ABSTRACT

Title of Dissertation: OPTIMIZING DESICCATION AS A BIOFOULING CONTROL STRATEGY FOR WATER-COLUMN CULTURED OYSTERS, *CRASSOSTREA VIRGINICA*, IN THE CHESAPEAKE BAY

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Oyster aquaculture is flourishing in the US and internationally, delivering wide-ranging benefits to coastal communities, economies, and ecosystems. While the oyster aquaculture industry has grown substantially, a number of ongoing challenges limit industry growth. The issue of biofouling is paramount among these impediments to industry growth and farmers employ a range of techniques for biofouling control, including chemical immersion, physical methods, paints or coatings, and biological control methods. Desiccation, or the periodic aerial exposure of oysters and cages, is a popular biofouling control strategy and previous research has demonstrated the effectiveness of 24 consecutive hours of desiccation at a weekly frequency (compared to 24 hours bi-weekly, monthly, or seasonally), but this treatment has yielded a growth penalty in addition to biofouling control. The optimal interval (duration) of desiccation has not been thoroughly investigated and shorter intervals applied at a weekly frequency may yield different results. Therefore, this dissertation set out to investigate the effects of multiple

weekly desiccation intervals (0-, 4-, 8-, and 24-hours) on eastern oyster (*Crassostrea virginica*) production and biofouling control. In *Chapter 2*, I examined responses of the biofouling community and oysters to three weekly desiccation intervals (0-, 8-, and 24-hours) at three commercial oyster farms in the Chesapeake Bay over 4 months. In *Chapter 3*, I conducted longer term (7 month) monitoring of the response of the biofouling community and oysters to four weekly desiccation intervals (0-, 4-, 8-, and 24-hours) at a single site in the Choptank River. In *Chapter 4*, I examined molecular (heat shock protein expression) and physiological (filtering rate, gametogenic stage, glycogen content) responses of oysters to four weekly desiccation intervals (0-, 4-, 8-, and 24-hours) in the Choptank River over 3 months. Results indicate broad-scale effectiveness of all desiccation treatments in reducing total biofouling coverage, although species- and site-specific responses were observed. Oyster growth was inconsistently affected by desiccation treatment, but reduced growth was observed in the 8- and 24-hour treatments in two of the three years of field investigations, perhaps influenced by reduced time spent in the water feeding and a delay in the onset of filtering post-desiccation. However, the timing of the greatest reduction in growth rates with desiccation suggests that concomitant stress of desiccation plus gametogenesis may have elevated the oysters' stress response (reduced growth). Indeed, gametogenic stage and glycogen content were influenced by desiccation interval and oysters in the 24-hour treatment were the most likely to spawn. A high-level stress response via the upregulation of heat shock proteins (HSPs) was absent in oysters from the 8- and 24-hour treatments, indicating depressed HSP expression (and cellular protection) among the most stressed oysters. Future research into the importance of environmental factors during desiccation (air temperature, wind, humidity, etc.) could yield useful information to allow oyster farmers to target desiccation during optimal conditions, potentially limiting oyster exposure time in order to

minimize oyster stress. Fouling reduction was significant in all treatments (4-, 8-, and 24-hour) and most consistent in the longer (8- and 24-hour) treatments. Therefore, a tradeoff is present between biofouling control and yield. Farmers prioritizing minimal biofouling may opt to desiccate for 8 or 24 hours weekly, while farmers seeking to maximize growth rates and minimize oyster stress may opt to desiccate for 4 hours weekly. Individual farmers must consider their own preferences regarding suitability of this husbandry technique, but results suggest that desiccation is an effective approach to biofouling control and can be applied with minimal stress to the oysters.

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Preface

This dissertation includes three original research chapters that were collaboratively written with my academic advisor, Dr. Louis Plough. Funding for these chapters was acquired with a team of colleagues and assistance with methods and data interpretation was provided by committee members and other colleagues. Funding sources and special acknowledgements are provided at the end of each research chapter.

Dedication

To my family.

Acknowledgements

It's taken a village to reach this point and I'm immensely grateful for the unending generosity provided to me over the past five years.

To my advisor, Louis Plough, for taking me on as a student and elevating the breadth and depth of my work. I'll be forever grateful for the generosity you've shown me, and my abilities as a scientist and writer are substantially improved because of your mentorship.

To Bill Walton, my longest-serving committee member, mentor, and unending source of perspective. You've taught me to keep an eye towards the practical side of research, enhancing applicability of results to the community of oyster growers we are all so enthused about.

To my advisory committee members for lending your expertise to enhance my research and my capability as a scientist. I'm thrilled at the diversity of perspectives encompassed within this committee and appreciate all of you dedicating your time and efforts to create a thoroughly inter-disciplinary support system for my research.

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You are all truly extraordinary and I'm lucky to know you. Thank you!

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Chapter 1: Biofouling as a challenge to oyster aquaculture operations

Introduction

Growth and importance of shellfish aquaculture

Shellfish aquaculture is a rapidly growing industry, both globally and within the United States (FAO, 2020). While wild capture seafood harvest remained relatively constant between 1990-2018, global aquaculture production increased by 527% during the same time period (FAO, 2020) and shellfish aquaculture comprised 56% of all coastal and marine aquaculture production in 2018 (FAO, 2020). Oyster aquaculture is a major contributor to the recent growth of shellfish aquaculture across the United States, with marked increases in coastal states including Alabama (Grice & Walton, 2020), Maine (GMRI, 2016), Maryland (van Senten et al., 2019), Massachusetts (Massachusetts Division of Marine Fisheries, 2019), New Jersey (Calvo, 2018) and Virginia (Hudson, 2018), among others. Oyster aquaculture presents an opportunity to produce a low-carbon source of nutritious food for a growing population that also yields ecological and socio-economic benefits (Dealteris et al., 2004; Engle et al., 2021; Kellogg et al., 2018; Michaelis et al., 2020; Oehlenschläger, 2012; Ray et al., 2019; van Senten et al., 2019).

