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Efficacy of magnesium enriched artificial substrate for oyster restoration

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Efficacy of Magnesium Enriched Artificial Substrate For Oyster Restoration

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Dedication

I would like to dedicate this thesis to my mother, who instilled in me from an early age the importance of going to college, working hard, achieving as much as possible, and just for being an amazing mom. I tell her every day she's the best mom on earth. I love you mom.

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Abstract

The eastern oyster, *Crassostrea virginica*, is a keystone species that has undergone a large (> 95%) population decline due to overharvest, pollution, and disease. Restoration efforts focus on alternative larval attachment substrates such as concrete, to supplement the loss of natural oyster shell. Magnesium is a component of bivalve shells and its presence in the environment has shown to be important to the growth of mussels, but the same relationship has not been studied in oysters. Assuming that magnesium can be assimilated from the substrate on which the organism is growing, or that ions of magnesium are leached into the water, magnesium supplements to concrete could have a similar benefit. Supplements in the form of magnesium carbonate can be incorporated into cement. A site in the northern neck of the Chesapeake Bay was used to test the effect of different artificial substrates (natural shell, concrete, and concrete enriched with magnesium carbonate) on mortality, growth, and recruitment exhibited by larval oysters and spat. Shell substrate types were tested for their effects on water chemistry in aquaria over the course of 8 weeks to understand the potential for leeching of nutrients into the environment. Spat on magnesium enriched substrates were not found to have a significant statistical difference from other spat for mortality, growth, or recruitment after running Kruskal-Wallis, ANOVA, and Poisson ANOVA analyses respectively (all p val > 0.05). Magnesium supplemented shells in aquaria impacted the pH (-0.14 pH units), alkalinity (+9 ppm $CaCO_3$), and magnesium (+36 ppm) concentrations in water chemistry over 8 weeks. Spat on all substrates were found to have grown to average sizes larger than expected for the region of the Chesapeake the study was conducted (33-37 mm

compared to 24 mm in previous observations), but within the range of growth seen throughout the entire Bay. Although magnesium enriched substrates did not have a significant impact on oysters' recruitment, growth, and survival relative to other substrates, spat did show higher recruitment and growth, and lower mortality than spat attached to concrete, indicating potential biological significance. The potential exists that higher concentrations of magnesium incorporation into artificial substrates could have a greater impact on attached spat and should be examined in future research.

Introduction

The eastern oyster, *Crassostrea virginica*, is the epitome of a keystone species. Their complex life history results in their ecosystem services of filter feeding and cleaning the water, creating reef structures that provide habitat for other oysters along with other invertebrates and fish, and protecting shorelines from erosion caused by wave action, their impact on habitats such as the Chesapeake Bay can be felt far beyond the local reef metapopulation (Bahr & Lanier, 1981). Unfortunately, the eastern oyster's historical reef habitats and population have been decimated from environmental factors such as diseases, and anthropogenic factors such as overharvesting and pollution (Beck et al. 2011). As the eastern oyster is also a species of critical economic importance, the interest in saving the bivalve extends beyond the typical motivations of saving endangered species. Restoration efforts have been undertaken, with mixed results (Baggett et al. 2015).

Oyster Anatomy and Life History

The eastern oyster and its evolutionary relatives (including clams, mussels, and scallops) belong to the class Bivalvia, that share common morphological traits regarding their general shell structure (Bahr & Lanier 1981). Bivalves exhibit halved shells attached together by an adductor muscle and hinged by conchiolin that are divided into three general layers; the innermost foliated layer, the middle prismatic layer, and the outermost periostracum (Bahr & Lanier 1981, Lombard et al. 2013). A mantle cavity within the margins of the shell contains the tissue of the oyster, including the ciliated gills that allow for filter feeding and respiration through pumping water through the shell (Bahr & Lanier 1981).

Oysters are dioeceous (separate sexes) but can change their gender on a yearly basis due to their simple reproductive systems (Bahr & Lanier 1981). After functional gonads develop within three months oysters can reproduce based on water temperature cues of 17-25°C depending on location, with cooler temperatures triggering spawning in oysters found further north. (Bahr & Lanier 1981). Based on summer water temperature cues of 17-25°C males begin the spawning process by releasing sperm into the water, which contains pheromones that stimulate females to release eggs (Bahr & Lanier 1981). Gametes meet in the water column (Fig. 1) through random chance after being released in great numbers ranging from 10,000 to 66,000,000 and fertilize to eventually develop into free swimming larva (Davis & Chanley 1956, Bahr & Lanier 1981).

The first larval stage capable of real movement is the trocophore stage in which cilia develop and shells are formed, with the cilia eventually becoming a ring of motor and feeding cilia called the larval velum, leading to the veliger stage (Bahr & Lanier, 1981). Next, a foot develops, enameling the larva to attach to a substrate, and the larva transitions to the pediveliger stage; pigmented eyespots development begins around this time as well and signifies that the larva is ready to attach and become an adult (Bahr & Lanier, 1981). The process from fertilization to attachment averages 2 weeks but can take as little as a week or as long as two months depending on factors such as food availability and temperature, with warmer waters triggering more rapid metamorphosis (Bahr & Lanier, 1981).

Once the oysters attach to a substrate the process of shell formation begins, in which over the course of an oyster's first year their shell will typically grow around 30 mm (Munroe et al. 2016). Shell growth typically occurs at a linear rate, except during winter months when it is minimal, leading to larger shells being seen in oyster spat that settle earlier in the year (Munroe et al. 2016). Larger oysters have been found to take longer to prepare to spawn but produce

more eggs when they do (Choi et al. 1993, Choi et al. 1994). Though it is uncommon, eastern oysters can live for up to 20 years (Buroker 1983).

Oysters attach, and ultimately build reefs, in areas that allow steady water flow and consist of suitable substrate, with anything but soft sand and mud being acceptable to oyster larvae (Bahr & Lanier, 1981). Individual oysters tend to settle aligned parallel to the direction of the water current which maximized their filter feeding, settling in close proximity to one another in forming reefs (Bahr & Lanier, 1981). These reefs can eventually grow into large structures that can cover a wide area when not affected by pollution or overharvest, with historical accounts claiming reefs were navigational hazards to ship traffic in the Chesapeake Bay (Bahr & Lanier, 1981). By volume the reefs are typically around 60% live oysters, 20% dead oysters, and 20% sediment and other organisms (Bahr & Lanier, 1981).

The eastern oyster feeds by propelling the surrounding water through their shell cavity with their cilia and filtering out particles to eat (Bahr & Lanier, 1981, Newell et al. 2005). Their diet consists of plant matter, algae, zooplankton, phytoplankton, and oyster larva, with the exact size of the particles that can be filtered effectively being as small as 2 micrograms (Bahr & Lanier, 1981; zu Ermgassen et al. 2013). Oysters do not filter continuously but instead go through stages of filtration and digestion and filter more strenuously when food concentrations are lower (Bahr & Lanier, 1981). Eutrophic conditions have been shown to benefit the eastern oyster, as increased availability of food positively correlates to juvenile growth rates and survival (Wall et al. 2013).

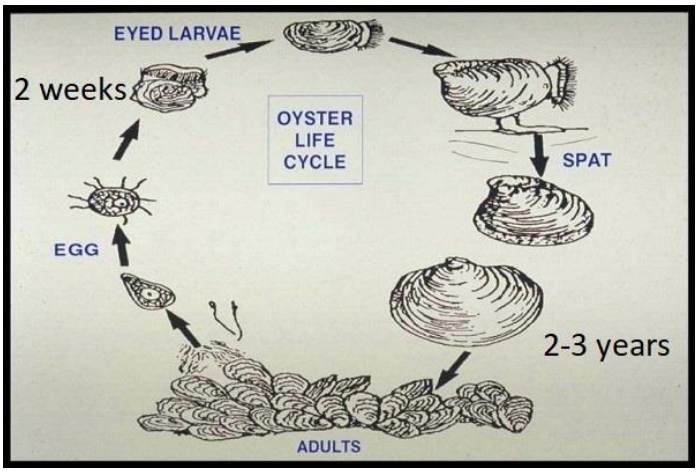

Figure 1. Oyster life history cycle diagram. Credit: Auburn University School of Fisheries

Oyster Shell Construction

Oyster shells are composed of calcium carbonate in the form of either calcite or aragonite crystals (Fig. 3) that differ in their exact shape (Lasseter et al. 2016). These crystals are the mineral component that along with a poorly understood organic component allow oysters to build up their shell throughout their lives; pH, temperature, salinity, hardness, and nutrient concentrations among other factors of water quality also affect shell construction (Lasseter et al. 2016). The innermost foliated layer is the thickest layer and provides support through calcite sheet layers (Lombard et al. 2013). The outer periostracum provides the shell with corrosion resistance (Lombard et al. 2013). Research in this area suggests that the hardness of the shell is directly related to water temperature, with warmer water leading to thicker shells, both when comparing Canadian oysters to oysters native to warmer climes and when artificially raising the temperature of tanks containing native northern oysters (Dame 1972, Lord & Whitlatch 2014). The shell of the eastern oyster strengthens significantly with age, with an average compressive force around 1000 N when a year old but reaching over 3000 N by age 6 (Lombard et al. 2013).

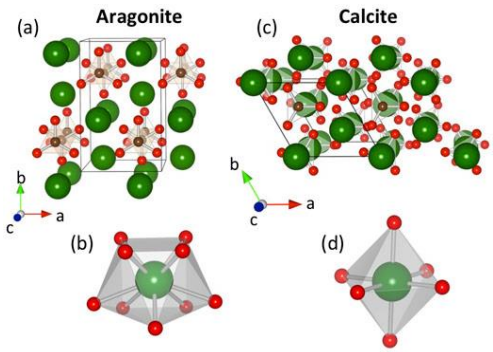

Figure 2. *The structures of Aragonite and Calcite crystals. Green spheres are calcium, brown represents carbon, and red spheres indicate oxygen. Credit: Soldati et al. 2016.*

The forms of calcium carbonate present in an oyster shell can vary with age and shell layer of the individual animal (Haley et al. 2018, Stenzel 1964, Lasseter et al. 2016). Younger oyster shells tend to be predominately the more soluble aragonite, while older oysters' shells are primarily calcite, but trace amounts of aragonite are still found in adult shells (Miller et al. 2009, Stenzel 1964). The exact reason for this shift in composition is unclear, but trace amounts of aragonite remain in the middle prismatic layer of the shell (Lasseter et al. 2016). The inner foliated layer is composed of calcite sheets and the outer periostracum consists of an organic matrix (Lasseter et al. 2016, Mount et al. 2004).

