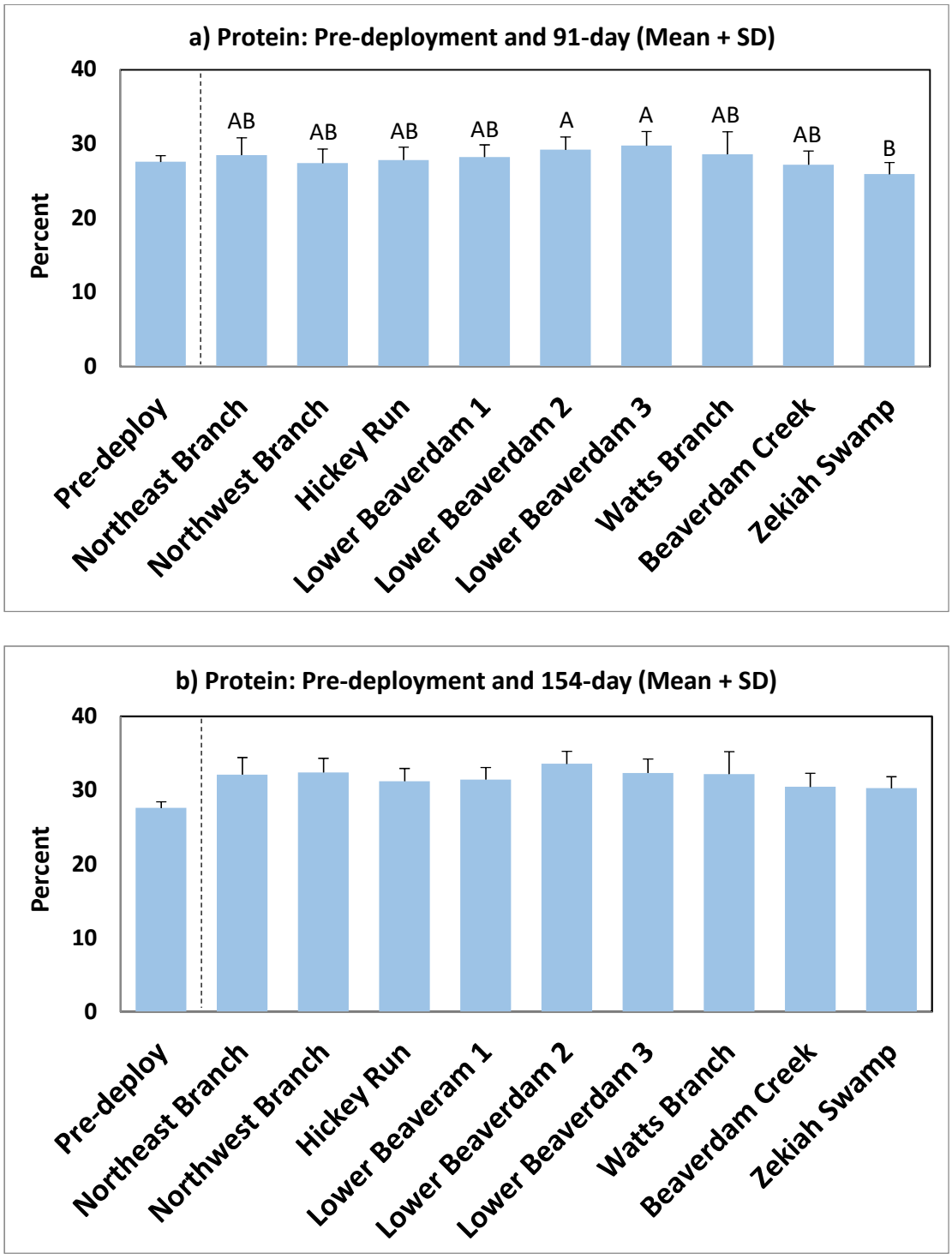

Figure 3.9. Protein content (% dry wt) in mussel whole-body homogenates for 2017 (mean + SD): a) pre-deployment and 91-day mussels; b) pre-deployment and 154-day mussels. Columns with different letters are significantly different; refer to Tables 3.5 and 3.6 for sample size and explanation of statistics. Pre-deployment mussels are not compared statistically with post-deployment mussels in this analysis.

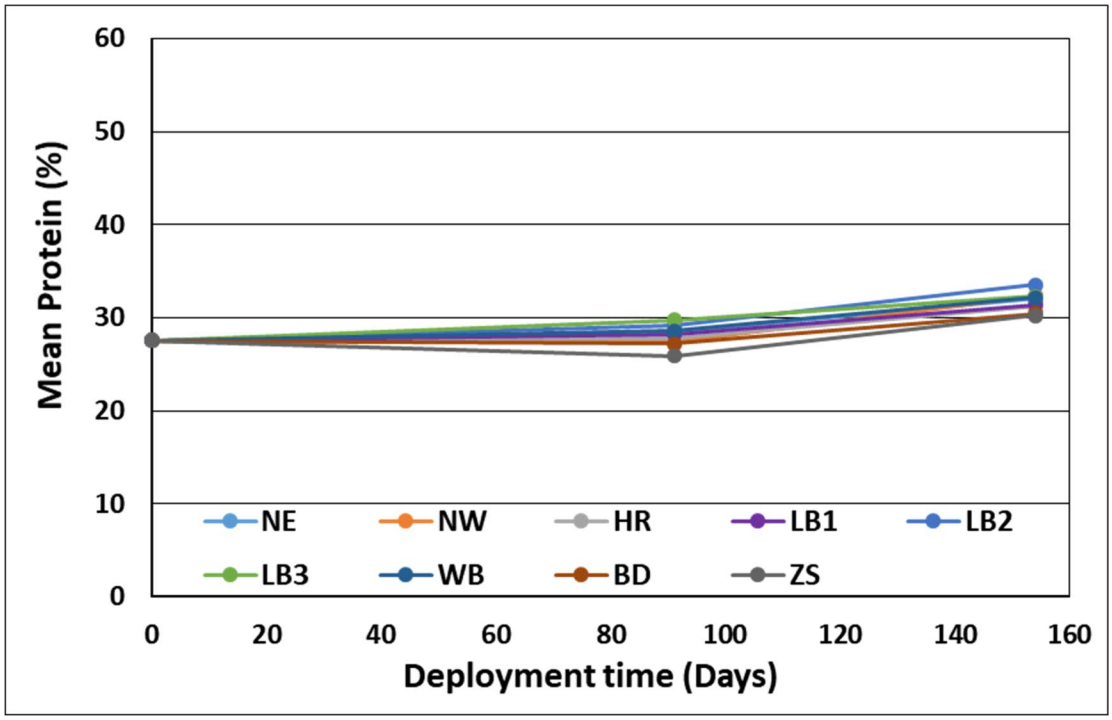

Figure 3.10. Variation of mean protein content (% dry wt) in mussel whole-body homogenates for 2017 from pre-deployment, 91-day, and 154-day mussels. Pre-deployment mussels are shown as one point as they were all collected from Zekiah Swamp. (NE = Northeast Branch; NW = Northwest Branch; HR = Hickey Run; LB = Lower Beaverdam Creek (1, 2, and 3); WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.)

Lipid

Statistically significant differences in lipid concentrations were found between batches of pooled mussels from the 91-day deployment at several locations (ANOVA, $p < 0.001$) (Tables 3.5, 3.6; Figure 3.11). Beaverdam Creek mussels showed significantly lower lipid content than all other Anacostia locations except Lower Beaverdam 2. Lipid % in 91-day mussels on a dry-weight basis ranged from 2.8% to 4%, comparable to 2016 results.