Oyster aquaculture takes two general forms: water-column aquaculture where oysters are containerized and suspended within the water column, and submerged-land aquaculture where oysters are deployed, grown, and harvested directly from suitable bottom substrate. Oysters grown on water-column oyster farms are generally destined for the half-shell market (Parker et al., 2020), which prizes shape and appearance, paying more per piece than oysters sold as shucked product, which are typically grown on submerged-land aquaculture operations (GMRI, 2016; Hensey, 2020; Hudson, 2018; Parker et al., 2020). In 2015, half-shell oysters comprised

59% of US oysters harvested by volume, but 71% of the total value of US oysters harvested. In contrast, shucked product oysters comprised 41% of total volume, but just 29% of the value of US harvested oysters (GMRI, 2016). While half-shell oysters obtain more value per piece compared to shucked product, the break-even cost per oyster is substantially higher for water-column cultured oysters than for bottom-cultured oysters due to elevated production costs (Parker et al., 2020). Labor and marketing expenses comprise the greatest costs for water-column oyster farms (Engle et al., 2021; Hensey, 2020). While many production processes contribute to labor costs on water-column oyster farms, a major source of these costs is derived from the removal of biofouling from oysters and cages, a costly and energy intensive process (Adams et al., 2011).

Significance of biofouling in aquaculture

Biofouling, or the aggregation of marine plants and animals on cages and the cultured animals themselves, presents a challenge to aquaculture operations worldwide, including oyster farms where fouling increases operational costs and reduces yield (Adams et al., 2011). Oysters, along with the submerged cages and infrastructure used by water-column oyster farmers, are rapidly colonized by a diverse assemblage of epibiotic and endolithic organisms (Dealteris et al., 2004; Marenghi et al., 2009). Epibiotic fouling organisms (epibionts) include marine plants and animals that colonize the external valves of the oysters, while endobiotic fouling organisms (endobionts) live within the valves, under the external surface, and are comprised primarily of worms and sponges (Dürr & Thomason, 2009). Of a number of species of worms that co-habit oyster farms, one of primary significance is the endobiotic annelid *Polydora websteri*, or mudworm. The mudworm bores into the valves of oysters, resulting in a distinctive "U-shaped" burrow which is visible on the interior cavity of the shell when the oyster is shucked (Haigler,

1969; Morse et al., 2015). Phyla and classes of species commonly comprising the biofouling community in marine shellfish aquaculture farms include Chordata (Ascidiacea), Turbellaria (Polycladida), Annelida (Polychaeta), Algae, Porifera, Mollusca (Bivalvia), Cnidaria (Hydrozoa and Anthozoa), Arthropoda (Maxillopoda), and Bryozoa (Fitridge et al., 2012; Bannister et al., 2019). Biofouling in shellfish aquaculture operations can be so severe that biodeposits from biofouling organisms can contribute prominently to total biodeposition loads under shellfish farms (McKindsey et al., 2009; Stenton-Dozey et al., 2001), supplying up to 86% of total biodeposits locally (Giles et al., 2006), and adds to concerns regarding the interaction of aquaculture farms and the environment (Burkholder & Shumway, 2011). Biofouling reduces water flow (Gormican, 1989), increases weight on gear (Ramsay et al., 2008) and reduces growth and/or condition of oysters (Stefaniak et al., 2005; Wargo & Ford, 1993). In terms of economic impacts, biofouling can also lead to devaluation of shellfish based on the degree of biofouling visible on the valves (Campbell & Kelly, 2002) or by contributing to brittle shells (Carver et al., 2010; Hensey, 2020), and biofouling control can compromise 5-20% of total farm operating costs (Lane & Willemsen, 2004; Watson et al., 2009).

Since biofouling is a major issue for aquaculture operations, considerable research has been conducted into fouling control techniques that comprise a range of methods. A suitable solution to control biofouling in oyster production must balance efficacy and practicality in that it must be safe, effective, and applicable at a commercial-scale (Creswell & McNevin, 2009). Physically dislodging biofoulers, desiccation (aerial exposure), chemical dips, biological control, and paints or coatings have all been examined as methods to control fouling with varying degrees of effectiveness and will be discussed in greater detail below. However, a unique aspect of desiccation, or the periodic aerial exposure of cages and oysters, is that in addition to

demonstrating significant fouling reduction across multiple sites and time periods, desiccation is readily deployable at commercial scale through the use of compatible gear types such as flippable floating cages (FFC) and adjustable longline systems (ALS) (Davis et al., 2013; Walton et al., 2012, 2013).

Desiccation shows strong promise for fouling control and is already in use as a biofouling management strategy by shellfish aquaculture farmers across the United States (Adams et al., 2011; Davis et al., 2013). Desiccation may be applied passively through siting oyster farms within the intertidal zone, or may be applied manually by exposing oysters and cage material to the air at a frequency (how often) and interval (how long) decided by the farmer. Studies on desiccation frequency have concluded that weekly exposure is necessary to control biofouling (Chapman, 2019; Gamble, 2017; Kirk, 2019), however the appropriate duration of desiccation exposure (e.g. 4-, 8-, or 24-hours) has not been thoroughly investigated. Further, a reduction in growth has been observed among oysters desiccated weekly for the industry-standard interval of 24 hours (Chapman, 2019; Gamble, 2017; Kirk, 2019), which is an important tradeoff to consider. Biofouling control is one salient aspect of oyster aquaculture, but farmers must ultimately consider their yield, including the time it takes their oysters to reach market size. Biofouling control techniques, or any other husbandry method which adds time to the growout process must be thoroughly evaluated and opportunities for greater efficiencies should be examined (Sievers et al., 2017). Therefore, a major goal of this dissertation is to gain a better understanding of the optimal duration of desiccation and physiological impacts of desiccation on oysters (i.e. stress response) to better equip industry members to make informed decisions about desiccation as a fouling control strategy.