Surprisingly little is known about the exact method through which oysters construct their shells at a cellular level beyond that the primary component is calcium carbonate (Lasseter et al. 2016). The two working theories are the matrix model, in which a matrix secreted from the mantle of the shell helps grow calcium carbonate crystals, and the cell mediated theory, in which hemocytes form the crystals that are deposited at the mineralization front (Zhang et al. 2012). Genomic data of the oyster shell has indicated that there are no self-assembling silk fibroin proteins such as those found in arthropod silk, which would be needed for the matrix model to prove accurate (Furuhashi et al. 2009, Zhang et al. 2012). There is however some evidence for the cell mediated theory, as there is a diverse number of proteins similar to animal

cells, including genes that code for fibronectin, laminin, and collagen that are likely organized by hemocytes (Zhang et al. 2012). Hemocytes that appear to be involved in shell construction have been found at the site of shell mineralization, and contain calcium carbonate crystals (Mount et al. 2004).

Oysters can also incorporate metals from their surrounding environment (Fig. 4) into their shells based on the concentrations of these micronutrients present in the water. (Lasseter et al. 2016). Certain metal ratios have been found in consistent amounts based on recent studies, with silver and iron and copper and boron appearing in consistent ratios, as well as two groups of trace metals (Lasseter et al. 2016). These trace element groups are exclusive of one another despite collectively amounting to 3% of the shell composition occur in proportion to one another, and members of each group occur in proportion to one another (Lasseter et al. 2016). These trace metals can also be found at higher concentrations in the shell when the metals are found in higher levels in the surrounding environment, and their exclusivity of each other is suggested to indicate metabolic control of their incorporation on the part of the oyster (Lasseter et al. 2016). In the case of magnesium, this incorporation leads to the formation of magnesium carbonate (Surge & Lohmann, 2008). This assimilation is possible as magnesium is present in ocean water in mean ratios of 0.324 Ca/Mg (Thompson & Wright, 1930).

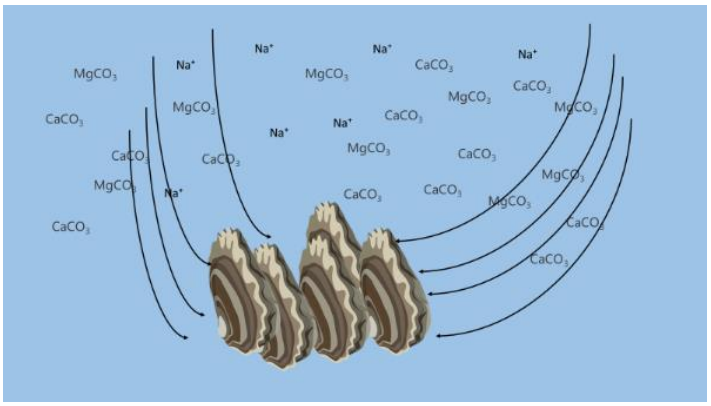

Figure 3. *Representation of oysters incorporating ambient ions and elements in ocean water.*

Oysters as a Keystone Species

The eastern oyster is a prolific filter feeder, with the Chesapeake Bay population capable of filtering the entire volume of the Bay in under a week before increased human interference began in 1870 (Newell 1988). Historically this would have had a major impact on estuaries with large oyster reefs, keeping the water clear of suspended particulate and algae and greatly improving water clarity (zu Ermgassen et. al. 2013). Individual oysters have been found to have a maximum filtration rate of 0.17 (±0.07) m3 g^{-1} Dry Weight day^{-1} (Ehrich & Harris 2015). This filtration ecosystem service has seen an 85% median reduction in the last century, with 12 of 13 examined estuaries impacted, including all in the Chesapeake (zu Ermgassen et al. 2013). Without this filtration eutrophication in native habitats is more likely to occur and general water clarity is diminished; and estuaries in the Gulf of Mexico and Chesapeake Bay have likely undergone significant ecological shifts away from their natural states in the last century due to the loss of these oysters as water bodies take longer for oysters to filter (Wall et al. 2011, zu Ermgassen et al. 2013).

The filtering capability of oysters has another positive benefit for the surrounding environment, as 30% of what is filtered is not assimilated by the oysters, and in turn these excretions provides food for nearby benthic organisms throughout the intertidal food web (Tolley & Volety, 2005; Newell, 1988). Providing food is just one of the benefits oysters provide other invertebrates and fish that colonize their reefs, as these structures are considered comparable to coral reefs for the habitat opportunities provided to aquatic wildlife (Tolley & Volety, 2005). Xanthid crabs feed on oysters, and mud crabs will consume oyster spat, while invertebrates such as porcelain crabs and the bigclaw snapping shrimp are filter feeders that are common among reefs to benefit from food missed by the oysters (Tolley & Volety. 2005).

Oyster reefs can also provide structural habitat benefits for organisms such as fish, as species such as the frillfin goby, naked goby, striped blenny, and skilletfish have all been found to lay their eggs within oyster reefs crevices (Tolley & Volety. 2005). Oyster reefs are an amalgam of living and dead oysters, with the dead more commonly used by fish for nesting purposes, while the living oysters provide benefits to filter feeders; but without living oysters to replenish the reef population many of these species would suffer for loss of food and habitat (Tolley & Volety. 2005).

Protection of shorelines from wave erosion is another service oyster reefs provide their native habitats (Theuerkauf et al. 2016). Reefs can stabilize sediment in the area, and slow sea level rise in areas where the reef is substantial enough (Theuerkauf et al. 2016). The shoreline protection of these reefs has been found to protect the retreat of marshland by an average of 1 meter per year relative to unprotected marshland (La Peyre et al. 2015). These reefs can also help to store excess N and P, as oysters both filter out excess phytoplankton when their numbers are high enough, and help store those elements in sediments (Newell et al. 2005). As a result of the losses oysters have sustained, eutrophication events are more common in the Chesapeake Bay today than in the past with the amount of Nitrogen content in the Bay increasing 2.5 times its initial levels since the 1940's (Kemp et al. 2005, Newell et al. 2005, Wall et al. 2011).

Oyster Population Decline

Oysters have seen a rapid decline in their population for a number of reasons, but human overharvest is a primary one. Worldwide populations of oysters are estimated to have declined by 85% of their historical numbers (Beck et al. 2011). The eastern oyster specifically inhabits reefs that exhibit conditions defined as fair to poor, with the Chesapeake Bay in the

poor category (Beck et. al 2011). This categorization is defined as having lost 90-99% of the reef's historical numbers, due to the fact that the Chesapeake is one of the most harvested from regions in the world with captures over 5500 metric tons per year (Beck et al. 2011). This makes the Chesapeake Bay one of only six areas that produces harvests this large, all but two of which are in the United States: the North and South Gulf of Mexico, the Yellow Sea, Virginia, Florida, and Carolina (Beck et al. 2011). Dredging of oyster reefs, in which the surface of reefs are scraped by a large net with a steel frame towed by a boat, has been particularly harmful as it both allows for larger harvests further from shore, and also destroys the structure of decades and century old reefs to an extent that their quality as habitat for future juvenile oysters was diminished as well (Fig. 2, Beck et al. 2011, Rothschild et al. 1994).

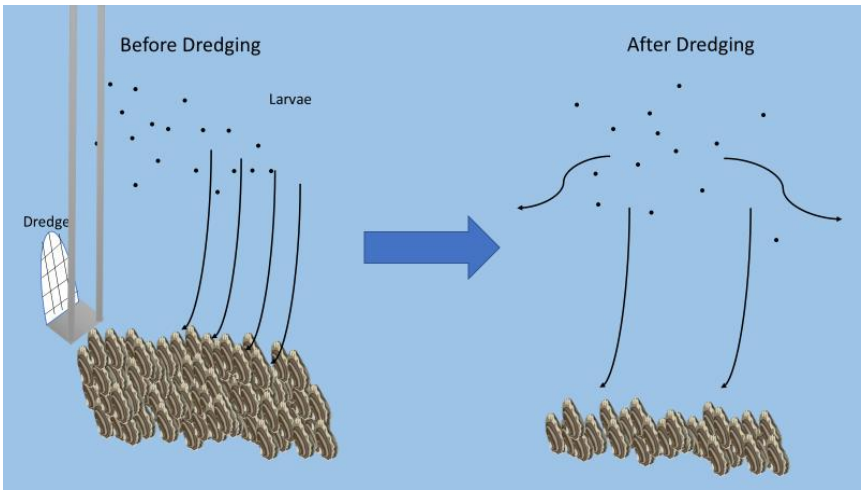

Figure 4. *A representation of an oyster dredge degrading potential larval oyster attachment habitat.*

Increased carbon dioxide in the Earth's atmosphere has adversely influenced the world's oceans and inhabitants including oysters through ocean acidification (Riebesell et al. 2010). When carbon dioxide dissolves in ocean water, it reacts to become carbonic acid, which in turn will release Hydrogen ions in becoming bicarbonate and finally carbonate (Riebesell et al. 2010). These hydrogen ions have caused the pH of oceans worldwide to drop by 0.1 point since the mid-1700's, and further decline over the next century could reach a 0.5 unit decline (Miller et al.

2009, Matoo et al. 2013, Boulais et al. 2017). Estuaries frequently inhabited by oysters could see these effects even more acutely as some Atlantic coastal areas can see pH ranges of 8.2 to under 7.0 due to microbial degradation of the organic matter releasing carbon dioxide in those environments (Boulais et al. 2017, Wallace et al. 2014).