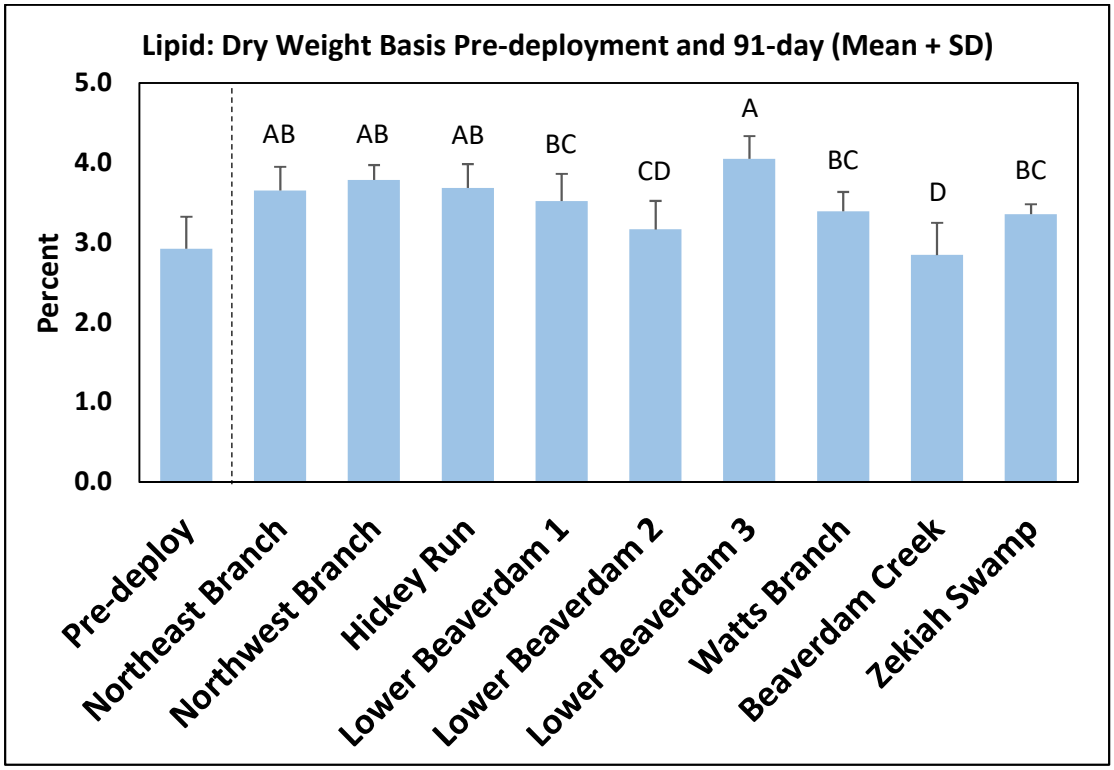

Figure 3.11. Lipid content (% dry wt) for 2017 in pooled whole-body homogenates comprised of 3 mussels (mean + SD) for mussels prior to deployment (pre-deploy) and on retrieval after 91-day deployment. Columns with different letters are significantly different; refer to Tables 3.5 and 3.6 for sample size and explanation of statistics. Pre-deployment mussels are not compared statistically with post-deployment mussels in this analysis.

Table 3.5. Biochemical data on a percent wet weight basis for 2017 mussels deployed for 91 and 154 days in eight locations in Anacostia tributaries and Zekiah Swamp.

	NE (n=10)	NW (n=6)	HR (n=10)	LB1 (n=10)	LB2 (n=10)	LB3 (n=10)	WB (n=10)	BD (n=10)	ZS (n=8)	Statistical Analysis[a]
0-91 Days										
Carbohydrate	44.7 (32.2, 73.4)	41.4 (27.1, 55.2)	46.3 (32.6, 80.0)	49.9 (29.7, 62.0)	36.5 (26.6, 60.6)	50.1 (36.3, 65.4)	43.2 (35.2, 72.0)	41.2 (31.1, 65.3)	54.1 (47.4, 62.2)	K-W $p = 0.068$
Protein	28.5 ± 2.3 AB	27.4 ± 1.9 AB	28.8 ± 1.7 AB	28.2 ± 1.7 AB	29.2 ± 1.7 A	29.8 ± 1.9 A	28.6 ± 3.1 AB	27.2 ± 1.9 AB	25.9 ± 1.6 B	ANOVA $p = 0.008$
Lipid	3.7 ± 0.3 AB	3.8 ± 0.2 AB	3.7 ± 0.3 AB	3.5 ± 0.3 BC	3.2 ± 0.4 CD	4.0 ± 0.3 A	3.4 ± 0.2 BC	2.8 ± 0.4 D	3.4 ± 0.1 BC	ANOVA $p < 0.001$
0-154 Days										
Carbohydrate	39.6 ± 9.3 AB	40.3 ± 9.4 AB	45.2 ± 8.6 AB	46.4 ± 9.9 AB	40.3 ± 6.6 AB	47.9 ± 7.5 A	34.8 ± 7.6 B	35.9 ± 10.2 AB	40.3 ± 5.8 AB	ANOVA $p = 0.010$
Protein	32.1 ± 3.2	32.4 ± 5.6	31.2 ± 2.5	31.4 ± 2.2	33.6 ± 2.1	32.3 ± 3.8	32.2 ± 3.6	30.4 ± 1.9	30.3 ± 1.5	ANOVA $p = 0.437$
Glycogen 91 Days										
Mantle Glycogen	3.6 ± 2.4 B	2.3 ± 2.2 B	4.1 ± 1.5 AB	6.5 ± 2.0 A	4.9 ± 1.8 AB	6.0 ± 2.2 A	3.3 ± 1.8 B	2.9 ± 1.7 B	6.8 ± 2.1 A	ANOVA $p < 0.001$

NE = Northwest Branch; NW = Northwest Branch; HR = Hickey Run; LB1 = Lower Beaverdam Creek 1; LB2 = Lower Beaverdam Creek 2; LB3 = Lower Beaverdam Creek 3; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

[a] Groups with different capital letters are significantly different at $p < 0.05$ using Tukey's test following ANOVA or Dunn's method following Kruskal-Wallis (K-W) test.

Table 3.6a. Statistical comparisons of 91 vs. 154-day carbohydrate and protein data on a percent dry weight basis for 2017 mussels from Anacostia tributaries and Zekiah Swamp. Mean ± 1 standard deviation or median with minimum and maximum. Statistically significant comparisons at $p<0.05$ are in bold.

	CARBOHYDRATE			**PROTEIN**		
	91-day	154-day	t-test	91-day	154-day	t-test
NE	49.9±13.3 n=10	39.6±9.3 n=9	p=0.07	**28.5±2.3** **n=10**	**32.1±3.2** **n=9**	**p=0.011**
NW	40.5±10.0 n=6	40.3±9.4 n=6	p=0.980	27.4±1.9 n=6	32.4±5.6 n=6	p=0.064
HR	50.1±12.9 n=10	45.2±8.6 n=10	p=0.330	**27.8±1.7** **n=10**	**31.2±2.5** **n=10**	**p=0.002**
LB1	47.2±11.5 n=10	46.4±9.9 n=10	p=0.870	**28.3±1.7** **n=10**	**31.4±2.2** **n=10**	**p=0.002**
LB2	39.1±9.6 n=10	40.3±6.6 n=8	p=0.780	**29.2±1.7** **n=10**	**33.6±2.1** **n=8**	**p<0.001**
LB3	50.5±9.5 n=10	47.9±7.5 n=10	p=0.500	1.5±0.02 n=10	1.5±0.05 n=10	p=0.086[a]
WB	**46.6±12.3** **n=10**	**34.9±7.7** **n=10**	**p=0.020**	**28.6±3.1** **n=10**	**32.2±1.1** **n=10**	**p=0.026**
BD	1.6±0.1 n=10	1.5±0.1 n=10	p=0.110[a]	**21.2±1.9** **n=10**	**30.4±1.9** **n=10**	**p<0.001**
ZS	**55.7±11.2** n=8	**40.3±5.8** n=8	**p=0.004**	**27.6±0.8** n=8	**30.3±1.5** n=8	**p<0.001**

[a] log-transformed data

Table 3.6b. Statistical comparison of 2016 carbohydrate and protein data in pre-deployment, 91-day, and 146-day mussels at each location. Mean ± 1 standard deviation or median with minimum and maximum. Statistically significant comparisons at p<0.05 are indicated with different letters. Data failing parametric assumptions were analyzed by the Kruskal-Wallis (K-W) test followed by Dunn's method.