Impacts of biofouling

Fouling impacts on labor costs and oyster marketability

The agglomeration of biofouling organisms can affect the productivity and profitability of water-column oyster farms by increasing production costs and reducing marketability of the final product (Adams et al., 2011; Campbell & Kelly, 2002; Lane & Willemsen, 2004). A survey of 1,375 shellfish growing operations (510 respondents) in the United States found that 43% of respondents indicated that biofouling affected the marketability of their product and 79% indicated that biofouling increased operational costs (Adams et al., 2011). Of respondents from the Chesapeake Bay region studied (Virginia), 89% stated that biofouling increased operational costs. In the same study (Adams et al., 2011), biofouling control accounted for an average of 14.7% of total farm operating costs (multiple species), whereas in oyster aquaculture specifically, direct costs of fouling control (cost of removing the fouling from infrastructure and oysters) have been estimated at 20% of final market price (Watson et al., 2009). Indirect costs derived from biofouling (maintenance, repairs, slower growth, etc.) remain largely unquantified, making the total cost of biofouling unknown, but potentially significantly greater than current estimates which rely on direct costs alone (Fitridge et al., 2012).

Biofouling impacts on production

Biofouling can generate negative impacts on shellfish, farm infrastructure, or both, which can lead to devaluation of shellfish, reduced growth or yield, and increased costs of production (Adams et al., 2011; Campbell & Kelly, 2002; Stefaniak et al., 2005; Watson et al., 2009; Willemsen, 2005). Impacts of biofouling to shellfish include physical damage, mechanical damage, and competition for resources (Fitridge et al., 2012). The impacts of biofouling on farm infrastructure include a reduction in water quality within cages and added weight on gear

(Fitridge et al., 2012). Many of these impacts are interrelated and should not be viewed as discrete, independent impacts. Each of these issues present challenges to aquaculture operations and are discussed in greater detail below.

Biofouling impacts on shellfish

Biofouling can compromise shellfish physiology and valve integrity, evidenced by numerous studies which have found substantially reduced growth and meat weight of shellfish grown in fouled culture gear compared to those grown in non-fouled culture gear (Claereboudt et al., 1994; Fitridge & Keough, 2013; Lodeiros & Himmelman, 1996; Sievers et al., 2013). Endobionts such as sponges and spionid polychaete worms (i.e. *P. websteri*) create a network of tunnels and blisters within the valves (Carroll et al., 2015; Haigler, 1969; Stefaniak et al., 2005; Warburton, 1958) which can lead to brittle, fragile, and weakened shells (Carver et al., 2010), leaving the shellfish more susceptible to predation (Buschbaum et al., 2007). Further, shellfish inhabited by endobionts secrete additional nacre (shell material) in response to the excavation (Hoeksema, 1983; Stefaniak et al., 2005), a costly energetic investment (Palmer, 1992). Reduced growth and condition have been observed among eastern oysters, *Crassostrea virginica,* infested with the boring sponge *Cliona celata,* which may result from the oysters divesting energy away from somatic growth toward shell maintenance (Carroll et al., 2015; Handley & Bergquist, 1997). Larger oysters may experience greater negative impacts from boring organisms compared to smaller oysters, perhaps due to elevated costs of maintaining larger shell as the oyster grows (Carroll et al., 2015; Palmer, 1992). It is possible that apparent physical effects of the fouling organisms are, in fact, due to multiple, interacting stressors acting on the shellfish at once.

Valve obstruction by biofouling species can affect an oyster's ability to physically open its vales or create a feeding current, introducing interference competition even if the fouling

species is not actively competing for the same resources. As filter feeders, oysters must be able to open their valves for aerobic respiration and feeding. Heavy biofouling colonization around the posterior or anterior convergence of the valves can inhibit valve opening (Chellam, 1991; Fitridge et al., 2012; Ye et al., 2019) which reduces respiration rates (Miyauti, 1968). In Mediterranean mussel (*Mytilus galloprovincialis*) culture, shell growth was reduced due to interference competition introduced by tubular hydroid (*Ectopleura crocera*) overgrowth (Sievers et al., 2013). Reduction in flow caused by interference competition limits the water exchange required for bivalve feeding and reduces access to critical resources (i.e. phytoplankton, oxygen) necessary for growth (Burrell, 1986).

As sessile species, the shellfish and associated fouling community access and utilize resources from their shared waterbody and direct competition for resources can negatively impact shellfish production. Despite being commonly mentioned in literature (i.e. Bannister et al., 2019; Fitridge et al., 2012), studies directly examining the differential use of food resources by fouling species and the shellfish of interest are limited, with variable results (Daigle & Herbinger, 2009; Lesser et al., 1992; C. J. M. Lodeiros & Himmelman, 1996; Ropert & Goulletquer, 2000). For example, Lesser et al. (1992) found differences in the size of particles ingested by blue mussels (*Mytilus edulis*) versus associated epibionts, indicating a lack of substantial competition. In contrast, Daigle and Herbinger (2009) observed significant overlap in the species and particle size of phytoplankton consumed by blue mussels and tunicates (*Ciona intestinalis),* and in the same study, also observed elevated mortality and reduced growth among fouled mussels. Competition for diatoms in *Crassostrea gigas* fouled by mussels (*M. galloprovincialis)* ultimately affected the nutritional composition of the final oyster product through a reduction in eicosapentaenoic acid (EPA) fatty acid content (Fujibayashi et al., 2021).

Biofouling organisms can also increase local oxygen demand, which can negatively impact shellfish physiology or growth (Giles et al., 2006; Nizzoli et al., 2005; Ropert & Goulletquer, 2000). This fouling/oxygen interaction may be particularly important for shellfish aquaculture operations located in areas with low oxygen conditions, which is exacerbated during warm months when biofouling colonization is strong (Calder, 1966; Murphy et al., 2011; Scavia et al., 2021). While results regarding competition for resources among different species of shellfish and biofouling organisms have varied, data suggest competition can negatively impact shellfish in aquaculture settings.

Biofouling impacts on farm infrastructure

Biofouling can exert negative impacts on the growing conditions for shellfish due to their ability to rapidly colonize cages or other farm infrastructure and generate high biomass (Watson et al., 2009). This rapid colonization and growth can lead to mesh occlusion and a reduction in water flow through cages, which has been documented in finfish aquaculture (Gormican, 1989; Madin et al., 2010). A reduction in water transfer can lead to depletion of food available to the shellfish in the cage's interior, as seen in pearl oysters (*Pinctada margaritifera* and *P. maxima*) in Yukhira et al. (1998). The potential reduction in in water transfer due to biofouling and the buildup of waste byproducts within the cage is a commonly mentioned concern but direct evidence of this is currently minimal for shellfish aquaculture, although research in this realm is ongoing at present (i.e. Campbell et al., *in prep*).