Though estuary pH varies naturally, oysters were shown to grow and survive best at pH conditions of around 8.2, while increasingly acidic conditions harmed juvenile oysters at the pH of 7.5 now common in summer months in oyster inhabited estuaries (Beniash et al. 2010). Juvenile oysters were found to spend more energy maintaining homeostasis, and by extension having smaller shell and tissue mass when pH ranges were more acidic (Beniash et al. 2010, Amaral et al. 2011, Waldbusser et al. 2013). Shells also dissolved more quickly when under more acidic conditions with both living oysters and dead oysters, displaying how juveniles have to spend more energy to build up their shells in lower pH than would be needed under more basic conditions (Beniash et al. 2010, Waldbusser et al. 2011). This increased energy expenditure also affects juvenile's shells structurally, as the hardness was reduced and shells were found to fracture more easily (Beniash et al. 2010). Further evidence for the negative effects of pH is a 30% mortality rate higher for oysters in acidic water than more basic water (Beniash et al. 2010). Gametogenesis in adult oysters is also adversely affected by ocean acidification, with gonad development delayed relative to oysters inhabiting higher pH levels beginning at pH of 7.1, and inhibited completely at a pH of 6.7 (Boulais et al. 2017).

Another anthropogenic cause for the oyster's precipitous population loss stems from non-CO^2 forms of pollution that have altered the fragile balance of conditions needed for oyster larvae and juveniles to thrive. Sedimentation caused by human interference upstream of oyster estuaries, such as deforestation and agriculture, is one factor that can harm oyster recovery through burying reefs faster than they can grow (Colden & Lipcius, 2015). Sedimentation is

likely to increase in the future due to climate change leading to more frequent and powerful storms that create more sediment (Najjar et al. 2010). Adult oysters tolerate sediment burial up to 70% after which survivorship decreases; the expectation is that juveniles would suffer more under similar conditions (Colden & Lipicus, 2015).

Oysters and native barnacles and mussels have developed neutral or even positive relationships, as barnacles both feed on oyster larvae and appear to provide settlement cues to oyster larvae as well as settlement sites, while mussels display higher densities when local oyster densities are higher as well (Manley et al. 2010). This likely results from the added protection from predators that oyster reefs provide, as these invertebrates compete for food (Manley et al. 2010). Invasive species have more harmful effects however, as human ship travel has allowed for invasive invertebrate species to alter the ecosystem dynamics of the Chesapeake Bay and the eastern oyster.

Three such species have been examined for their effects on the eastern oyster: the Asian green mussel, charru mussel, and pink titan acorn barnacle (Yuan et al. 2016). These species all came to the east coast from their native Pacific waters within the last twenty years, typically through ship ballast water (Yuan et al. 2016). The presence of the mussels was found to negatively affect the survival of juvenile oysters by 20-25%, and the green mussel and the barnacle were found to negatively influence recruitment of oysters such that typical observed rates of over 1.3 oyster spat per cm^2 were lowered to under 1 spat per cm^2 on substrates with the invasive animals (Yuan et al. 2016). Researchers believe this occurs through invasive green mussels and acorn barnacles secreting chemicals that repel larval from attaching nearby (Yuan et al. 2016). The mussels also consume a large number of early-stage oyster larva through filter feeding (Yuan et al. 2016). Competition for food could also contribute to lowered survivorship of the attached oysters (Yuan et al. 2016).

Human interference through non-native species introduction is also believed to have led to disease epidemics among the eastern oyster (Burreson et al. 2000). The protozoan parasites *Haplosporidium nelson* (MSX) and *Perkinsus marinus* (Dermo) were discovered to be affecting the eastern oyster decades ago, and have severely reduced the restoration of the eastern oyster (Mann & Powell 2007). MSX is believed to have been passed to eastern oysters through introduced pacific oysters, *Crassostrea gigas*, in the late 1950's (Burreson et al. 2000, Guo et al. 2016). MSX exhibits high mortality rates of 90% in infected oysters, and primarily affects oysters in water temperatures above 20°C (Guo et al. 2016).

Dermo has likewise been observed for approximately 50 years, and has a particularly unusual effect of killing older, larger oysters that are typically past the point of dying through predation at a rate of over 70% of infected individuals (Mann & Powell 2007). Oysters become infected through filtering particles in the water containing the disease; these can come from feces of nearby infected oysters, and tissue decay of oysters that had been infected, potentially killing the majority of a reef quickly as oysters infect their neighbors (Bidegain et al. 2015). Selective breeding has begun to be ameliorate the issue such that survival in select stocks is approximately 70-80% (Mann & Powell 2007, Dégremont et al. 2015). Additionally, resistance may be localized; a population of North Carolina native oysters had a mortality of 40% when challenged with Dermo while Chesapeake Bay native strains experienced 100% mortality (Brown et al. 2005). Though oyster larvae cannot control the prevalence of disease in locations where they settle, they do exhibit several instances of conscious habitat selection.

Oyster Habitat Selection

Oyster spat display habitat preference regarding their attachment site, both in terms of substrate type and orientation (Bahr & Lanier, 1981, Theuerkauf et al. 2015). Oyster larvae are

believed to follow many potential settlement cues such as light, salinity, biofilm composition of the settlement site, and water current, as their swimming capabilities are limited (Bahr & Lanier, 1981, Campbell et al. 2011). Increased salinity has been shown to increase their swimming speed (Hidu & Haskins, 1978), and they show a preference to attach to adult oysters that is believed to come from biochemical markers on adult shells (Bahr & Lanier, 1981). Larvae also respond to the underwater sounds of a reef positively and settle more compared to unstructured settlement sites (Lillis et al. 2013). This larval awareness applies to factors such as orientation, in which oysters orient themselves parallel to the water flow; and in substrate firmness, in which oysters select substrates that are more stable than soft sand or mud (Bahr & Lanier, 1981). Biofilms on the attachment substrates of larvae can play a role in settlement choice, with older biofilms being preferred (Campbell et al. 2011). Oyster larvae also will display preferences for substrate composition when more than one acceptable landing spot is present, field tests have shown recruitment lagging on substrates such as porcelain, but exceeding natural shell on organized concrete structures such as oyster castles (George et al. 2015, Theuerkauf et al. 2015).

Another factor that seems to govern the possible recruitment and survivorship of oyster spat is interstitial space of the habitat. Past research indicates that greater degrees of open interstitial space lead to more predation from mud crabs on invertebrates in oyster reefs (Hesterberg, 2016). The degree of interstitial space in oyster reefs has been shown to potentially influence the survivorship of oyster spat as well, with smaller, harder to reach spaces potentially helping protect juveniles from decapod predators while larger spaces may provide greater access to predators, and animals of higher trophic states that consume the predators of oysters more readily (Nestelrode et al. 2007).

Oyster Restoration Efforts

Within the last decade the perception of oyster restoration has changed significantly. Restoration of the eastern oyster in the Chesapeake Bay was considered a lost cause by some researchers, due to factors such as unchecked disease, slower settlement rates than required for a population to be self-sustaining, and general poor restoration goals and management (Mann & Powell 2007). In more recent years however, novel methods of restoration have helped stem some of these issues. Restoration efforts have accelerated with the discovery that oyster populations that have been self-sustaining for longer than 5 years used high relief reefs to create a metapopulation that became the largest introduced population in the world (Schulte et al. 2009, Schulte et al. 2014). The area was also protected from fishing, highlighting the value of restoration efforts that incorporate regulations that protect oysters from anthropogenic removal (Schulte et al. 2009).

As natural oyster shell has become harder to procure and more expensive, alternative substrates have become a focal point for restoration research, with both artificial and natural sources being studied (Nestelrode et al. 2007, Theuerkauf et al. 2015). Surf clamshell's effectiveness has been shown to be minimal in terms of oyster preference (Nestelrode et al. 2007). Alternative substrate types that have been examined include concrete, porcelain, limestone, and river rock (George et al. 2015). The alternative substrates have not shown significant differences in recruitment of spat, compared to natural shell (George et al. 2015); however, the alternative substrates did demonstrate some variation in terms of mean oyster growth, with the size of porcelain spat lagging behind other substrates (George et al. 2015). Building off of the potential utility of concrete, structures called oyster castles (Fig. 5) have been created with the goal of providing an alternative substrate to act as a reef foundation

(Theuerkauf et al. 2015). These castles are composed of concrete, limestone, and oyster shell and designed with a parapet shape to mimic a simple castle, and were shown to match or improve recruitment and survivorship of attached oysters relative to natural shell substrates, potentially providing a new method of oyster restoration (Theuerkauf et al. 2015).

Figure 5. *A photo of an Allied Concrete oyster castle*

Measuring the success of oyster habitat restoration has not been standardized, but guidelines involving several factors with logical, measurable goals have been suggested. Oyster population enhancement, habitat enhancement locally and in surrounding areas, and water clarity improvement follow as acceptable metrics based on the oyster's ideal role as a keystone species (Baggett et al. 2015). Reefs should be able to grow large enough to be self-sustaining in order to influence their environment, which would in turn create new habitat for other local species and benefit the surrounding areas through factors such as erosion control and water clarity improvement (Baggett et al. 2015).

Future Areas of Research

Oyster restoration efforts have seen mixed results; while more successful practices such as oyster castles and use of alternative substrates are being discovered and implemented (Baggett et al. 2015, Schulte et al. 2009, Theuerkauf et al. 2015), any additional findings that

would allow oyster population growth to be augmented would still be valuable. One aspect of oyster research that has been little studied is the possibility that the addition of micronutrients to the artificial substrate, such as the addition of magnesium to concrete, could aid oyster health and by extension restoration efforts. Any potential relationship magnesium may have on direct oyster health has been little examined, save a few anecdotal inferences from studies focused in other areas that oysters seem to consciously incorporate magnesium in some environments and that a group of metals that include magnesium did not significantly affect oyster health (Lasseter et al. 2016).