	Carbohydrate				Protein			
	Pre-Deploy	**Day 91**	**Day 154**	**Statistics[a]**	**Pre-Deploy**	**Day 91**	**Day 154**	**Statistics[a]**
NE	45.0 ± 6.9	49.9 ± 13.3	39.6 ± 9.3	p = 0.111	27.6 (25.9, 28.7) B	26.7 (25.9, 28.7) B	29.0 (27.9, 37.2) A	p = 0.003
NW	45.0 ± 6.9	40.5 ± 10.0	40.3 ± 9.4	p = 0.464	27.6 (25.9, 28.7)	27.3 (25.0, 30.5)	31.3 (27.1, 42.7)	p = 0.040
HR	45.0 ± 6.9	50.1 ± 13.0	45.2 ± 8.6	p = 0.426	27.6 (25.9, 28.7) B	27.6 (25.8, 30.8) B	31.6 (28.2, 35.9) A	p = 0.001
LB1	45.0 ± 6.9	47.2 ± 11.5	46.4 ± 9.9	p = 0.869	27.6 (25.9, 28.7) B	28.4 (25.3, 30.3) B	31.9 (27.3, 34.0) A	p = 0.002
LB2	43.1 (35.0, 60.4)	36.5 (26.6, 60.6)	40.3 (31.9, 54.0)	p = 0.110	27.6 ± 0.8 B	29.2 ± 1.7 B	33.6 ± 2.1 A	p < 0.001
LB3	45 ± 6.9	50.5 ± 9.5	47.9 ± 7.5	p = 0.319	27.6 (25.9, 28.7) B	29.6 (26.8, 32.4) AB	32.5 (25.9, 38.4) A	p = 0.003
WB	43.1 (35.0, 60.4) A	43.2 (35.2, 72.0) A	34.8 (23.1, 46.4) B	p = 0.019	27.6 (25.9, 28.7) B	28.6 (24.1, 34.0) AB	32.3 (25.5, 36.7) A	p = 0.010
BD	43.1 (35.0, 60.4)	41.2 (31.1, 65.3)	30.8 (24.0, 50.6)	p = 0.141	27.6 ± 0.8 B	27.2 ± 1.9 B	30.4 ± 1.9 A	p < 0.001
ZS	45.0 ± 6.9 B	55.7 ± 11.2 A	40.3 ± 5.8 B	p = 0.003	27.6 ± 0.8 C	25.9 ± 1.6 B	30.3 ± 1.5 A	p < 0.001

NE = Northwest Branch; NW = Northwest Branch; HR = Hickey Run; LB1 = Lower Beaverdam Creek 1; LB2 = Lower Beaverdam Creek 2; LB3 = Lower Beaverdam Creek 3; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.

[a] Groups with different capital letters are significantly different at $p < 0.05$ using Tukey's test following ANOVA or Dunn's method following Kruskal-Wallis (K-W) test.

Glutathione and Catalase

Glutathione S-transferase (GST) activity in digestive gland tissue on a wet weight basis from 2017 mussels deployed for 91 days showed significant differences at several locations based on one-way Kruskal-Wallis test by ranks ($p < 0.001$; Table 3.7). Based on Dunn's method, Northeast Branch mussels had significantly lower GST activity than Lower Beaverdam 2 and Lower Beaverdam 3 mussels. There were no other significant differences among locations. GR activity was not significantly different between locations based on Kruskal-Wallis test by ranks ($p = 0.115$). The activity of glutathione peroxidase (GPx) was analyzed; however, the assay was suspected to have failed, thus data are not reported in the results of this study.

There was one significant difference in measured activity of catalase for 91-day mussels based on Kruskal-Wallis test by ranks ($p = 0.002$). Beaverdam Creek had significantly higher activity than Northeast Branch; otherwise, there were no significant differences among deployment locations. Catalase activity in digestive gland on a wet weight basis ranged from 137.4 μmol/min/mg at Northeast Branch to 244.6 μmol/min/mg at Beaverdam Creek (Table 3.7).

Table 3.7. The activity of GST, GR, and catalase (CAT) in wet digestive gland tissue of 2017 mussels deployed for 91 days. Results expressed in nmol/min/mg protein.

Site	n	GST	GR	CAT
NE[b]	10	457 (261, 789) B	5.6 (4.4, 8.0)	148.8 (36.8, 194.7) B n = 9
NW[b]	6	485 (161, 728) AB	5.4 (4.0, 6.6)	222.5 (146.6, 336.5) AB n = 6
HR[b]	10	586 (348, 1037) AB	6.1 (5.5, 8.3)	174.8 (102.4, 192.6) AB n = 7
LB1[b]	10	781 (390, 981) AB	5.2 (4.2, 6.7)	151.1 (65.0, 246.1) AB n = 10
LB2[b]	10	857 (402, 1040) A	5.9 (3.1, 12.7)	117.7 (13.1, 250.7) AB n = 10
LB3[b]	10	823 (526, 959) A	5.3 (3.3, 7.1)	235.2 (74.9, 269.1) AB n = 9
WB[b]	10	721 (402, 913) AB	4.0 (2.8, 10.4)	201.5 (78.4, 311.5) AB n = 10
BD[b]	10	537 (460, 813) AB	6.0 (4.5, 7.1)	254.0 (31.2, 345.0) A n = 10
ZS[b]	8	688 (541, 813) AB	4.8 (4.2, 5.9)	243.4 (142.1, 291.2) AB n = 8
Statistics[c]		K-W $p < 0.001$	K-W $p = 0.115$	K-W $p = 0.002$

[a] Median with range in parentheses.
[b] NE = Northwest Branch; NW = Northwest Branch; HR = Hickey Run; LB1 = Lower Beaverdam Creek 1; LB2 = Lower Beaverdam Creek 2; LB3 = Lower Beaverdam Creek 3; WB = Watts Branch; BD = Beaverdam Creek; ZS = Zekiah Swamp.
[c] Groups with different capital letters are significantly different at $p < 0.05$ using Tukey's test following ANOVA or Dunn's method following Kruskal-Wallis (K-W) test.

Chapter 4: Discussion

Evidence of Mussel Viability within the Anacostia

The deployment of *E. complanata* in Anacostia tributaries successfully demonstrated very good survival of the organisms after approximately 150 days in 2016 and 2017. There were significant increases in shell length over time at most locations, suggesting that at these locations, Anacostia tributaries provide adequate nutrient supply for the survival and growth of *E. complanata.* Gagné et al. (2001) report a shell growth rate of 0.044 mm/wk for adult (~70-mm) *Elliptio complanata* caged for 62 days in the St Laurence River, Canada. This value is comparable to the 2016 measured shell growth rate of 0.046 mm/wk in the present study in mussels deployed for 91 days at Zekiah Swamp, our reference site from which all mussels were collected. In 2016, mussels at Hickey Run showed significant growth at day 91 but not at day 146. In 2017, mussels at Watts Branch and the Zekiah Swamp reference location grew significantly at day 91 but not at day 154. This could indicate a limited nutrient supply at locations in which mussels exhibited less growth. At Zekiah Swamp, there is a large population of mussels that may cause competition for sources of food. At Hickey Run, there is very little stream flow which could impact the amount of nutrients available to benthic organisms.

Habitat suitability for freshwater mussels has been studied for several decades to assist with conservation efforts and is a critical aspect of potential efforts to augment or reintroduce or augment populations in a given area. Microhabitat factors (sediment size, depth, velocity) and macrohabitat factors (hydrological variability, drainage area, gradient) have been examined to predict mussel distribution. The main factors that

determine distribution may depend on geographic region. According to Strayer (1993), in the North Atlantic slope, stream size had little influence on the frequency of *E. complanata* and several other species in the area, while in other regions it has been found to be a dominant factor. However, other macrohabitat factors, mainly hydrological stability, was found to be a useful predictor of mussel distribution (Strayer, 1993).

Substrate particle size is another factor that has been studied for usefulness in determining mussel distribution. According to Lewis and Riebel (1984), particle size influences the time and depth that mussels can burrow; however, the authors conclude that burrowing can occur in a variety of substrate. Thus, substrate selection may not be directly associated with burrowing ability, but rather the hydrology of the area which affects the deposition and erosion of the substrate. Gagnon et al. (2006) found that the two largest contributors to diversity and richness of several freshwater mussel species in the Flint River (Georgia) Basin drainage were riparian wetland and catchment forest cover, and drainage network position. Areas with perennial flow are likely more suitable habitat choices than areas that stagnate during droughts. The mesohabitat factor of slackwater and riffle areas may influence which species will be found in which areas as well as in intermediate areas between slackwater and riffles (Gagnon et al., 2006). Ideal habitat requirements differ between species and must be considered for conservation efforts.