Fouling adds weight to submerged farm infrastructure, which can lead to broken equipment and require costly repairs or upgrades to mechanization of farm operations (Dürr & Thomason, 2009). Fouling has increased the drag force acting on aquaculture netting (Swift et al., 2006), which can lead to broken farm infrastructure, including anchoring and cage systems

(Dürr & Thomason, 2009). The impacts of broken farm infrastructure can necessitate costly repairs and hours of additional labor expenditures with the added risk of losing the shellfish crop if clips or lines have broken (Adams et al., 2011; Claereboudt et al., 1994). Further, increased weight of cages requires enhanced mechanization for retrieval, increasing the need for costly equipment (Boyd, 2009; Dürr & Thomason, 2009).

Fouling control methods

Given the well-documented impacts of biofouling on commercial shellfish aquaculture operations, extensive research has focused on developing effective treatments for biofouling control. Biofouling control treatments include chemical coatings on cages and shellfish as well as biological, immersive chemical, and physical treatments of the gear and shellfish. It is important that biofouling control methods are effective but do not negatively impact shellfish growth and health (Sievers et al., 2017), while lending themselves to application at a commercial-scale. Some aquaculture operations are primarily impacted by a single species or taxonomic group while other operations are affected by a broad spectrum of species (Sá et al., 2007; Sievers et al., 2019; Watts et al., 2015). The biofouling species present may also change over time or across seasons (Underwood & Anderson, 1994) and a given aquaculture operation may value a treatment option that is species-specific, or a treatment with demonstrated effectiveness across a range of species. Decisions on which treatment methods are used to control biofouling may also be influenced by the type of gear used in production (Adams et al., 2011).

Coatings

Coatings refer to treatments that coat either the shellfish themselves or cage material to inhibit colonization and/or growth of biofouling species. Photoactive release and silicone-based

release coatings can be applied to shellfish culture gear. Both have demonstrated effectiveness in reducing fouling colonization of hard clam (*Mercinaria mercinaria*) nets over a period of three to six months (Cassiano et al., 2012). In another examination of a silicone-based release coating, biofouling on bay scallop (*Argopectin irradians*) culture gear was reduced, but fouling on the scallops was elevated among the treated group, indicating that limiting the ability of biofoulers to colonize the culture gear may have encouraged fouling on the scallops themselves (Tettelbach et al., 2012). While less commonly applied, coatings have also been used directly on shellfish valves. For example, wax-based coatings reduced biofouling when applied directly to pearl oyster (*Pinctada imbricata)* valves (Ye et al., 2019). In general, while coatings can be effective in deterring fouling, they can also be difficult to apply at a commercial-scale, and anecdotal reports from industry members indicate that they require frequent re-application in order to maintain effectiveness.

Biological

Biological control involves the strategic introduction of certain species placed within culture gear to consume biofouling species but not the shellfish crop of interest (Fitridge et al., 2012). Multiple species of sea urchins, along with snails, have been successfully employed in biological control techniques, yielding reduced fouling and either no impacts or positive impacts to the shellfish. Green sea urchins (*Strongylocentrotus droebachiensis*) reduced biofouling in mussel (*Mytilus* sp.) aquaculture without impacting mussel growth (Sterling et al., 2016). Sea urchins (*Lytechinus variegatus* and *Echinometra lucunter*) also reduced biofouling from nets in a pearl oyster (*Pinctada imbricata*) aquaculture operation, but not from the pearl oysters themselves (C. Lodeiros & García, 2004). In a study of scallop (*Pectin maximus*) aquaculture, sea urchins (*Echinus esculentus* and *Psammechinus miliaris*) reduced biofouling (hydroids and

solitary tunicates) on both the nets and the scallop shells (Ross et al., 2004). The addition of periwinkle snails (*Littorina littorea*) to oyster (*Crassostrea gigas*) bags reduced algal fouling on bags and was associated with an increase in oyster mass (Cigarria et al., 1998). Biological control methods may prove to be a practical, relatively passive approach to biofouling control at commercial scale, and this is an active area of research (i.e. Mizuta et al., *in prep*).

Chemical

Chemical immersion treatment methods involve dipping the shellfish and cage material into a solution, often followed by a period of exposure to air (emersion). Freshwater (Denny, 2008; Morse et al., 2015), hot water (Bannister et al., 2019), brine (Carver et al., 2010; Hooper, 2001; Loosanoff, 1960), acetic acid (Piola et al., 2010; Sievers et al., 2019), hydrated lime (Gallo-Garcia et al., 2004), or combinations therein have all demonstrated effectiveness in controlling some biofouling species. Chemical dips should be applied with caution and only to shellfish which can completely close their valves (Leavitt et al., 2020). Further, they should not be applied during stressful times of the season (i.e. warmest summer months, coldest winter months; Leavitt et al., 2020). Some immersion dips generate considerable heat when created (i.e. brine), so caution should be taken to not unduly stress the shellfish through immersion. Consideration must also be given to the safety of the dip selected, given that the shellfish may be consumed as a raw product. While dips have demonstrated effectiveness in controlling some biofouling species, routine application of the process may be too cumbersome for wide-scale adoption by commercial industry.