Other bivalves such as mussels have been shown to benefit from magnesium (Dietz et al. 1994, Fritts et al. 2015.) The zebra mussel for example requires magnesium in the water to survive (Dietz et al. 1994). Magnesium deficiency is believed to disrupt cell junctions in the epithelial layer and make osmoregulation more difficult for bivalves such as freshwater mussels (Fritts et al. 2015). Whether oysters could receive a similar benefit from magnesium is unknown and exactly how oysters incorporate magnesium is not well understood, but this relationship of magnesium and bivalve health deserves further study, as the eastern oyster could benefit from increased magnesium incorporation similar to how mussels seem to. It is also possible that larval oysters would be able to detect (Lasseter et al. 2016), and by extension be attracted to, substrates containing higher levels of magnesium. Support for this theory could lead to more effective restored oyster reefs, as oyster larvae could be enticed to attach to more secure locations that exhibit ideal conditions for survival of the reef as a whole.

Study Objectives

The purpose of these experiments was to examine what effects the addition of magnesium to an artificial substrate may have on the recruitment and growth of juvenile oysters both in the field and in the lab. Using a study site on the Northern Neck of the Chesapeake Bay in Kilmarnock, VA, two artificial substrates and natural shell were left to examine recruitment, mortality, and growth on larval oysters that would attach and grow over a summer. In the fall, the shells and all attached oysters were taken from the field to the lab to examine if attachment to a particular substrate lent spat any additional resistance to simulated ocean acidification. Shells of spat were also tested for magnesium levels to determine if spat attached to magnesium substrates incorporated more of the micronutrient.

Methods – Field

The chosen field site was located in Kilmarnock, VA at Pitman Cove (37°41'39.5"N 76°21'09.5"W). A dock at the site is owned by the Camp Kekoka YMCA camp and was used to attach mesh oyster bags containing artificial and natural shells used in the study (Fig. 6). The dock extends approximately 25 meters from the shore. Each bag was hung from a dock plank on the side of the dock facing the cove inlet. The cove's water level ranges from 1-2 meters depending on tidal activity, and bags were hung so that they remained submerged underwater at all times. The water temperature in the cove during field visits in September and October ranged from 27.7°C to 19.9°C with a mean of 23.97°C. Cove pH in September and October ranged from 6.98 to 7.46, with a mean of 7.31. Lab salinity probes were found to be inaccurate even after recalibrating, recording values that were too low to be possible. NOAA provided data from nearby data collection sites display salinity ranges of 16 PSU to 22 PSU for the area during

the time period of data collection. Water turbidity for the site was high based on observation, with visibility generally not extending past a meter depth.

Figure 6. *Overhead view of field site cove and dock*

Three substrate types were examined in this experiment: natural oyster shell, concrete, and concrete enriched with magnesium carbonate. Natural oyster shells were donated or purchased from local restaurants and scrubbed clean using Simple Green cleaner, bleach, and toothbrushes. Cleaned shells were left to soak in bleach water for approximately 48 hours then removed and allowed to dry. Half shells were treated as individual shells for this experiment due to the difficulty of finding matching valves and reconnecting those valves with environmentally friendly glues. We assume the difference in surface texture between the two sides of an oyster shell will not affect the outcomes of the experiment. This assumption was not explicitly tested, but throughout the experiment larvae attached to the inner side of shells as well as the outer side. Both concrete and enriched concrete artificial shells were made by pouring wet concrete into silicone shell molds. Molds were created using wet Mold Max 30 (https://www.smooth-on.com/products/mold-max-30/) that was poured around a complete oyster shell (both valves) sitting upright in a cup. Molds were left to dry for 24 hours. Upon the

drying of the silicone mold, shells were cut out using a knife or scalpel so molds were not damaged to the point of losing their seal, leaving a mold in the exact shape of the removed shell. Five shells were molded.

For concrete shells, Portland cement, limestone sand, and water were mixed together in a bowl in 2:2:1 ratios using a dental spatula to stir, and then poured into shells molds. Shells were left to dry for 24 hours before being removed. Cement batches were produced in amounts of 200g each of cement and sand and 100g of water, with 4-5 molds being filled depending on the specific mold used. Total daily batches of 20 shells were made using 1 kg of cement and sand each and 500g of water. Concrete molds were not cured underwater as their hardness was sufficient upon removal from the molds.

Magnesium shells were produced similarly, with cement, limestone sand, magnesium carbonate powder, and water being mixed in ratios of 4:4:1:4. More water was needed due to magnesium carbonate powder being less water-soluble than cement or sand. Shell batches were produced 20 per day, with 800g of water, sand, and water being mixed with 200 grams of magnesium carbonate powder. Higher magnesium concentrations were attempted in batches but proved too brittle after removal from the molds. Shells were left to dry for 24 hours and then removed from the mold to be cured underwater in a plastic container filled with tap water for 1 week to allow the shell to better harden (Mamlouk & Zaniewski (1999): Materials for Civil and Construction Engineers, Addison Wesley Longman, Inc.).

Shells were numbered from 1-120 for each substrate type using sharpie marker on 3 sides of artificial shells and on the smooth side of natural shells. Fifteen randomly selected shells were placed in one of 24 oyster bags, eight for each substrate type. Bags were tied off on both ends and labeled with a laminated tag attached to the bag with a zip-tie. On the dock 1.8 meters of rope was used to tie a bag to each plank (Fig. 6). Removing the rope length tied to

either the plank or the oyster bag, bags were suspended approximately 1.4 meters below the dock, ensuring they were submerged regardless of tidal conditions or distance from shore while still suspended above the cove bed. Bag order along the dock was randomized so that each substrate was represented within a block of 3 bags in a random order, to ensure that certain substrates were not clustered in a single dock position and exposed to any different water, depth, or light conditions (Fig. 7).

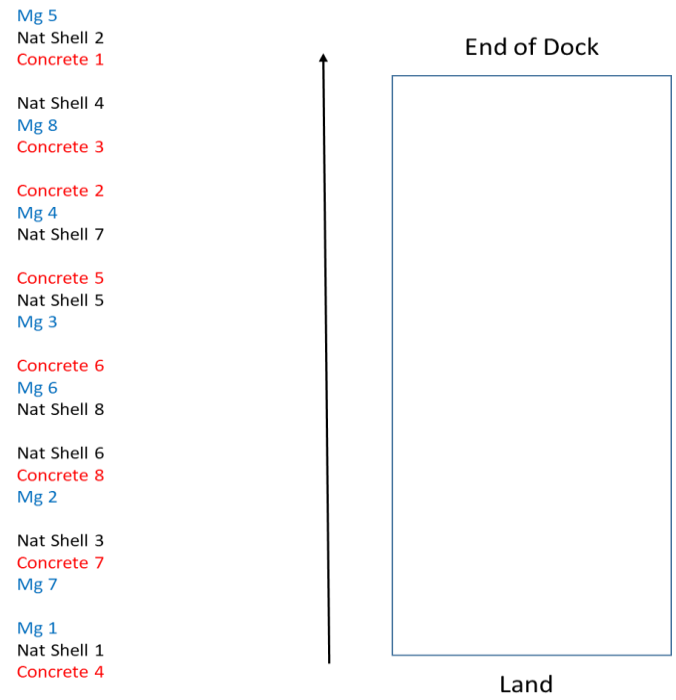

Figure 7. *Top – photo of bags hanging from dock. Lower - Representation of order of oyster bags hanging from the field site dock.*

Bags were left at the field site on May 15th for shells to accumulate biofilm and a microbiome conducive to oyster larval recruitment well before spawning was slated to occur. Based on historical spawning periods in the local area and local oyster gardener advice, an anticipated start date for data collection of July 8th was planned, with one earlier visit to remove excess biofilm from the shells occurring 2-3 weeks prior. Upon the preparatory visit on June 17th, bags were found to be falling apart and in need of replacement, and oyster spat were found. Spat were not tallied because not all bags were examined to reduce the chance of the unexamined bags falling apart. Bags were replaced on June 28th with a bag made of sturdier plastic mesh that did not need to be replaced again for the duration of the field season. A first official data collection visit occurred on June 28th to compensate for this early spawning event, with the next field visit occurring as planned on July 8th, and every 2 weeks thereafter through October 28th.

Field sampling consisted of removing each bag from the water and removing each shell from the bag. Shells were scrubbed clean of biofilm using toothbrushes and visually examined for any attached spat. If spat were found on a shell, a ruler was placed next to the spat and a photograph was taken of the spat and shell number. Photographs were ideally taken of each shell with spat for each visit, but this was not always the case due to time constraints or weather. On September 2nd rain caused some shells to not be photographed, and on September 16th and 30th time constraints caused some shells to not be photographed. On days in which not all shells were photographed, special attention was given to ensure that those shells were photographed during the subsequent visit. Only spat alive at the end of the field season were measured for growth, and as such missing photos had minimal impact on results. Visual tallies still allowed for accurate measurements of recruitment and mortality during the parts of the field season photos were not collected.

Total spat counts per shell were recorded in a field notebook. Dead spat (defined as spat whose hinges were open when examined) were tallied through visual surveys of shells in the field or through spat going missing when comparing recorded spat tallies from previous visits. Faded marks were remarked as needed with sharpie marker. Photos taken of shells were used to determine approximate spat attachment period, determine if spat went missing, and measure absolute growth of spat through the field season, or their final size upon death. Analysis of the photos occurred upon return from the field site, with absolute growth measurements being performed through the program ImageJ (Rueden et al. 2017) using the photographed ruler next to the shells as a scale. The program allowed digital measurement of the ruler in a photo to set a scale for measuring any 2 points within the photo. Spat in the photos were measured from the hinge of the shell to the top of the shell.