Elliptio complanata is widely distributed along the Atlantic Slope (IUCN 2018), suggesting that the Anacostia watershed is a viable location for reintroduction or augmentation of the population. According to Gagnon et al. (2006), *E. complanata* is a generalist species for riffle and slackwater environments, meaning that the species is

most often found in intermediate conditions between these two extremes. More research is necessary to determine additional preferred habitat conditions of *E. complanata.*

Freshwater mussels depend on a host to support larvae called glochidia. The most effective host for *E. complanata* in the mid-Atlantic region is the American eel (*Anguilla rostrata*) (Lellis et al., 2013). Fish barriers have previously limited passage of catadromous eels; since the removal of many of these barriers, *A. rostrata* have been documented in virtually all Anacostia tributaries investigated (MBSS; Fig. 4.1). Presence of the host species is encouraging for future efforts to reintroduce or augment *E. complanata* populations in the Anacostia watershed.

Since eels are now present within the system and can move freely between tributaries, more quantitative eel surveys must be conducted to determine which tributaries chosen for this study contain eels and adequate mussel habitat. Along with reintroducing mussels, stocking juvenile eels in tributaries of the Anacostia with adequate conditions for mussels and eels may assist in recruitment of mussel populations as demonstrated in Susquehanna River tributaries by USFWS and USGS (2014). Results from Morrison and Secor (2003) show that 70% of tagged eels recaptured after one year were within 1 km of the release site, suggesting home range behavior is conducive to the idea that stocking eels may assist in mussel reproductive success.

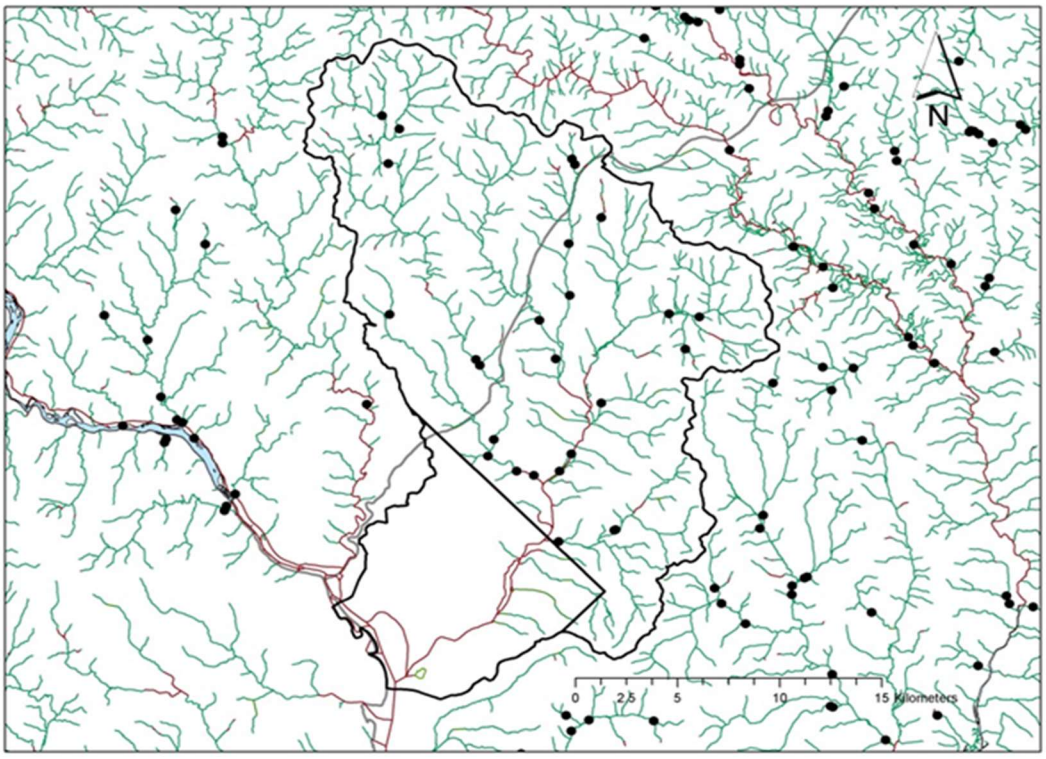

Figure 4.1. Presence of American eels (*Anguilla rostrata*) in the Anacostia watershed in the 1995-2002 MBSS dataset.

Biochemical Health Indicators

Carbohydrate stores are essential for sustaining mussels over winter so should generally increase during autumn (Gray and Kreeger, 2014). Results from this study are well within the 24 % to 57 % range in carbohydrate content reported by Gray and Kreeger (2014) for caged *E. complanata* in southeastern Pennsylvania streams. Overall, these data demonstrate the maintenance or increase of carbohydrate stores between the September and November retrieval dates. The ability of caged mussels to maintain carbohydrate concentrations through early November is encouraging for the possibility of mussels to overwinter in Anacostia tributaries. Although 2017 was very different in terms of weather, with more intense storm and high flow events, results suggest that energy

storage during autumn may be sufficient to maintain reserves throughout the winter months.

Several significant differences were found in mantle glycogen of mussels deployed in 2017 for 91 days. These differences could be attributed to varying food supply among tributaries, or to a stressor present at some locations causing the mussels to use more energy stores. Glycogen content at 91 days was highest at Lower Beaverdam 1, Lower Beaverdam 3, and Zekiah Swamp. This suggests that in early September these locations may be providing adequate nutrition for mussels to store higher amounts of glycogen. Northeast Branch saw the lowest glycogen content at 91 days. Glycogen content of caged mussels at Zekiah Swamp was compared to the glycogen content of eight mussels collected from the same location 84 days after the 91-day deployment. The purpose of this comparison was to determine whether caging the mussels creates a stressor that affects glycogen stores. There was no significant difference between caged and uncaged mussels at Zekiah; however, the uncaged mussels were collected at day 175, creating a time gap of approximately 84 days between the retrieval of caged versus uncaged mussels analyzed for glycogen content. No true comparison can be made between the 91-day caged mussels and the uncaged mussels collected much later; however, it is interesting to note that glycogen content was not different at the reference location during the time period.

Increasing glycogen stores during summer months when more food is available is critical for the survival of mussels as winter approaches (Patterson et al., 1999). For 91-day mussels in 2017, mean glycogen in wet mantle tissue among sites ranged from 2.3-7.3 mg/g. These are comparable to the results reported by Patterson et al. (1999) who

reported mean mantle glycogen values of 4.8 mg/g and 9.9 mg/g glycogen in two unionid mussel species (*Quadrula. pustulosa* and *Amblema plicata*, respectively) that were fed algae with adequate nutrients over a period of 30 days. These results were reported in mg/g of mantle tissue preserved in ethanol. Preserved tissue overestimates dry weights and underestimates wet weights (Patterson et al., 1997; Patterson et al., 1999). Palais et al. (2012) used digestive gland tissue from *Dreissena polymorpha.* The authors report a range from 7.5 mg/g to 36.1 mg/g wet weight in July and January respectively, demonstrating substantial seasonal variation of energy reserves (Palais et al., 2012). The results demonstrate considerable variability which is in agreement with the findings of the current study using mantle tissue.

For mussels deployed in 2016, at all times and locations the mean % protein on a dry weight basis fell between 24.2% and 31.7%, comparable to the mean protein content measured by Gray and Kreeger (2014) of 21-41% on a dry weight basis for *E. complanata* during the deployment interval of late-spring through mid-autumn. In 2017 the mean protein content ranged from 25.9% to 32.4%, again within the range reported by Gray and Kreeget (2014). Between day 91 and day 154, protein content increased slightly at each location, suggesting adequate maintenance of stores during the deployment period. As with carbohydrate and glycogen, this is encouraging for the prospect of long-term survival. Borković et al. (2005) report protein concentrations of approximately 27-37% on a wet weight basis for the Mediterranean mussel (*Mytilus galloprovincialis*) during winter and spring, with no statistical differences between sites or seasons.

Carbohydrate and protein stores showed variation over time for both sampling years at several locations. Protein showed elevation over time at most locations in 2016 and 2017. Carbohydrate stores at Northeast Branch and Northwest Branch were elevated in 146-day mussels compared with 91-day mussels. However, all other locations with variation in carbohydrate content did not indicate this trend. Carbohydrate content in 2017 was less variable in 2017 with only two locations showing significant fluctuations over time (Watts Branch and Zekiah Swamp). More research is necessary to associate these changes in biochemical content with environmental factors such as food availability, temperature, seasonal variation, or reproductive status.