Physical

Physical methods to control biofouling dislodge or stress fouling organisms to the point of decay. Powerwashing is a common fouling control method employed by the US shellfish aquaculture industry (Adams et al., 2011) and has demonstrated effectiveness in removing biofouling from culture gear and improving shellfish growth (Taylor et al., 1997). Desiccation, or periodic aerial exposure during months when biofouling organisms are colonizing, is another control practice which can be utilized passively via placement of gear within the intertidal zone (Littlewood et al., 1992) or prescriptively via the use of gear that can be raised or inverted (Davis et al., 2013; Gamble, 2017; Kirk, 2019; Mallet et al., 2009; Walton et al., 2012, 2013), and has seen increasing application among US shellfish producers (Adams et al., 2011). Based on recent research and reports, desiccation is a very promising approach because it reduces fouling by both endobionts and epibionts on oyster shells, and also reduces fouling on cage material, reducing weight of culture gear (Carver et al., 2010; Gamble, 2017). As a husbandry technique, desiccation is applied by exposing oysters and cages to the air at a frequency (how often cages will be desiccated) and an interval (number of hours cages will be desiccated) decided on by the grower. Previous studies have examined the issue of frequency and have revealed that weekly desiccation (24 hour duration) was effective in reducing biofouling across a range of biofouling species, but was also associated with a reduction in oyster growth (Chapman, 2019; Gamble, 2017; Kirk, 2019). Regular (weekly) exposure is critical when biofouling organisms are colonizing, as some fouling species (i.e. *Mytilus edulis*, *Crassostrea gigas, Perna canaliculus*, *Aulacomya maoriana*) can tolerate aerial exposure for over 100 hours as adults (Hopkins et al., 2016; Kennedy, 1976). Shorter weekly desiccation intervals have, to date, not been thoroughly investigated in field settings and research has indicated differential desiccation tolerance among biofouling species (Hopkins et al., 2016). Given the broad range of species controlled via regular

desiccation, along with the relative ease of application at commercial-scale and current industry appetite for utilization of this method (Adams et al., 2011), a thorough investigation of shorter weekly desiccation intervals is warranted to assess the potential for biofouling reduction without a reduction in oyster growth.

Desiccation: mimicking the stresses of the intertidal

Desiccation as a practice essentially relies on the stress of intertidal exposure to control fouling while minimizing a stress response in the oysters (i.e. reduced growth, elevated mortality). Periods of aerial exposure are stressful, inducing large shifts in temperature, anaerobic metabolism, and the buildup of harmful byproducts (Newell, 1973). As intertidal animals, oysters are accustomed to periods of emersion, but repeated and prolonged exposure can reduce growth (Littlewood et al., 1992). However, some research has found elevated growth in intertidal oysters compared to subtidal counterparts, perhaps due to reduced competition from biofoulers (Bishop & Peterson, 2006; La Peyre et al., 2017), indicating the potential to find an optimum balance that minimizes fouling and maximizes oyster growth. Time spent out of the water reduces the time available to the oyster for feeding and can lead to a reduction in metabolic rate of up to 90% (Shumway, 1982). During aerial exposure, oysters respond by closing their valves (Gainey & Shumway, 1988; Porter & Breitburg, 2016) and may shift to anaerobic metabolism (Meng et al., 2018). Anaerobic metabolism is associated with a reduction in glycogen reserves (de Zwaan & Wijsman, 1976; de Zwaan & Zandee, 1972), an important energetic storage reservoir which must be built up again upon re-immersion (Greenberg, 1977; Patrick et al., 2006). Energy allocation can change seasonally due to reproductive efforts, creating a potentially deleterious interaction between high temperatures and spawning (Berthelin et al., 2000; Cho & Jeong, 2005; Huvet et al., 2010). Thermal stress coupled with reproductive

stress and impaired immune responses have been associated with mass mortalities of *Crassostrea gigas* (Cho & Jeong, 2005; Huvet et al., 2010; Li et al., 2007; Wendling & Wegner, 2013). Independent and/or concomitant stressors associated with periods of emersion act upon the newly settled fouling organisms and help to control fouling but this may also contribute stress to the oysters, evidenced by reduced growth and elevated mortality documented among desiccated oysters (Bodenstein et al., 2021; Chapman, 2019; Gamble, 2017; Kirk, 2019).

Research focus

In this dissertation, I seek to understand the effects of various desiccation intervals on biofouling community composition and abundance as well as on oyster growth, survival, and physiology to refine desiccation as a fouling control strategy for the oyster aquaculture industry. This industry has enormous potential to provide a growing population with a nutritious source of protein that yields environmental and economic benefits. The issue of biofouling is a major impediment to industry growth, resulting in economic losses and reductions in yield and as such, optimizing biofouling control is expected to contribute considerable benefits to the industry. While periodic desiccation shows strong potential as a fouling control strategy, the causes of reduced growth and elevated mortality that have been associated with extended desiccation treatments must be evaluated to enhance suitability of this approach for fouling control. Data on the efficacy of shorter desiccation intervals in controlling biofouling are generally lacking, as are data on the physiological effects of desiccation interval on the oysters. In this dissertation, I seek to advance understanding of the impacts to biofouling and oyster physiology from weekly desiccation treatments of varying length (0-, 4-, 8-, and 24-hours), with the overarching goal of balancing fouling control while maintaining oyster yield. To achieve this, I performed a series of experimental investigations on the response of biofouling organisms and oysters to various

desiccation treatment intervals at four different sites within the mesohaline portion of the Chesapeake Bay over four years (2018-2021).

In Chapter 2 (*Efficacy and effects of three desiccation intervals on biofouling and* Crassostrea virginica *oysters at three commercial oyster farms in the Chesapeake Bay, MD*), I deployed flippable floating cages of oysters to three oyster farms in the Chesapeake Bay (located in the Chester River, Back Creek, and Honga River) and three desiccation treatments (0-, 8-, and 24-hours of weekly desiccation) were followed. Biofouling community composition and relative abundance were monitored at each site, as well as oyster growth and mortality, allowing an understanding of species- and site-specific responses to the three desiccation treatments. Consistent biofouling reduction was achieved in both the 8- and 24-hour treatments in Chapter 2, which informed development of Chapter 3 (*Impact of desiccation interval on biofouling and oyster performance in water-column cultured* Crassostrea virginica *in the Choptank River, MD*). In Chapter 3, I again deployed oysters to cages, but added a fourth (shorter) desiccation interval (0-, 4-, 8-, and 24-hours of weekly desiccation) and monitored the biofouling community composition and relative abundance across treatments over seven months at a single site in the Choptank River. I measured oyster growth, mortality, and condition as indicators of gross responses to the desiccation treatments, yielding an understanding of tradeoffs between biofouling reduction and crop yield. A clear reduction in growth in two desiccation treatments (8- and 24-hour treatments) generated additional research questions regarding the drivers of stress among desiccated oysters, leading to Chapter 4 (*Assessing physiological and molecular responses of* Crassostrea virginica *to weekly desiccation treatments*). In Chapter 4, I deployed oysters to the same four desiccation intervals as Chapter 3 (0-, 4-, 8-, and 24-hours weekly) and measured oyster physiological and molecular responses (filtering rates, gonad development,

glycogen content and expression of heat shock proteins) to understand oyster stress responses to the desiccation treatments. Finally, in Chapter 5 (*Conclusions and considerations for oyster farmers*), I synthesized results and discussed implications with respect to the growing oyster aquaculture industry and future research needs.