Shells were removed from the water and placed in new, cleaner bags before being removed from the field permanently and scrubbed thoroughly. Bags were placed in buckets containing water from the field site, both to keep oysters alive for the transport back to JMU and to inoculate the aquarium tank with the local aquatic microbiome upon return. Buckets had small holes in their lid to reduce water loss but allow oxygen exchange for the oysters. Oxygen bubblers were not used for the 3-hour drive, and mortality was low in the week following the removal (3/471). Additional water collections were taken back to the lab with 40 L carboys. Upon return to the lab, water from the field site was added to the 100 gallon aquarium to be used in the laboratory experiments and then shells with live spat on them were added. Shells without spat were dried and stored in the lab.

Recruitment per shell result histograms were heavily skewed to the right (primarily ones and zeros) and this distribution type made a Poisson ANOVA the appropriate test to determine if there were differences in larval preference for attachment substrate. Spat mortality per shell

was not normally distributed but did exhibit equal variance (Levene's test p value 0.81) and a Kruskal-Wallis test was used over an ANOVA to test for differences between exhibited mortalities. Ties were present and a Nemenyi post-hoc was used to determine where differences were present and control for Type I error inflation. For spat growth a one-way ANOVA was used with a Tukey post-hoc test to determine where significant differences occurred between spat growth on the three substrates. All statistics were run in R 3.4.3 (R Core Development Team 2017).

Methods - Lab

pH Experiment

The 100-gallon lab aquarium was filled with water taken directly from the field site. A single large aquarium was used to house all bags instead of several aquaria being used to house 2-3 bags. This arrangement aimed to ensure that the exact same water conditions were experienced by all oysters and fit the structural parameters allowed by the lab space. Using multiple aquaria would have created the likelihood that the exact same pH conditions could not have been achieved using a single pH controller and CO_2 tank for every tank due to the design of controllers and CO_2 tank manifolds (splitters).

A total of 371 liters (98 gallons) of collected water was used to fill a 100-gallon aquarium and another 113.5 liters was stored in an uncapped carboy container in the lab to allow refilling of the aquarium as needed from evaporation. Using water from the collection site allowed seeding on the lab aquarium with microbes found in the field. When field site sourced water was depleted, RO water mixed with Instant Ocean Salt ® (in ratios suggested by the package instructions of 40 g per liter of water) was added to the tank to supplement water loss caused

by evaporation. A main difference between the lab water and cove water was it lacked the microorganism composition of the cove water. This organismal difference was less than ideal, but as the field site was 320 km away from the lab, frequent trips to collect water from the field site were impractical.

Water was initially cycled through the aquarium using 4 Aqueon ® water filters. Filter pads were removed so oyster food (Shellfish Diet 1800® at 0.5 ml every other day) was not filtered out but to still allow for water movement and oxygen cycling. Over the course of the first month, two filters stopped functioning, at which time an Imagitarium ® aquarium power head was used to circulate water within the tank. The aquarium was not controlled for temperature and kept at room temperature with a mean of 21.7°C, within the range of the temperatures recorded at the field site mean of 23.97±3.9°C but above the water temperatures expected to occur in October and through the winter months. This increased temperature could have led to oysters being more metabolically active than they typically would have been in December, but this would also have simulated the metabolism activity seen when the local water is warmer and lower pH would be more commonly experienced. Salinity of the Chesapeake water in the aquaria was measured to have a mean value of 23.26 ppt, prior to a major equipment failure that will be described below.

A Neptune Apex Aquacontroller ® regulating a solenoid attached to a CO_2 tank was used to control the pH (Fig. 8). A 22.68 kg. CO2 tank was attached to a Milwaukee ® CO_2 regulator with the solenoid. The regulator fed CO_2 into the tank through CO_2 proof tubing attached to a ceramic diffuser stuck to the side of the tank (methods adapted from Waldbusser et al. 2011, Keppel et al. 2016). The regulator had a bubble counter attached, and a flow rate of approximately 40 bubbles per minute was used for CO_2 input based on user experimentation. The Aquacontroller monitored pH, temperature, and salinity continuously, and was

programmed to turn on the solenoid when pH was higher than the intended study pH treatment level. Probes were suspended over the middle of the tank to ideally measure the water quality.

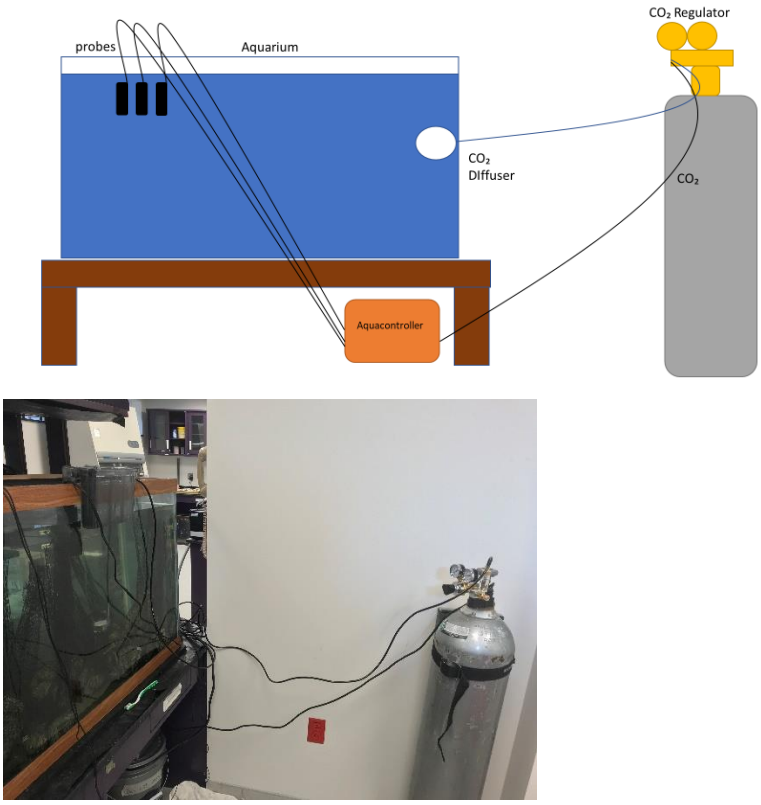

Figure 8. *Top – schematic of aquarium setup. Lower – Photo of aquarium with tubing connecting to a CO_2 tank and regulator, aquacontroller not pictured.*

Oysters were allowed one week without CO2 input to acclimate to the tank. During this time the pH of the tank slowly rose from an initial 7.46 to a final pH of 7.57. Initial pH treatment was set to 7.45, matching the pH seen at the field site. Aquacontroller programming was established through a program synced to the controller from a computer to keep the pH between 7.42 and 7.48. Every 10 days the pH was to be lowered by an intended mean of 0.2 units, with a +/- of 0.03 pH units programmed as an acceptable range. This would continue until all oysters were dead. The pH level dose that killed half of the initial number of oysters taken from the field would be used to calculate the LD50, or the amount of pH change needed to kill 50% of the total spat for each substrate.

Oysters in the tank were examined every other day to determine a status of alive or dead. Shells were kept in bags in the tank, with the same group of shells as in the field, to allow easier data processing and ensure oyster shells could be easily removed from the tank for examination. Oysters that were dead were cleaned from their shell to prevent water fouling and avoid confusion with remaining oysters. Once all spat on a shell were dead, the shell was removed from the tank. Removed upper shell valves were kept and frozen to be examined later through mass spectroscopy to determine the percentage of magnesium present in spat attached to each substrate. Final durations of survival were to be calculated for each spat, and a mean survival duration for each bag was calculated.

Unfortunately, equipment failure occurred three weeks into the study, and only the first pH treatment was performed. Shortly after the daily check, both the regulator and pH controller failed. The regulator drastically increased the amount of CO2 entering the tank, lowering the pH. The pH controller, which should have turned off the regulator also failed. The reason for the pH controller failure is unknown, it had been functioning well until the failure. The reason for the failure of the regulator is also unknown. The aquarium was found the next morning at a pH of 5.7, with the regulator billowing in CO2 at a rate far above 40 bubbles per second (the water in the bubble counter had evaporated) before being turned off. To raise the pH quickly, 1.36 kg of baking soda was added to the tank, to raise the pH to 7.0. The regulator was no longer trusted and not used again; the tank was allowed to idle until a replacement could arrive. The replacement regulator did not arrive for 3 weeks. In the interim, the pH began to rise to a level not seen when the tank was previously allowed to idle with no pH control, rising above 8 within a week. Without a usable regulator, pH lowering was attempted through adding white vinegar, but the amounts needed to make any noticeable difference in tank pH were uncomfortably high. Adding leaves was the next attempted method of lowering the pH but this

proved ineffective. Aquarium pH had stabilized at 8.6, within the reported tolerance range of the eastern oyster (Calabrese & Davis 1966), and at this point the aquarium was left alone until the regulator arrived.

Every two days from November 29th through December 24th oysters were checked for status of alive or dead and cleaned out to prevent fouling. Despite 8.6 being within oyster pH tolerance ranges, there were 20-50 dead oysters (5-10% of the total spat population) in each check. Between December 22nd and December 24th, 2017, total counts of oysters surviving on each substrate dropped below 50 individuals, from initial starting points of between 126 – 183 for concrete and natural shell respectively. At this point, alternate methods of research were examined, as the experiment sample size was declining to the point of being unable to continue. No results were obtained from a pH below 7.4, which was only the starting point of the experiment, what little data there was not robust enough to report.

Shell Chemical Composition and Water Chemistry Analyses

The shells of any spat to die over the course of the experiment were collected so they could be analyzed for their chemical composition, specifically for magnesium concentrations. Dead spat shells were analyzed through scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDX) to determine the chemical composition of the shells (Dr. Harry Hu, JMU Chemistry). SEM/EDX uses a focused beam of electrons to map the surface of a solid substance, and chemical composition information can be gleaned from backscattered electrons and light from the beam. Four shells formerly attached to each substrate type were randomly chosen from a thoroughly mixed pile containing all shells larger than 35 mm to allow compositions from the oldest oysters to be measured and analyzed. Sample sizes were small due to time needed to analyze them, and larger shells were chosen to provide a clearer picture

of incorporation rates using the oldest oysters. Due to the lab experiment issues and subsequent additions of baking soda and vinegar, the outer portion of the shells were feared to be possibly contaminated and the middle shell layer was analyzed instead. Inner shell layer analysis was performed on two spat attached to magnesium enriched shells for an informal comparison. Analyzed fragments, 1 taken from each shell, were coated in gold so the samples could be made conductive; gold was used over other metals because it is not present in oyster shells and could not skew any composition results.