Results of lipid content analyses on pooled mussels show no major decreases between pre-deployment and 91-day samples. Analysis on individual mussels and after both deployment periods would be beneficial to determine any decreases after the approximate 150-day mark and to compare these results with other biochemical health parameters. For this study, lipid analyses were performed at UMBC on pooled mussels so that contaminant concentrations in mussel tissue could be normalized to the lipid content of the same samples. Our mean lipid percentages (2.9% to 4.0% dry weight) is lower than the range reported by Gray and Kreeger (2014) of 8% to 16% lipid of dry tissue weight. Results from the current study are slightly higher than levels reported by Raeside et al. (2009) of 0.42% to 1.03% among individual *E. complanata* using a different analytical method (samples were dried with anhydrous sodium sulfate rather than freeze dried). The discrepancy may be the result of differences in methods of lipid isolation and purification among the studies.

Antioxidant Enzyme Activity: Glutathione and Catalase

Antioxidant response enzymes such as glutathione and catalase are often used as biomarkers for pollution. These analyses were performed on 2017 mussels deployed for 91 days. However, 2017 contaminant data were not available at the time of thesis preparation, so no comparison could be made between glutathione and organic contaminant body burdens in mussels after deployment in the various Anacostia tributaries.

In the present study the mean GST activity in 2017 *E. complanata* deployed for 91 days ranged from 457-857 nmol/min/mg protein. These results are comparable to those reported by Palais et al. (2012) of 336-395 nmol/min/mg protein in the zebra mussel (*D. polymorpha*). Gagné et al. (2004) found that caged *E. complanata* exposed to a municipal effluent showed increased GST activity in the digestive gland after one year of exposure. According to Gowland et al. (2002), who report a range of approximately 10 to 28 nmol/min/mg protein GST in digestive gland of *M. edulis*, GST activity in mussels is best correlated with exposure to the more lipophilic and carcinogenic congeners of PAHs, possibly due to the active feeding from suspended particles in the water column.

The mean GR activity in digestive gland tissue was between 4.9 and 6.6 nmol/min/mg protein, similar to the activity reported in Faria et al. (2009) in *D. polymorpha* exposed in a laboratory study to PCBs and metals, which ranged from approximately 3 to 6 nmol/min/mg protein. Elevated activity of CAT has been associated with PCB exposure in *M. edulis* (Krishnakumar et al., 1997). Responses induced in their laboratory exposures were comparable to responses in mussels field collected from PAH and PCB contaminated sites. Results of a study by Borković et al. (2005) showed site-

specific correlations between GST and CAT activity in *M. galloprovincialis*. In the current study, similar agreement between GST and CAT activity was seen in the Anacostia tributaries (Table 3.7). Interestingly, the Zekiah Swamp reference location which is known to be low in contaminants did not exhibit lower levels of enzyme activity than the markedly contaminated Anacostia deployment sites.

The activity of antioxidant enzyme biomarkers may be influenced by many factors including seasonal variability, locality, intrinsic biological processes of mussels, environmental changes, and contaminant changes (Borković et al., 2005). Moreover, Cossu (2000) reported that it is as yet unknown whether these biomarkers are able to predict short- or long-term environmental contamination. The current study did not investigate seasonal variability as enzyme measurements were only made at one time point during a single deployment season. Therefore, further research is certainly warranted to determine their usefulness as tools for detection of contaminant exposure in *E. complanata*.

Abundance and Variability of Contaminants

Results from 2016 contaminant data in mussels and passive samplers deployed for 91 days successfully demonstrate the utility of mussels as a bioindicator for total contaminant loadings in the water column within Anacostia tributaries. Total concentrations measured in water showed good agreement with total concentration in composite mussel tissue (Ghosh et al., 2018). When available, the results from 2017 chemical analysis will be compared between sampling locations in the same way as in 2016, including the two additional 2017 locations in Lower Beaverdam Creek. This preliminary investigation into continuing sources of contamination in the Anacostia

watershed will be useful in decision making for remedial actions. Contaminant results for 2017 will be completed later in 2019 and will be included in a manuscript to be submitted for publication.

PAH Concentrations in Mussels

In 2016, total PAHs in pooled mussels deployed for 91 days were highest at Hickey Run (HIR) (Fig. 4.2; Ghosh et al., 2018). Hickey Run was also the site that experienced no significant increase in shell length in mussels deployed for 146 days. The Hickey Run deployment location is unlikely to be considered viable for mussel reintroduction due to lack of adequate substrate and suitable habitat until further restoration projects have been completed based on the Hickey Run Subwatershed Action Plan (AWRP, 2009). Hickey Run, historically contaminated by oil and grease, was removed from the impaired list for oil and grease contamination in 2002; however, there are still severe water quality concerns for this subwatershed (AWRP, 2009). Overall, total PAH concentrations were elevated in all of the urbanized Anacostia tributaries relative to Beaverdam Creek and the Zekiah Swamp reference location.

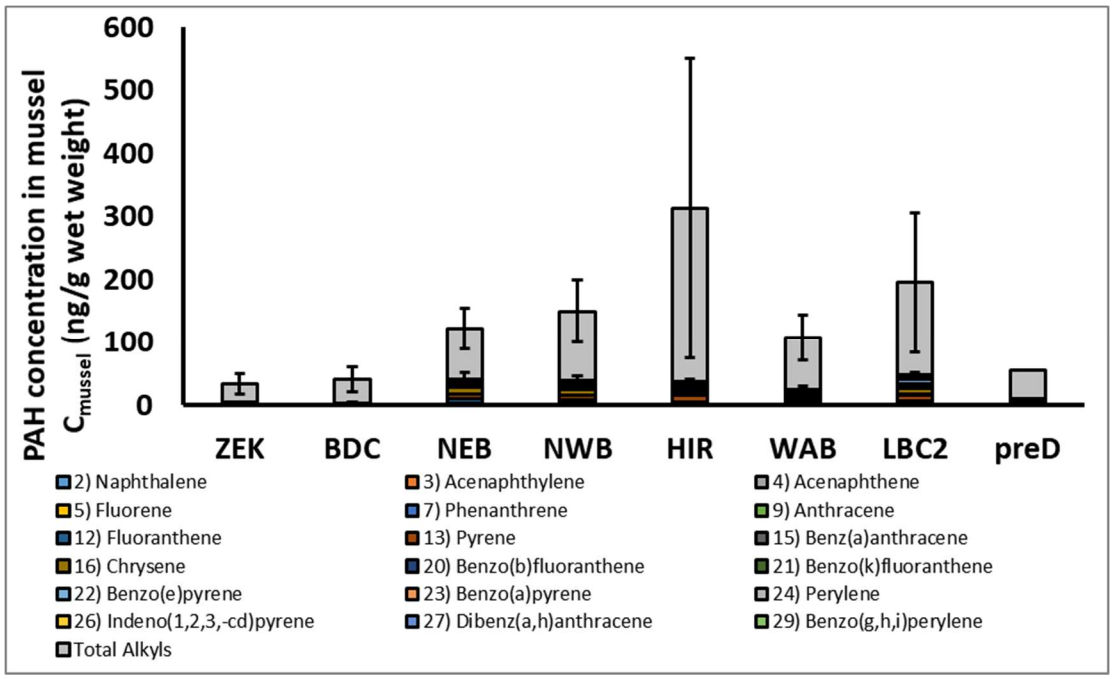

Figure 4.2. Comparison of PAH and alkyl concentration in mussels deployed for 91 days in 2016. Data for 12 composite mussel replicates. Error bars represent standard deviation (from Ghosh et al., 2018).

PCB Concentrations in Mussels

In 2016, total PCBs in pooled mussels deployed for 91 days were highest at Lower Beaverdam Creek (LBC2) (Fig. 4.3; Ghosh et al., 2018). Lower Beaverdam Creek includes several areas that are concrete-lined and channelized, altering the hydrology of the tributary. This along with the high level of imperviousness within the catchment area causes pollution concerns for the area. Lower Beaverdam Creek is also the most industrialized subwatershed in the Maryland portion of the Anacostia watershed (AWRP 2009). Total PCB contamination is associated with industrial runoff due to the historical use of these compounds in electrical equipment, paints, pigments and other industrial products produced before the ban of PCBs in 1979 (USEPA, 2018). As with PAHs, PCB

body burdens were elevated in mussels from all Anacostia tributaries investigated with the exception of Beaverdam Creek.