This body of work contributes novel information regarding the effectiveness of shorter (4- and 8-hour) weekly desiccation intervals in controlling biofouling, and presents data on the tradeoffs that may be associated with longer desiccation intervals (8- and 24-hours). Results also contribute valuable information on desiccation as a stressor to *C. virginica*, advancing understanding of cellular and physiological responses to desiccation under summer conditions. Overall, this work complements and builds upon existing literature and research into desiccation as a management strategy, and provides data that can be used by oyster growers to make informed decisions regarding farm management to facilitate strong yield and superior crop quality.

Chapter 2: Efficacy and effects of three desiccation intervals on biofouling and *Crassostrea virginica* oysters at three commercial oyster farms in the Chesapeake Bay, MD

Abstract

Oyster aquaculture is a growing industry both globally and nationally. Highly productive coastal waters support high density caged culture of oysters, but also sustain many other macrofaunal species which can settle on oysters and cage materials (biofouling) causing problems for aquaculture operations. Desiccation, or periodic air drying, of farm infrastructure and oysters has been used to control colonization and growth of biofouling organisms in oyster farms worldwide, but the necessary interval of desiccation has yet to be thoroughly assessed. Given the known stresses associated with periods of emersion, an understanding of the effects of different weekly desiccation intervals is needed. This study investigated the use of desiccation in controlling biofouling and associated impacts to oyster growth and mortality at three oyster farms within Maryland's portion of the Chesapeake Bay, including one low salinity site in an upper Chesapeake Bay tributary and two sites in the middle portion of the Chesapeake Bay. Three desiccation treatments were applied at each site: control (no desiccation), 8 consecutive hours of desiccation per week, and 24 consecutive hours of desiccation per week. Oysters were deployed between July - December 2018 and the percent coverage by individual biofouling species was monitored, along with endobiont worm prevalence, oyster growth, and oyster mortality. Total biofouling was significantly greater on non-desiccated oysters but minimal differences in biofouling coverage were observed among oysters desiccated for 8 or 24 hours weekly. Seven biofouling species were observed among the sites, and all except for a single

macroalgal species (*Ulva intestinalis*) were controlled by the 8- and 24-hour desiccation treatments. No clear treatment-derived differences in oyster growth or mortality were observed. Overall, results suggest desiccating weekly for either 8 or 24 consecutive hours may be a suitable biofouling management strategy for gross biofouling reduction on oyster farms.

Introduction

Globally, aquaculture is the most rapidly growing form of food production (FAO, 2018), with shellfish aquaculture comprising 56 percent of marine and coastal aquaculture in 2018 (FAO, 2020). Aquaculturists experience a wide range of obstacles and the issue of biofouling is paramount among them, increasing operational costs for water-column oyster farmers (Adams et al., 2011). Water-column oyster farming is expanding and in some regions, represents the dominant form of aquaculture (Grice & Walton, 2020), yielding high value per piece in half-shell oyster markets (GMRI, 2016). Unlike submerged-land aquaculture where oysters are grown directly on the bottom, water-column oyster farms rely on cages or bags to containerize oysters during the growout cycle. Oysters, cages, and the associated infrastructure used on water-column oyster farms can quickly become colonized by marine plants and animals, collectively referred to as biofouling (Dealteris et al., 2004; Fitridge et al., 2012; Marenghi et al., 2009; O'Beirn et al., 2004; Shinn et al., 2021). The agglomeration of these species at high densities can result in negative impacts to the oysters within the cages and contributes a substantial problem for commercial oyster farmers (Adams et al., 2011). Although experiencing strong growth, the oyster aquaculture industry is not without challenges, and biofouling is a major concern that merits additional research.

Biofouling affects nearly all aspects of water-column oyster aquaculture, including labor costs (Adams et al., 2011), shellfish growth and condition (Carroll et al., 2015; Wargo & Ford,

1993), and market demand (Campbell & Kelly, 2002; GMRI, 2016). Estimates indicate that aquaculturists spend 5-20% of their total operating costs on biofouling control alone (Lane & Willemsen, 2004; Watson et al., 2009). Biofouling presents a challenge to oyster farmers by reducing water flow (Gormican, 1989), increasing weight on gear (Ramsay et al., 2008), and reducing growth and/or condition of oysters (Stefaniak et al., 2005; Wargo & Ford, 1993). Biofouling can also lead to devaluation of shellfish based on the degree of biofouling visible on the valves (Campbell & Kelly, 2002).

In coastal marine environments, the biofouling community and the dominant macrofaunal fouling organisms can vary spatially (Sá et al., 2007; Sievers et al., 2014; Watts et al., 2015), temporally (Underwood & Anderson, 1994), and with respect to elevation and tidal height (Littlewood et al., 1992; Thomsen et al., 2007). Even within a single embayment, the fouling community composition and relative fouling pressure can vary spatially (Fraschetti et al., 2005; Mallet et al., 2009; Sievers et al., 2019). The distribution and abundance of marine organisms in estuaries is affected by variability in recruitment of macrofaunal organisms (Underwood & Fairweather, 1989). Larval supply and transport (Satumanatpan & Keough, 2001), habitat availability and suitability (Hyder et al., 2001), predation (Roughgarden et al., 1988), and climactic and environmental stochasticity (Ysebaert & Herman, 2002) are all drivers of variability in the composition of the marine macrofaunal community (Marques-Silva et al., 2006). Settlement may occur constantly throughout the year or may peak during a certain period or periods (Underwood & Fairweather, 1989). The spatial, seasonal, and inter-annual variability among the species that comprise the fouling community in a given area presents a challenge to oyster growers seeking a single husbandry technique to consistently control biofouling.