Separately from the aquarium containing the oysters, four shells of each substrate type were kept in 10-gallon aquaria filled with reverse osmosis water to examine the effects of each substrate on water chemistry over time. Instant Ocean salt was added to the tanks to bring salt concentrations to levels comparable to the field site, approximately 20 ppt. Treatments were 3 tanks each of natural shell, concrete, magnesium, and broken magnesium, and 1 empty control tank. Magnesium shells broken into pieces with a hammer were meant to simulate the effects of weathering that these shells encountered in the field, to see if that resulted in any measurable effect on water chemistry.

Salinity and temperature were recorded with salinity raised to around 20 ppt to match the concentrations at the field site. Alkalinity, pH, magnesium concentrations were recorded weekly for 8 weeks using Hanna instruments probes for all measures except magnesium, sent to an outside laboratory for analysis. Samples were sent to Air Water & Soil Laboratories to test magnesium concentrations more rigorously than an over the counter test kit would allow. Initially it was planned to test for calcium concentrations as well, but for the first month of the study calcium concentrations were too low to be in range of the Hanna colorimeter, and accurate measurements were unavailable. Results were analyzed for averages and trends due to small sample sizes.

Results

Field Study

Recruitment

From late June to late October 2017, a total of 868 oyster spat attached to shells in the 24 bags left at the field site (Fig. 9). Two hundred seventy-eight oyster larvae attached to natural shell substrate, 273 spat attached to concrete shells, and 317 spat were found attached to concrete shells enriched with magnesium. By substrate, an average of 2.32 oysters were found attached to each natural shell, 2.28 oysters attached to each concrete shell, and an average of 2.65 on magnesium shells. At the end of the field season there were 183 living natural shell spat, 126 spat still alive on concrete, and 162 spat surviving on magnesium-enriched shells. On natural shell substrate, 71 out of the 183 surviving spat (38.8%) attached to the inner portion of the shell. While there seemed to be a preference for the outer portion of the shell, the smoother inner portion still drew larval recruits.

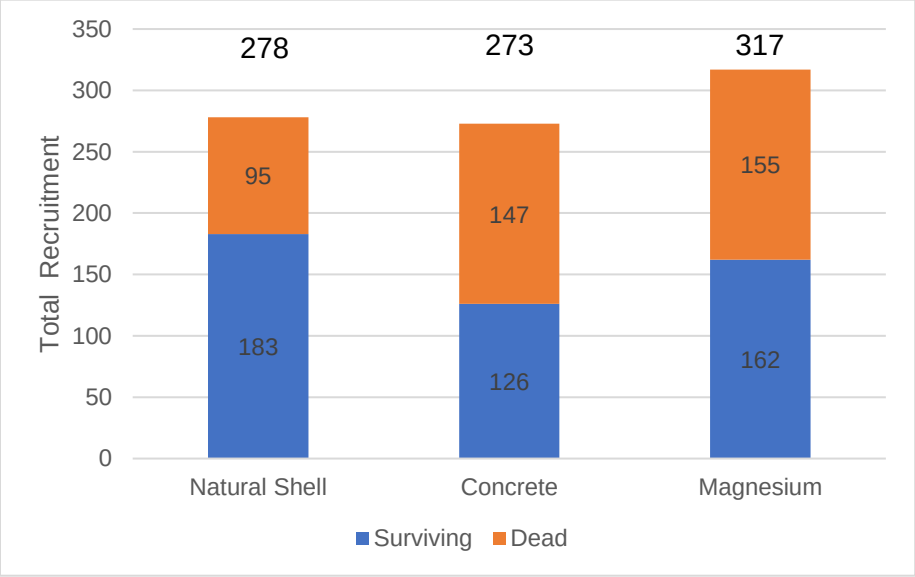

Figure 9. *Total larvae to recruit to each substrate, counts of spat found dead on top of live spat counts at the end of the field season.*

New recruits were found during each site visit throughout the field season, including one new spat on the final visit in late October, an unusually late time for a new oyster to attach. Initially, more larvae settled on magnesium substrates, but by mid-September there were more surviving spat on natural shell substrates (Fig. 10). Magnesium and concrete substrates saw an asymptotic number of recruits up to early August, as expected, and then began steady declines in total surviving spat. During this decline, the substrates still gained new recruits but showed net losses for the remainder of the field season. In contrast, recruitment on the natural shell substrate lagged but added spat until early September, reaching its recruitment plateau a month later than the other substrates and surpassing magnesium. From that point onwards, natural shell exhibited higher surviving spat counts than the other substrates.

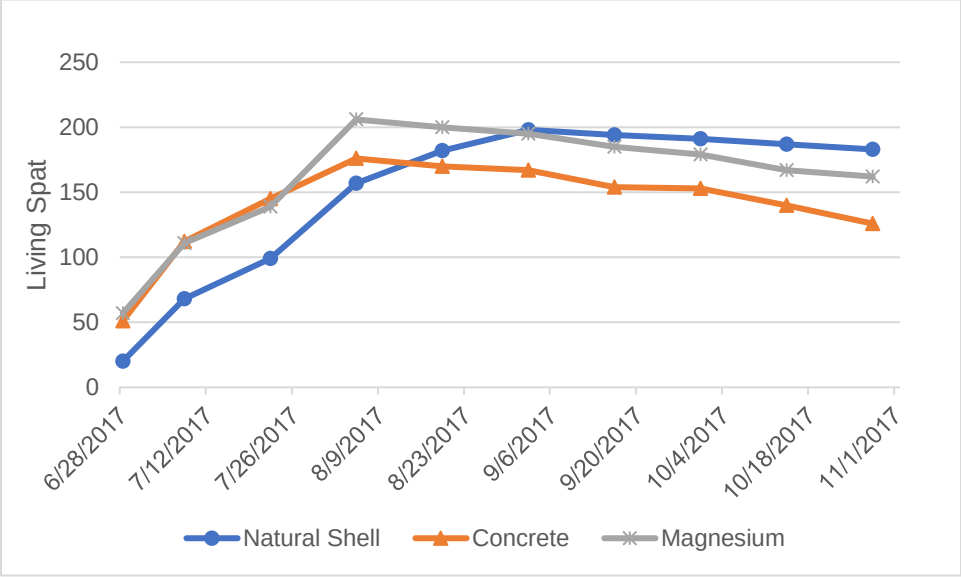

Figure 10*. Spat recruitment over time, showing living spat counted during each field visit.*

Shapiro-Wilk normality tests were performed for total strike counts on the three substrates and were found to have non-normal distributions (p-values > 0.05). A Poisson

ANOVA was performed because histograms of all recruitment data showed right skewed distributions (Fig. 11). With recruitment on concrete substrates as a baseline (control), spat were 1.007 times more likely to attach to natural shell substrates (p value = 0.93), and 1.149 times more likely to attach to magnesium substrates (p value = 0.071). Concrete was chosen as the control to more directly compare the two artificial substrates.

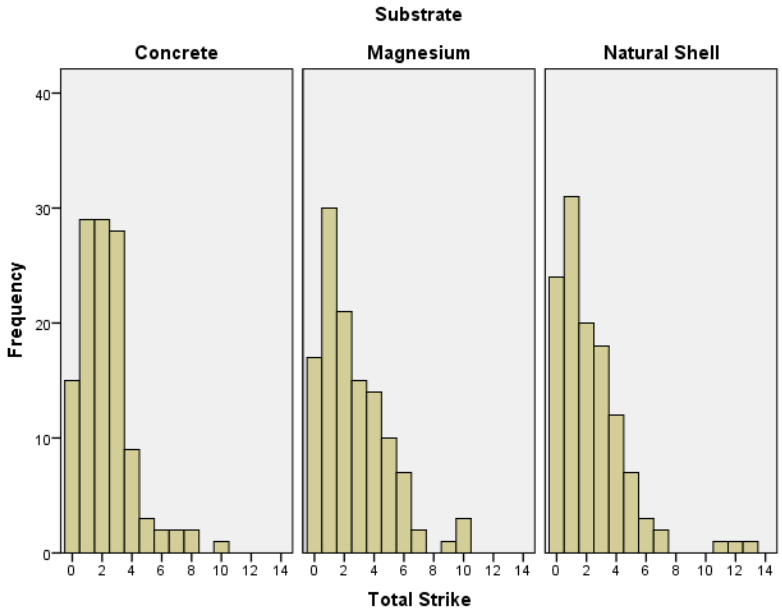

Figure 11. *Histogram of larval recruitment distributions for 3 tested substrates.*

Mortality

The number of spat found dead during each visit, including the spat that disappeared entirely between visits, was totaled for each shell and divided by the number of all spat to attach to each shell to calculate mortality. The average spat mortality for each substrate was 33.9% for natural shell, 54.0% for concrete, and 49.4% mortality for magnesium (Fig. 12). The data were non-normally distributed (all Shapiro-Wilk p values < 0.05) and a Kruskal-Wallis test was performed to test for differences between the mortality exhibited by each substrate type.

There were significant differences between spat mortality among the three substrates (Kruskal-Wallis $\chi 2 = 19.48$, df 2, p-value of 0.02). A Nemenyi post-hoc test showed that the mortality of spat on natural shell was significantly lower than mortality for spat on concrete and magnesium substrates (p-values of < 0.0001 and 0.049 respectively). There were no statistically significant differences in mortality between spat on magnesium and concrete (p value = 0.14).