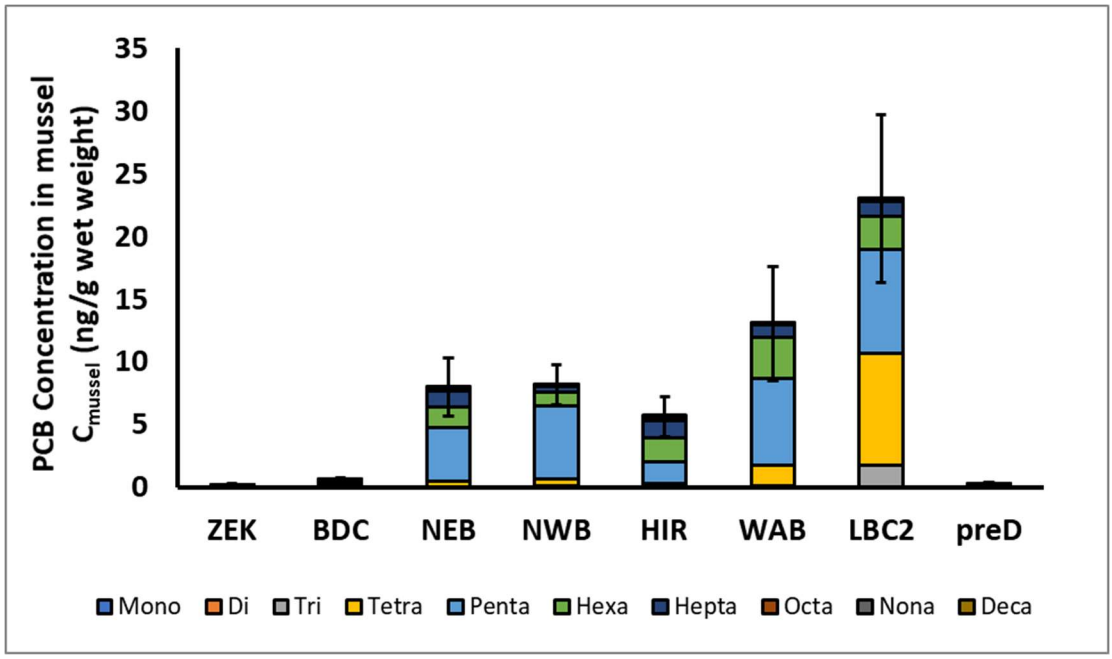

Figure 4.3. Comparison of PCB concentrations in mussels deployed for 91 days in 2016. Data for 12 composite mussel replicates. Error bars represent standard deviation (from Ghosh et al., 2018).

Organochlorine Pesticide Concentration in Mussels

In 2016, the OC pesticides measured in pooled mussels deployed for 91 days varied by compound and by site (Fig. 4.4 and 4.5; Ghosh et al., 2018). Total chlordane concentrations were highest at Northeast Branch (NEB), Northwest Branch (NWB), Watts Branch (WAB), and Lower Beaverdam 2 (LBC2). Chlordanes were historically used for termite control in residential areas. Though chlordane was banned in 1983, the chemical and its breakdown products can persist for decades in terrestrial and aquatic soils and sediments. Mean mussel body burdens of dichlorodiphenyltrichloroethane

(DDT), dichlorodiphenyldichloroethane (DDD), and dichlorodiphenyldichloroethylene (DDE) are all shown in Fig. 4.5. Total DDTs (sum of DDT, DDD, and DDE) were highest at Beaverdam Creek (BDC). However, only DDE was actually highest at Beaverdam Creek, while DDD was highest at Hickey Run, and DDT was highest at Hickey Run and Watts Branch. The differences in ratios of DDT to its breakdown products may be an indication of how long DDT has persisted in each subwatershed. For example, at Watts Branch and Hickey Run, the ratio of DDT to DDE is larger which may suggest that there are newer sources of DDT still contributing to the system. On the other hand, Beaverdam Creek appears to have the oldest DDT remnants due to the smaller ratio of parent DDT to persistent degradation product DDE. As stated above, these data are from 2016 and cannot be reconciled to 2017 antioxidant enzyme activity; however, Moreira and Guilhermino (2005) found elevated GST activity in *Mytillus galloprovincialis* exposed to pesticide runoff from agricultural lands.

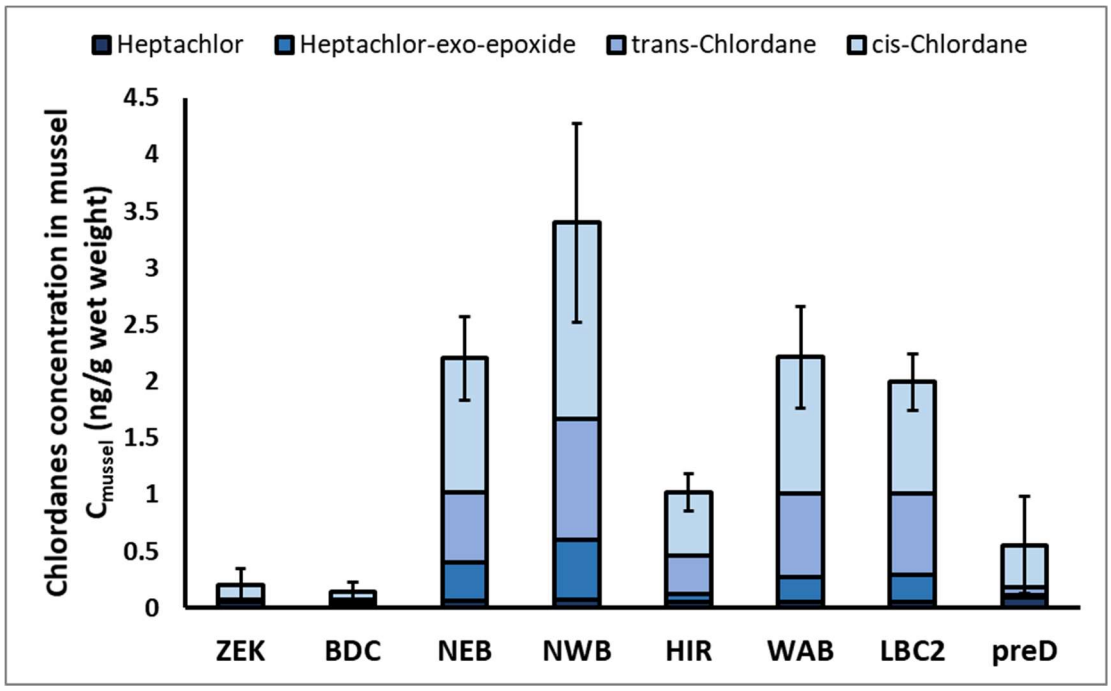

Figure 4.4. Comparison of chlordane concentration in mussels deployed for 91 days in 2016. Data for 12 composite mussel replicates. Error bars represent standard deviation (from Ghosh et al., 2018).

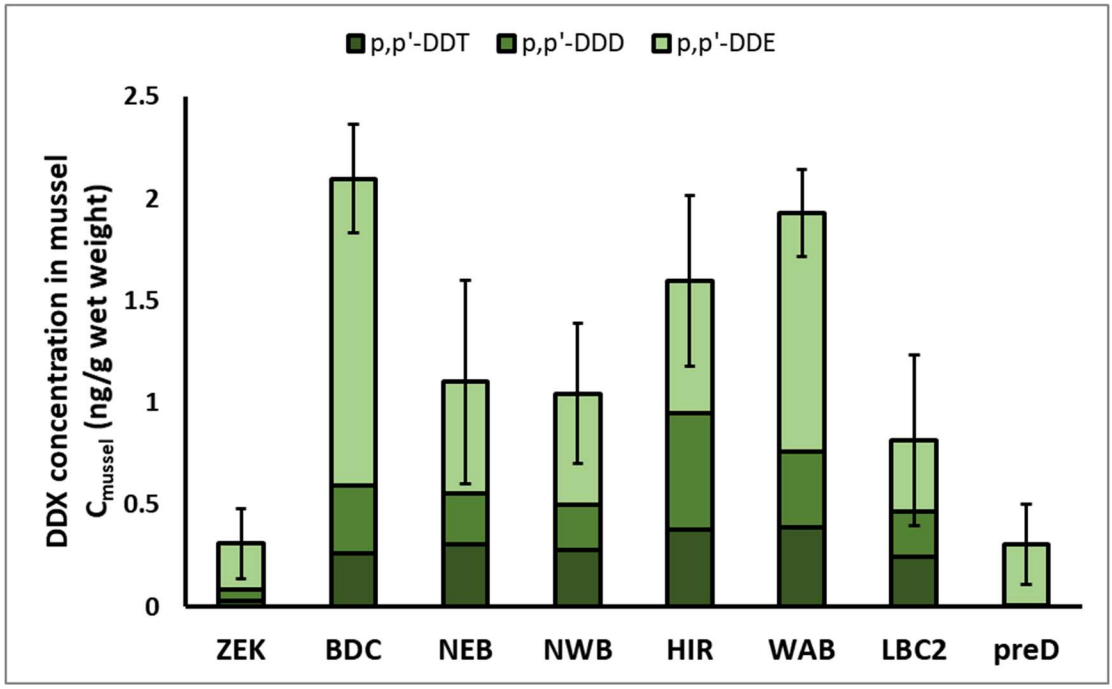

Figure 4.5. Comparison of DDX concentration in mussels deployed for 91 days in 2016. Data for 12 composite mussel replicates. Error bars represent standard deviation (from Ghosh et al., 2018).