A number of treatment options for controlling biofouling among water-column cultured oysters have been examined with varying levels of success and feasibility. Biological options (e.g. Cigarria et al., 1998; Lodeiros & García, 2004; Sterling et al., 2016) essentially restore a degree of ecological balance through the strategic introduction of predators which will consume biofouling organisms but not the oysters themselves. Chemical dips (e.g. Carver et al., 2010; Denny, 2008; Gallo-Garcia et al., 2004; Hooper, 2001; Loosanoff, 1960; Morse et al., 2015; Piola et al., 2009; Sievers et al., 2019) control biofouling by stressing biofoulers to the point of decay through forced immersion in acidic, alkaline, fresh, brine or otherwise inhospitable conditions from which biofoulers cannot recover. Preventative methods (e.g. Cassiano et al., 2012; Tettelbach et al., 2012; Ye et al., 2019) reduce likelihood of colonization through coating the cage material and/or shellfish with a substance that prohibits or reduces colonization. Finally, physical methods (e.g. Mallet et al., 2009; Taylor et al., 1997) physically dislodge biofoulers or subject biofoulers to stressful emersion conditions to reduce both colonization and subsequent survivorship. Each of these strategies have their own costs and benefits in terms of effectiveness and feasibility (reviewed in Bannister et al., 2019; Fitridge et al., 2012), and treatment effectiveness can vary from experiment to experiment (or farm to farm) due to spatial and temporal variability of fauna and differences in environmental conditions. For example, alkaline dips are effective in controlling tunicates (Carver et al., 2010; Denny, 2008), but not other species (Piola et al., 2010); and brine dips can be effective against some algal species and worms (Morse et al., 2015; Sharp et al., 2006), but not against solitary tunicates (Carver et al., 2003). Thus, the appropriate fouling control method may be dictated by idiosyncrasies of the site, season, gear, or species assemblages being targeted.

As one of the most commonly applied methods of biofouling control in the US (Adams et al., 2011), desiccation has yielded promising results in both the realms of feasibility and effectiveness. Desiccation is unique among biofouling control tactics in that gear types are available which allow the process of desiccation to be applied relatively passively. Cages can be inverted, or lines can be raised, allowing farm staff to move on to other activities while the biofouling control process takes place without requiring continued attention. This is in contrast to some other biofouling control strategies which require at least one person to actively employ the treatment (i.e. dips, powerwashing, coatings), preventing them from engaging in other husbandry processes. While early results of desiccation studies revealed little to no fouling reduction on the oysters themselves (e.g. Mallet et al., 2009), these studies employed floating bags which were flipped, allowing one side of the bag to be exposed but keeping the oysters submerged. Recent advances in gear types (e.g. flippable floating cages and adjustable longline systems) allow both the cages and oysters to be exposed, facilitating drying of the oysters and leading to fouling control on the shells in addition to the cage.

To apply desiccation as a husbandry technique, a grower must decide on a desiccation frequency (how often cages will be desiccated) and interval (number of hours cages will be desiccated). Studies to date have focused on manipulating the desiccation frequency (weekly, bi-weekly, monthly, etc.), while maintaining a consistent interval of 24 consecutive hours (Chapman, 2019; Gamble, 2017; Kirk, 2019). Results have clearly demonstrated the need for weekly desiccation during periods of biofouling colonization. These seminal studies have also documented a reduction in growth or meat weight among oysters desiccated for 24-hour intervals at a weekly frequency (Chapman, 2019; Gamble, 2017; Kirk, 2019). However, weekly frequencies also confer the greatest biofouling reduction and yield largely consistent fouling

control results among sites (Chapman, 2019; Gamble, 2017; Kirk, 2019; Mallet et al., 2009). Given the potential for stress to the oysters during prolonged periods of emersion (Littlewood et al., 1992; Newell, 1973), an examination of the interval necessary to achieve biofouling reduction without sacrificing growth is warranted.

This study examined the effect of desiccation interval treatments on biofouling and oysters grown in floating cages. We hypothesized that biofouling on oysters would be reduced in both the 8- and 24-hour treatments, and that oysters in the 24-hour treatment would grow less than those in the 0- and 8- hour treatments. Gross fouling (percent cover) and species-specific patterns of fouling at three oyster farms in the Chesapeake Bay that differ in environmental conditions were monitored during the summer and fall. Oyster growth and mortality were also monitored to assess any bio-physiological tradeoffs associated with prolonged desiccation. Multivariate statistical techniques were employed to describe the relationship among the fouling community and environmental variables at each site. This species-specific assessment of the response of the biofouling community to desiccation across space and time has yielded novel information regarding persistent fouling organisms in the region and their ability to be controlled using desiccation. Data on oyster growth and mortality has allowed an assessment of tradeoffs of desiccation as a management technique, information which is critical to farmers looking to minimize fouling without sacrificing crop productivity.

Methods

Site descriptions

Three tidally-influenced Chesapeake Bay commercial oyster farms served as study sites for this experiment. Sites were selected to represent a range of environmental conditions common to Maryland oyster farms (Figure 1, Table 1). Site A (39°02’56”N; 76°12’34”W) is

located in the Chester River and is exposed to the north, east and south. The site has a 30-year mean surface salinity of 9.2 (Maryland Department of Natural Resources, 2021) and is located approximately 2 nautical miles from the confluence of the Chester River and the Chesapeake Bay mainstem. Site B (38°18’49”N; 76°13’24”W) was the most protected site, located in Back Creek, along the Honga River with a 30-year mean surface salinity of 13.3 (Maryland Department of Natural Resources, 2021). Site C (38°16’49”N; 76°04’59”W) is located in the Honga River near its confluence with the mainstem of the Chesapeake. The site has a 30-year mean surface salinity of 12.9 (Maryland Department of Natural Resources, 2021). All locations were permitted for oyster aquaculture.