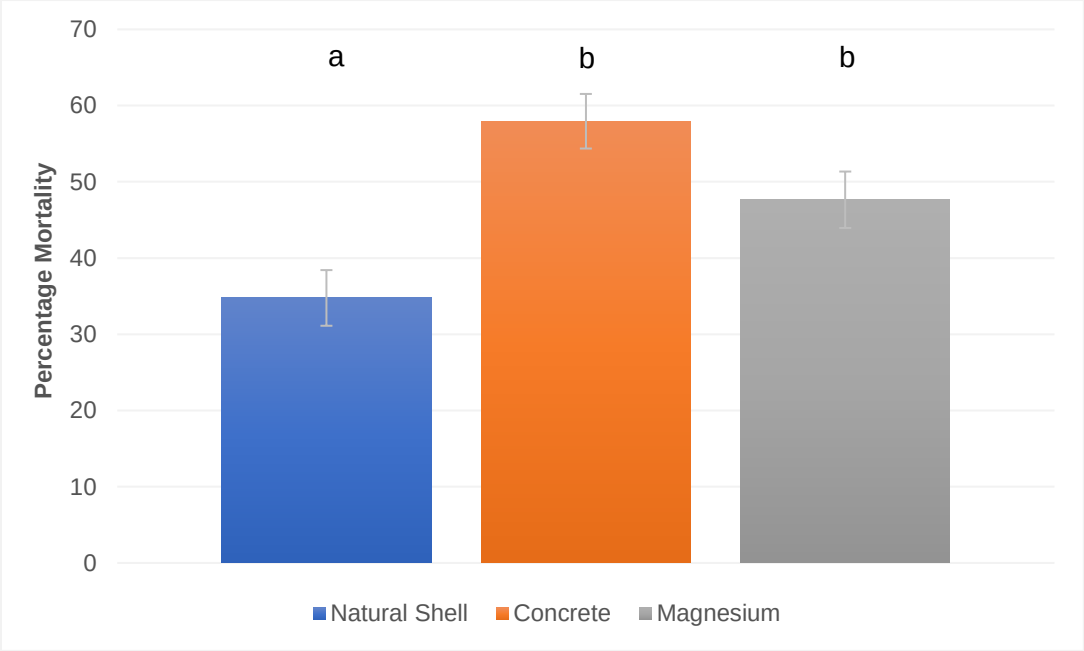

Figure 12. *The average mortality percentage per shell for each substrate, with standard error bars for each substrate.*

Spat Growth

The absolute growth of the surviving spat (n = 471), defined as the length from the bottom hinge of the shell to the top of the shell, was measured using the freely available software, ImageJ (Rueden et al. 2017, Schindelin et al. 2012). The absolute growth of surviving spat was averaged 37.27 ± 12.09 mm for spat on natural shell, 33.27 ± 11.35 mm on concrete, and 34.92 ± 12.55 mm for spat attached to magnesium shells (Fig. 13). The growth data were normally distributed (all Shapiro-Wilk p values > 0.05), and a one-way ANOVA test with a Tukey

post-hoc test was used to determine if there were differences in the mean absolute spat growth between the three substrates (ANOVA F=4.946, p=0.007). The variances for the three substrates were recorded as 128.75 for concrete spat, 146.22 for natural shell spat, and 157.46 for magnesium spat growth, with a ratio of 1:1.22 for the variances between concrete spat and magnesium spat. The post-hoc test suggested significant differences in spat growth between natural shell and concrete (p = 0.005) but no significant differences between growth on natural shell and magnesium or magnesium and concrete spat (p = 0.13, 0.39 respectively). Variances were unequal between groups however, which could affect post hoc results. The unequal variance could account for the result of magnesium not being significantly different from either of the other substrates but concrete and natural shell being significantly different from each other.

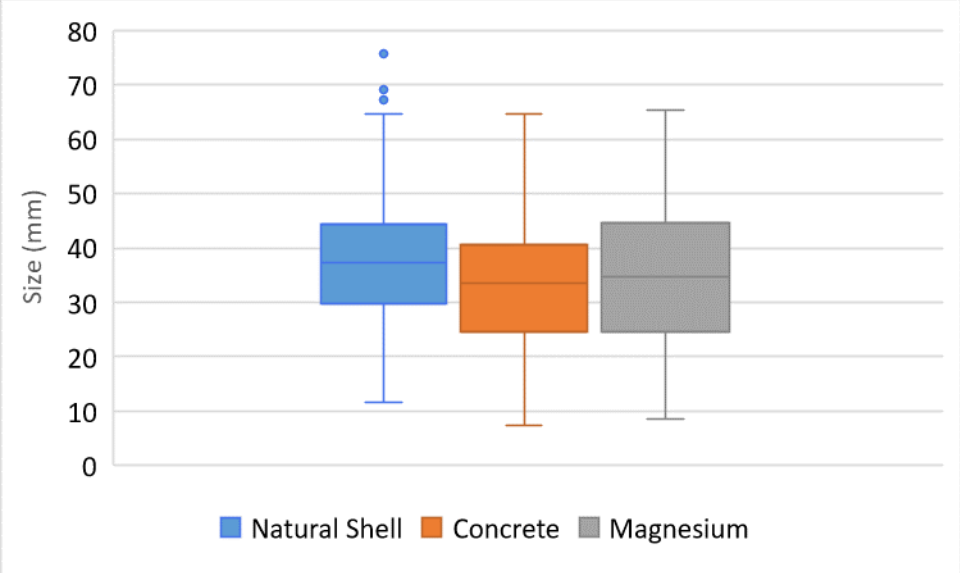

Figure 13. *Boxplots displaying spat growth distributions for three substrates.*

Water and Shell Chemistry Results

Natural and artificial shell effects on water chemistry in aquaria were measured over the course of 8 weeks. For all substrates salinity did exhibit a slight increase (Table 1-4) despite there being no manipulation after initially adding salt to the tank. For tanks containing natural shells the mean pH decreased from 8.21-8.02 and alkalinity increased from 86 -97.3 ppm $CaCO_3$ as shells remained leeching in the tanks for 2 months (Table 1). Concrete shells exhibited similar trends with pH decreasing from 8.3-8.09 and alkalinity increasing from 91-103 ppm $CaCO_3$ over time (Table 2).

Intact magnesium shells displayed a lower pH from an average of 8.23 to 8.09 from beginning to the end of the study, with an increase in alkalinity levels from an average of 92-103 ppm $CaCO_3$ (Table 3). Magnesium shells that were broken saw a mean decrease in pH from 8.23-8.07 and a mean increase in alkalinity from 84.67-97.67 ppm $CaCO_3$ (Table 4). Broken magnesium shells exhibited the highest net increase of alkalinity levels among the four substrates.

All substrates exhibited overall increases in magnesium concentrations in their aquaria (Table 1-4). Natural shell and broken magnesium shell substrates exhibited the largest increases of magnesium concentrations (Table 1,4) with an average increase of 48 and 49 ppm respectively. Concrete shells exhibited the smallest increase (Table 2) with an average increase of 21 ppm.

Table 1 *Initial and Final Water quality characteristics recorded from natural shell tanks used in the study.*

	Initial	**Final**	**Within Ocean Ranges**
Temperature	21.17°C	21.43°C	Yes
Salinity	20.77 ppt	21.63 ppt	Yes
pH	8.21	8.02	No – higher than Bay
Alkalinity	86 ppm	97.3 ppm	Yes

Mg Concentrations	512.33 ppm	560.67 ppm	No – lower than Bay

Table 2 *Initial and Final Water quality characteristics recorded from concrete tanks used in the study.*

	Initial	**Final**	**Within Ocean Ranges**
Temperature	21.6°C	21.7°C	Yes
Salinity	20.07 ppt	21.87 ppt	Yes
pH	8.3	8.09	No – higher than Bay
Alkalinity	92.33 ppm	110 ppm	Yes
Mg Concentrations	502.67 ppm	524 ppm	No – lower than Bay

Table 3 *Initial and Final Water quality characteristics recorded from intact magnesium shell tanks used in the study.*

	Initial	**Final**	**Within Ocean Ranges**
Temperature	21.63°C	21.57°C	Yes
Salinity	20.37 ppt	21.33 ppt	Yes
pH	8.23	8.09	No – higher than Bay
Alkalinity	92 ppm	103 ppm	Yes
Mg Concentrations	488.33 ppm	524 ppm	No – lower than Bay

Table 4 *Initial and Final Water quality characteristics recorded from broken magnesium shell tanks used in the study.*

	Initial	**Final**	**Within Ocean Ranges**
Temperature	21.23°C	21.57°C	Yes
Salinity	19.13 ppt	20.7 ppt	Yes
pH	8.23	8.07	No – higher than Bay
Alkalinity	84.67 ppm	97.67 ppm	Yes
Mg Concentrations	474 ppm	523 ppm	No – lower than Bay

SEM/EDX analysis of the middle layers of the shells revealed there was no significant magnesium concentrations in any of the spat's shells. Some magnesium was present in trace

amounts that were too low to be considered reliable (< 0.5%) based on SEM/EDX accuracy levels, and so spat from all substrate types were only recorded as consisting of 3 elements – calcium, carbon, and oxygen (Table 5). Observations of a small number of inner layers of spat shells attached to magnesium (n=3) indicate that there were higher concentrations of magnesium on the inner layer of the shell (3% of composition) but this was not tested for frequently enough and is instead a future area of study.