Conclusions and Future Directions

Results from the deployment of *E. complanata* over two sampling seasons largely support the second hypothesis of this study; that indicators of health in deployed mussels were not statistically different across locations including the reference location. This suggests that reintroduction or augmentation of this species to several Anacostia tributaries may be feasible. Growth and health indicators of mussels over the deployment period were encouraging for long term survival.

The energy requirements of mussels vary seasonally and with spawning periods, but the specific body burdens of carbohydrate, protein, and lipid needed to sustain these organisms over the long term are poorly understood. The interpretation of seasonal fluctuations in biochemical health parameters is useful to determine if mussel health is sub-optimal after relocation (Gray and Kreeger, 2014). Results from the current study indicated no significant reductions in carbohydrate or protein stores over the deployment periods; however, 91 and 146 (or 154) days may not have been long enough to observe fluctuations in energy use in response to contaminants or other environmental changes or stressors. Year-round deployment and seasonal monitoring of biochemical content and oxidative stress indicators would provide more insight into how mussels respond to conditions in the various Anacostia tributaries. More research is necessary to determine which tributaries have appropriate habitat conditions and adequate nutritional resources to sustain mussels over the long-term.

A condition index for individual mussels using whole body dry weight and shell volume would be a useful measure of whether organisms were losing tissue mass over time (Gray and Kreeger, 2014). For the current study, a condition index involving dry

weights of whole individuals was not possible due to the need to pool 3 mussels per sample prior to freeze drying for chemical analyses. Residual individual tissue was freeze dried for biochemical analyses at UMD after pooling the samples. Any future studies should either modify methods to allow calculations of whole-body dry weights prior to tissue processing for chemical analysis, or should deploy additional mussels for the sole purpose of determining mussel growth and calculating condition index.

Enzyme results from 2017 will be compared with chemical data from 2017 when available; however, this will not necessarily determine which if any contaminants induce a specific type of enzyme activity. Although these biomarkers have been found to be useful in other species, and in laboratory settings, confounding factors in the environment (e.g., complex chemical mixtures, limited/variable food availability, sex/reproductive status, and water temperature/hardness/pH) could affect stress responses in *E. complanata*. A controlled laboratory exposure to different contaminants would allow a better understanding of which contaminants trigger stress responses in this mussel species, as well as the necessary exposure concentrations and durations to produce these responses. Cheung et al. (2004) exposed the Asian green mussel (*Perna viridis*) to known concentrations of individual contaminants as well as mixtures of contaminants. The authors found correlations between digestive gland GST and CAT and exposure to the PCB Aroclor 1254. Activities of GST and GR were also correlated with exposure to the PAH benzo[*a*]pyrene (B[*a*]P). Controlled laboratory exposures of *E. complanata* to persistent contaminants of concern at concentrations measured in Anacostia tributaries would establish whether analysis of these enzymes is actually capable of detecting real-

world exposure and potentially assist in interpreting results of the current study (Cheung et al., 2004).

Overall, results of the survival, growth, and biochemical content analyzed for mussels in this study are encouraging for the potential of long-term survival and growth of *E. complanata* in Anacostia tributaries as part of a larger remedial strategy for the watershed. Although more research is necessary, this successful caged deployment of mussels will help scientists and managers gain a better understanding of continuing bioavailable contamination and the potential areas for reintroduction within the Anacostia watershed.

Appendix A – Water Quality Data

Water quality data collected in 2016, expressed as range of each parameter.

Site	Temperature (°C)	Conductivity (mS)	DO (%)	pH
NE	14.3-29.5	0.25-0.53	73.2-206.3	6.51-9.15
NW	14.8-27.5	0.21-0.58	80.3-151.0	7.26-8.05
HR	18.7-25.6	0.47-1.23	45.7-193.8	6.87-8.05
LB	14.3-27.9	0.23-0.66	68.0-135.0	6.67-7.90
WB	14.6-27.2	0.29-0.67	85.0-141.0	7.18-8.05
BD	10.4-24.1	0.07-1.10	72.0-134.6	4.09-7.22
ZS	11.3-26.1	0.07-0.18	6.9-113.1	2.90-7.04

Water quality data collected in 2017, expressed as range of each parameter.

Site	Temperature (°C)	Conductivity (mS)	DO (%)	pH
NE	13.3-28.4	0.28-0.50	48.7-208.1	6.56-9.46
NW	12.7-27.9	0.28-0.61	40.2-130.7	6.83-8.11
HR	12.9-26.0	0.35-1.26	35.5-98.6	6.60-7.80
LB1	11.7-26.8	0.27-0.66	20.0-110.4	6.50-8.29
LB2	11.6-27.6	0.29-0.93	29.1-109.8	6.71-8.12
LB3	12.0-28.6	0.24-0.65	201-148.9	6.81-8.89
WB	13.3-27.0	0.13-0.55	36.0-121.5	6.75-8.47
BD	10.6-22.6	0.09-0.15	61.6-131.0	6.14-7.26
ZS	10.0-27.5	0.10-0.16	48.1-98.2	5.85-7.16

Appendix B – Carbohydrate Standard Operating Procedure

Materials:

Scale
Weigh paper
2mg freeze dried tissue
20mL borosilicate test tubes
Test tube rack
Vortex
Pure lab water, DI and distilled
Small beaker for pure lab water
5% phenol
Concentrated sulfuric acid
50mL conical tubes for sulfuric acid (3)
Conical tube rack
Cold soluble starch for standardization (Sigma Chem 9765)
50mL glass bottle with lid
Hot plate
Thermometer
Large beaker
Pipette tips (1 box of each)

- 1-5mL (clear tips in clear bag)
- 100-1000µL (blue tips in blue box)
- 20-200 µL (yellow tips in blue box)

Micropipettes

- 1-5mL
- 100-1000 µL
- 20-200 µL

Microplate
Microplate reader with 490 nm wavelength

Methods:

1. Weigh 2mg freeze dried tissue into tared test tube. Record weight to nearest 0.01mg.
2. Add sulfuric acid to 3 50mL conical tubes. Label.
3. Using the 100-1000 µL pipette, add 1mL lab pure water to sample. Vortex 10 seconds.
4. Add 1mL 5% phenol to sample. Vortex 10 seconds.
5. **Under a fume hood, slowly,** with test tube directed away, add 5mL concentrated sulfuric acid from conical tube to sample.
6. Allow to cool 15 minutes. Vortex carefully, directing test tube away.
7. Allow to stand 10 min.
8. Prepare standard solution by adding 50mg starch to 50mL of lab pure water to the glass bottle.
9. Fill large beaker ½ way with tap water. Place on hot plate.
10. Turn hot plate to 8. Use thermometer to check water temperature.
11. When water in beaker reaches 80ºC, place capped bottle with standard solution into the beaker.
12. When starch is dissolved (~5min), prepare 6 standard dilutions ranging from 0µg/mL to 1000µg/mL (see dilution sheet).
13. Pipette 1mL from each standard into another set of 6 test tubes.
14. Add 1ml 5% phenol solution, and 5mL sulfuric acid using steps 4-6 as for samples.
15. Pipette 200µL from each sample in triplicate into microplate wells (starting with well 1G).
16. Pipette 200µL from each standard in triplicate into microplate wells (starting with well 1A).
17. Read at 490nm on plate reader.