Table 5 *Chemical Composition Percentage of Oyster Shells*

Spat Shell	**Ca %**	**C %**	**O %**
Natural Shell 1	37.02	10.82	52.15
Natural Shell 2	39.31	10.38	50.31
Natural Shell 3	41.04	11.06	47.91
Natural Shell 4	50.71	7.34	41.95
Concrete 1	42.33	10.13	47.54
Concrete 2	43.55	9.59	46.86
Concrete 3	44.19	9.19	46.62
Concrete 4	51.56	7.88	40.56
Magnesium 1	46.87	7.97	45.16
Magnesium 2	49.99	7.89	42.12
Magnesium 3	43.85	9.2	46.95
Magnesium 4	33.98	12.25	53.77

Discussion

Field Study

Based on local advice and previous research conducted at the field site, the expectation was that there would be one larval spawning period in early-mid July that would end by the beginning September, with little recruitment after mid-August (Mandirola 2017). The initial spawning occurred early however, approximately around mid-June. It seems likely a second spawning occurred based on positive recruitment to natural shell continuing through early September, with new straggler recruits being found even on the final field visit in late October (Fig. 10). The slight overall preference larvae exhibited for magnesium concrete substrates over natural shell and normal concrete is in line with studies performed in the Chesapeake Bay region and the Gulf of Mexico. These studies have found that larvae can recruit to concrete substrates at levels equal to or even greater than natural shell (George et al. 2015, Theuerkauf et al. 2015, Dunn et al. 2014). However, a study at the same field site as this study discovered larvae having a clear preference for natural shell over two different type of concrete substrates however (Mandirola 2017). As alternate substrate's utility in oyster restoration is still a recent area of research, factors that might influence a larval oyster's settlement site preference is little understood and could be influenced by several factors such as biofilm differences for each substrate, chemical differences of substrate surfaces detected by larvae, or hereditary or regional differences in preference among larvae.

Any larval preference for magnesium could be explained by larval ability to detect magnesium in the surrounding water (Lasseter 2016). There is not a statistically significant preference for magnesium substrates, but more oysters did attach overall and there is potential for substrates with even higher magnesium concentrations proving more appealing to oyster

larvae (Fig. 9). Testing for effect size using the Cohen's d formula (d = (M2 - M1)／SD pooled) using the average and standard deviation spat recruited to each concrete shell (2.28 and 1.8 respectively) as M1 and SD found that an average of 3.73 spat would have to recruit to magnesium enriched shells to exhibit a large effect size of 0.8 relative to concrete recruitment. It is unknown at what concentration of magnesium that could be predicted to occur, or if shells could be made with that level of magnesium without being too frail for the field.

Other micronutrients could also be added to concrete to examine any potential recruitment effects on larvae. Elements such as iron, copper, nickel, silicon, and potassium are found in oyster shells in trace amounts, and like with magnesium some are examples of what appear to be incorporation of certain elements through metabolic control by oyster larvae (Lassester 2016). Magnesium shells are inexpensive to produce, with approximately 400 shells being made from one 2 kg container of magnesium carbonate powder. The total cost of producing 400 shells was less than $100 between the cost of magnesium carbonate, the cement, and aggregate (limestone sand consisting of 1.5% magnesium). While magnesium enriched shells did not provide statistically significant benefits relative to traditional concrete, their relatively low cost could make this substrate a worthwhile investment for the benefits they do provide, including a potential slight increase in recruitment numbers and growth.

Spat mortality results show spat recruiting to magnesium supplemented substrates showed no significant difference in mortality than spat recruiting to concrete; they did exhibit lower mortality albeit not to a statistically significant level compared to concrete (Fig. 12). Prior research on survival exhibited by spat on natural shells and artificial substrates yielded similar spat mortality on natural shell, but lower mortality on concrete (Theuerkauf et al. 2015). Spat in the aforementioned study exhibited mortality rates of 10% on concrete oyster castles and 40% on natural shell/concrete mixes, lower than the 49-54% mortality exhibited by spat in this study.

This result could be related to the differences between artificial shells and larger artificial constructs, but the exact cause is unknown. The lower mortality exhibited by magnesium could have biological significance as more artificial substrates are used to supplement restoration efforts, and even marginal improvements in mortality could benefits those restoration efforts. To determine if reefs were reaching self-sustaining population growth levels would likely take several (3-5) years to confirm however, requiring longer monitoring than most restoration project receive (Schulte et al. 2009).

While spat on natural shell grew significantly longer than spat growing on concrete, oysters grown on magnesium substrates were not significantly different from either substrate (Fig. 13). Due to the unequal variances in mean size exhibited by the three substrates these post hoc results may need to be taken with skepticism, especially based on the result that magnesium spat did not differ significantly from either substrate but, counterintuitively, concrete and natural shell did still differ significantly from one another. The unequal variances between groups is a possible explanation for this post hoc result and is another indication that this research topic requires further study before any conclusions can be reached about the impact magnesium enriched substrates may have on spat growth. As the benefits, if any, of magnesium incorporation in oysters has not been researched it is difficult to say if adding more of the micronutrient would lead to oysters that grew any larger.

Past research has indicated that spat on concrete will exhibit similar growth rates to natural shell counterparts (George et al. 2016). Whether spat were actually incorporating the magnesium could aid interpretation of results, but based on the findings of the SEM/EDX analysis the wrong layer of the shell was analyzed, and more tests are needed to verify if spat attached to magnesium enriched shells incorporated the nutrient at a higher rate. Magnesium is generally found in highest concentrations of the prismatic layer of oyster shells, but this was

not the finding of our analysis, in which no magnesium was present (Carriker et al. 1980, Table 4). Future analyses will focus on the inner layer of the spat shell, in which some evidence was found of higher (3%) concentrations in the small number of shells examined.

Past research that worked to model the growth of oysters focused on fitting recorded growth data to von Bertalanffy growth models of the formula $L_t = L_\infty (1-e^{-kt})$ in which L_t is the length at age t, L_∞ is the asymptotic length, and k is the Brody coefficient. (Coakley 2004). Coakley recorded Bay-wide L_∞ values of 90.85 with k = 0.55 and t_o = -0.51, with regional values in the Potomac (near the study site for this research) to be L_∞ = 148.97 with k = 0.237 and t_o = -0.831. This 2004 study found oysters near the Potomac River generally grow to a mean size of 27.18±4.09 mm. In comparison to the results of Coakley, oysters in this study grew to be larger than would be expected for the particular studied region of the Bay, but still within the Coakley model's range of overall potential growth of oysters in their first year (Coakley 2004). This difference in growth exhibited by this study's spat likely stemmed from the earlier than usual initial spawning event in June, allowing spat a longer growing season than normal. Oysters in other parts of the Chesapeake that were also studied for the Coakley model did grow to mean sizes comparable to the spat in this study, and the oyster spat sizes seen in this study are near the upper range overall of what models and prior observations expect to see from first year spat (Coakley 2004, Grabowski et al. 2004, Albright et al. 2007).

Lab Study

Shells left in the tanks did not have any major effect on water temperature and salinity, which was expected as nothing was done to manipulate those factors after the initial tank setup (Table 1-4). All four substrates tested lowered the pH in their respective aquaria, though all

leveled off at pH above 8.0 (Table 1-4). Even water that held concrete shells (that were the sturdiest substrate and least prone to weathering based on personal observations) exhibited a decline in pH and increase in alkalinity and magnesium concentrations. What is of note is the decrease in pH of the water in the concrete tanks, in spite of concrete leeching calcium carbonate which should theoretically act as a buffer against pH decreases (Feely et al. 2004, Davis et al. 2017). The reason for this is unclear at this time, but results seem to imply that concrete substrates could have little impact of mitigating the effects of ocean acidification in areas they are installed.

The initial hypothesis was that broken magnesium shells would simply exhibit similar but stronger trends than intact shells due to increasing the exposed surface area and exposing the inner layers of the shell. This hypothesis was not validated as broken magnesium did exhibit slightly lower pH (0.02) and a slightly greater mean alkalinity increase (11 ppm compared to 13 ppm) but a greater difference between the two substrates was expected (Tables 3,4). The water in all tanks exhibited higher final alkalinity and pH when compared to the water of the Chesapeake (Wong 1979). The alkalinity more closely resembled levels in the open ocean than the Chesapeake Bay (Wong 1979). pH was also higher than typically seen in the Bay, but the effects of these shells in an environment larger than a 10-gallon aquarium would likely be insignificant for their local ecosystem. In the event shells did provide a small local increase in pH levels, it would be beneficial in light of ocean acidification issues in the Chesapeake and worldwide. While artificial shells do seem to impact their local water chemistry in varying ways, none of these would cause effects that would be harmful to local oysters. We also recognize that field coastal ecosystems would see an exchange of water over the course of a day that was not possible in this lab experiment. This would likely decrease the effects of these shells on

their local water chemistry even further with water exchange and a larger volume of water nullifying the effects of these shells on their environment.

Water from each substrate aquarium exhibited an increase in magnesium concentrations over the duration of the experiment (Tables 1-4). It cannot be said with any certainty if the concentration of magnesium in the water of the natural shell substrate was affected by the source of the shell used, as the region shells were collected from could play a role in magnesium levels (Lasseter et al. 2016). Our natural shells were donated from two local restaurants and the exact source of the shells is unknown. Concrete shells did leech magnesium to the water in the tank, but at an average of 21 ppm compared to an average of 36 ppm for magnesium enriched shells and 49 ppm for broken magnesium shells. The sturdiness of concrete shells likely affected how much magnesium could leech from the shell as with previously mentioned water chemistry characteristics. Water containing broken magnesium shells exhibited a higher average increase in magnesium concentrations relative to intact shells, likely due the increased surface area from being broken and increased exposure of weaker inner layers of the shells that had been unaffected by curing.

As with other results, exploring the effect of increased magnesium concentrations or different micronutrients could yield more significant differences, but extensive further research is needed at this stage to support these possible next steps. Future steps involving magnesium could center on whether increasing the concentration of magnesium in artificial substrates had any effect on larval recruitment. Shells used in the study were approximately 8% magnesium, while magnesium carbonate is fairly water insoluble and therefore more difficult to mold than regular concrete, that concertation could likely be increased to around 12% and still produce viable substrates if cured underwater properly. Different layers of the shell need to be analyzed for a clearer picture of whether spat actually incorporate magnesium, observations indicated

that the inner layer of this experiment's oyster spat contained higher levels of magnesium (3%) than typically found in an oyster shell (Lasseter 2016). Both the inner layer and uncontaminated spat's outer shell layers should be analyzed through SEM/EDX to clarify this observation's validity. Spat attached to magnesium could also be examined for added health benefits, through acidification resistance trials such as what was attempted in this project or compressive force experiments in which shells were broken to determine if spat attached to magnesium enriched substrates had stronger shells.

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