Generate standard curve. (R^2 values should be 0.95 or higher.) If samples exceed standard curve, dilute and rerun. % CHO = (Mean of 2 Readings (µg/mL)) / (Subsample Weight (mg) * 1000 (µg/mL)) * 100%

Appendix C – Mantle Glycogen Standard Operating Procedure

Reagents

1. 100 mM sodium citrate buffer @ pH 5
2. 1% amyloglucosidase – add 250 µL to 25 ml sodium citrate buffer @ pH 5
3. 12 N H2SO4
4. Glucose standards (prepare on day of test, see below)
5. Nanopure H20

Methods:

1. Weigh mantle tissue (~40 mg) and place in 2 mL centrifuge tube.
2. Add 5-10% weight/volume sodium citrate buffer to samples.
3. Macerate/homogenize mantle tissue in tube using a pellet pestle.
4. Place in boiling water bath for 5 min. Cool to room temperature.
5. Centrifuge @ 10,000 g for 5 min.
6. Add 10 µL 1% amyloglucosidase in duplicate for each sample to a 96-well microplate. These are the treated rows of sample.
7. Add 10 µL sodium citrate buffer in duplicate for each sample to the microplate in separate rows. These are the untreated rows of sample.
8. Add 200 µL supernatant from samples to treated rows and 200 µL of supernatant to untreated rows. Incubate at 25°C overnight.

Day 2:

1. Prepare 'G.O. Reagent' as indicated in kit.
2. Prepare Glucose standards in H20:

Standard	Glucose (µL)	H_20 (µL)
A	0	500
B	10	490
C	20	480
D	30	470
E	40	460

3. Prepare dilution plates of samples in duplicate.
   Dilution from rows treated with amyloglucosidase: 2 µL sample in 198 µL H20 (may need to change based on sample activity)
   Dilution from untreated rows – target: 2 µL sample in 198 µL H20

Final Plate

1. Transfer 30 µL from dilution plates to final plate in duplicate.
2. Transfer 30 µL of standards to final plate in duplicate.

3. Add 60 µL of G.O. reagent to all wells. Incubate for 30 minutes at 37°C.
4. Stop the reaction with 60 µL 12 N H_2SO_4. Read at 540 nm.

Appendix D – Protein Standard Operating Procedure

Materials:

10mg homogenized and freeze dried tissue
Scale
Plastic bottle for working NaOH solution
Sterilized beaker for working reagent
Beaker with DI water
Solution basin
15mL test tubes
15mL borosilicate test tubes with caps
0.1 mol/L NaOH (8mL per sample)
Homogenizer
Sonicator
Oven
Vortex
Centrifuge
96-well flat bottom microplate (#15041)
BCA protein assay kit (Thermo Scientific)
Microplate with 580nm filter
20-200 µL micropipette
20-200 µL multichannel micropipette
1-5mL micropipette
20-200 µL yellow pipette tips (blue box)
20-300 µL pipette tips (grey box)
1-5mL pipette tips
Plate shaker
Sealing Tape for 96-Well Plates (#15036)
Plate reader

Methods:

1. Prepare up to 24 samples at a time (per plate) of freeze dried and ground tissue.
2. Using weighing paper, measure 10mg tissue. Record weight to 0.01mg. Carefully add to labeled test tube.
3. Using a 1-5mL micropipette, Add 4mL 0.1M NaOH to test tube.
4. Homogenize 10 seconds.
5. Sonicate for eight bursts (10 seconds per burst).
6. Add 4 more mL of 0.1M NaOH.
7. Vortex and pour into borosilicate test tube. Transfer any residual liquid/foam using a 20-200 µL micropipette and yellow pipette tip. Transfer the sample label to the new tube.
8. Cap test tubes loosely and place in oven at 60ºC for 45min. When complete, change oven temperature to 37ºC.
9. Prepare 8 standards from BCA kit stock, ranging from 0 µg/mL to 800 µg/mL (see dilution sheet).
10. Prepare working reagent in sterilized beaker using 25mL Reagent A : 0.5mL Reagent B. (50:1 BCA Reagent A : BCA Reagent B)
11. Pipette 25 µL standards in triplicate to first 3 rows of microplate. Cover plate with foil.
12. Vortex each sample and allow to cool 15min.
13. Centrifuge at max speed for 10min.
14. Pipette 25 µL of supernatant in triplicate to microplate, starting in 4^{th} row. Change tips each time.
15. Pour working reagent into solution basin.
16. Pipette 200 µL of working reagent to each well using the multichannel micropipette. Change tips each time.
17. Cover plate with sealing tape. Mix on a plate shaker for 30 seconds.
18. Incubate plate at 37ºC for 30 minutes.
19. Allow to cool to RT.
20. Read microplate at 562nm.
21. Compare samples to standard curve. If too high, dilute and re-run plate. (R^2 of standard should be .95.)

$$\% \text{ Protein} = \frac{(\text{Mean of 2 Readings (µg/mL)}) * \text{Final Volume (mL)}}{(\text{Subsample (mg)} * 1000\ (\text{µg/mg})} \quad 100\%$$

Appendix E – Glutathione Standard Operating Procedures

Buffers:

Potassium buffer stock solutions:
1 M monobasic KPO4 (H2KO4P; MW=136.09 g/mol): 34.02 g in 250 ml nanopure water
1 M dibasic KPO4 (HK2O4P; MW=174.18 g/mol): 26.12 g in 150 ml nanopure water

Sodium buffer stock solutions:
1 M dibasic HNa2O4P (MW=141.96 g/mol) = 14.196 in 100 ml nanopure
1 M monobasic H2NO4P (MW=156.01 g/mol) = 15.601 g in 100 ml nanopure

GR buffer:
100 mM KPO4 + 1 mM EDTA @ pH 7.5
How to make 100 mM KPO4 plus EDTA:
21.5 ml 1 M dibasic KPO4
3.25 ml 1 M monobasic KPO4
Adjust to around 200 ml
Add 0.093 g EDTA
Adjust to pH 7.5 and 250 ml (final volume)

Glutathione Extraction Buffer:
Need 500-600 µl per sample
80 mM Tris, 200 mM NaCl, and 1 mM DTT (1,4-dithiothreitol)
For 200 ml:
2.52 g Tris, 2.34 g NaCl, and 0.0308 g DTT
Adjust to pH 7.2 and 200 ml

GST buffer:
1M PBS @ pH=6.5
8.24 ml 1 M dibasic NaPO4
16.8 ml 1 M monobasic NaPO4
Adjust to 250 ml final volume and pH=6.5

Stock CDNB:
Target ~ 0.243 g in EtOH and add EtOH such that the concentration is 60 mM

Stock Glutathione Solution (GSH):
10 mM GSH

Glutathione S-transferase Methods:

1. Weigh 40-60 mg wet digestive tissue gland and place in 1.5 mL microcentrifuge tube.
2. Add 600 µl glutathione extraction buffer and macerate digestive gland with a small pestle.
3. Centrifuge samples at 10,000 g for 30 minutes.
4. Make reaction mix (200 µl):
   4.95 mL PBS @ pH 6.5, 0.9 mL GSH @ 10 mM, 150 µl 60 mM CDNB (add the CDNB right before adding to microplate in step 3).
5. Add reagents to 96-well microplate in duplicate, adding the CDNB to the reaction mix, vortexing and quickly adding reaction mix to wells as a last step directly before running on a plate reader.
   -For each sample well, add 10 µl sample, 90 µl GST buffer, 200 µl reaction mix.
   -For each "blank" well, add 100 µl and 200 µl reaction mix
6. Read at 340 nm on plate reader for 10 20-second intervals for 3 minutes.

Glutathione Reductase (GR) Methods:

1. Use the same digestive gland sample as for GST.
2. Prepare DTNB, GSSG, and NADPH in test tubes. (Note: Amount of each of these will depend on the number of samples, multiplied by 2 for duplicates plus blank and positive control. The target concentration stays the same, but the target volume will change. Never weigh anything less than 1 mg NADPH for a 2 mL total volume. The 2 mL total volume will be good for 10 samples. Always make 1 mL extra.)
3. Using 2 ml GR buffer (for 10 samples), use target weights below:
   3.96 mg DTNB (target 5 mM)
   2.7 mg GSSG (target 2.22 mM)
   1.1 mg NADPH (target 0.667 mM)
4. Add reagents to microplate in duplicate as shown below. (Note: Sample and buffer in each well must always equal 50 µl, everything else is the same.)
   -30 µl DTNB
   -90 µl GSSG
   -30 µl NADPH (Add this last before reading because it starts the reaction)
   -Blank: 50 µl GR buffer.
5. Read at 412 nm on plate reader for 12 26-second intervals for 5 minutes.

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