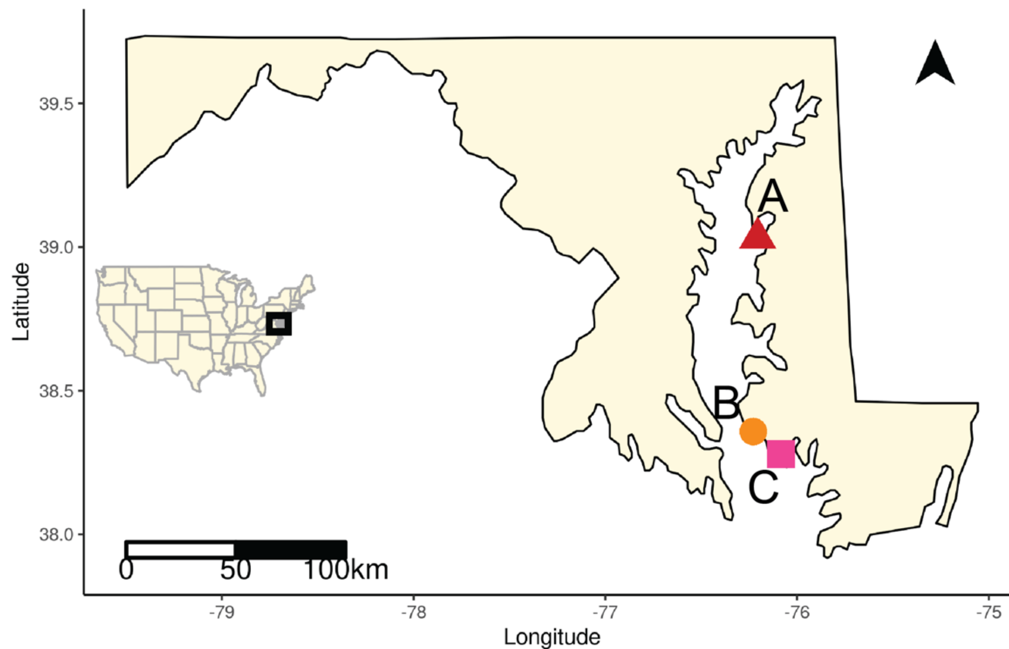

Figure 3. Locations of oyster farms in Maryland’s portion of the Chesapeake Bay where desiccation investigations were conducted during July-December 2018. Site A is represented by the red triangle, Site B is represented by the orange circle, and Site C is represented by the pink square.

Table 3. Site descriptions for the three sites where field investigations were conducted in 2018. Locations are provided in latitude and longitude. Salinity (1988-2018 mean and 95% confidence interval) and Chlorophyll a (µg/l, 1988-2018 mean and 95% confidence interval) were retrieved from Maryland Department of Natural Resources Eyes on the Bay Fixed Station Long Term Monitoring Program (MD DNR, 2021).

Site	Location	Salinity		Chlorophyll a (µg/l)	
		Mean (1988-2018)	95% CI	Mean (1988-2018)	95% CI
A	39°02’56”N; 76°12’34”W	9.2	8.9-9.5	13.7	12.9-14.5
B	38°18’49”N; 76°13’24”W	13.3	13.1-13.6	8.5	8.2-8.8
C	38°16’49”N; 76°04’59”W	12.9	12.6-13.2	11.8	11.2-12.4

Environmental monitoring

Salinity and temperature data were retrieved from the MD Department of Natural Resources Eyes on the Bay (EOTB) Fixed Station Long Term Monitoring Program (Maryland Department of Natural Resources, 2021). Buoys are proximal (3 - 4 miles) from each site (Site A: Station ET4.2, Site B: Station CB5.1, Site C: Station EE3.0). Weather conditions during the study period were accessed using NOAA National Centers for Environmental Information Local Climatological Data (https://www.ncdc.noaa.gov/cdo-web/datatools/lcd). Stations are located nine to sixteen miles from each site (Site A Station ID WBAN:00124, Sites B and C Station ID WBAN:93720). Air temperature (°C), total precipitation (mm), cloud cover (0-8), and relative humidity (%) were assessed for the periods of emersion at each site. Cloud cover data were reported using the NOAA Local Climatological Data sky condition units, where 0 corresponds to clear skies, 1-2 is defined as few clouds, 3-4 is defined as scattered clouds, 5-7 is defined as broken clouds, and 8 is defined as overcast (NOAA, 2022).

Oyster preparation and deployment

Two-year old hatchery-reared diploid oysters (*Crassostrea virginica*) were sourced from the University of Maryland Center for Environmental Science (UMCES) Horn Point Laboratory Demonstration Oyster Farm in the Choptank River, MD. All existing biofouling was removed from oysters prior to deployment to ensure that any fouling colonization could be ascribed to the period of study. Oysters were scrubbed using an oyster knife, rigid bristle brush, and freshwater to dislodge epibionts. Oysters were then placed in a 5% ethanol / 95% artificial seawater bath (deionized water + Crystal Sea® Marinemix, Baltimore, MD, USA salted to 13) for 24 hours prior to deployment to expel worms living within the valves. Preliminary experiments demonstrated that a 24-hour ethanol/seawater bath was effective in causing worms to vacate their burrows (modified from Morse et al., 2015).

Smaller, "experimental-sized" versions of the commercially available flippable floating cage system (e.g. Flow N Grow™, HI-Flow, OysterGro®) were constructed for this study (Figure 2). When inverted, these cages allow both the cage material and oysters to sit out of the water. Each cage was constructed of 25.4mm vinyl-coated wire mesh and measured 50.8cm x 50.8 cm x 7.6 cm. Two parallel lengths of capped schedule 20 polyvinyl chloride (PVC) were attached to the top of each cage to provide adequate flotation. Oysters were deployed in July 2018 to three desiccation interval treatments. Sixty (60) oysters (mean (± SD) shell height = 64 mm ± 6.7mm) were deployed to each cage, with three technical replicate cages deployed to each desiccation treatment per site for a total of nine cages per site at each of the three sites (Figure 1). Weekly, industry partners flipped (inverted) cages to allow desiccation to take place in the field. At each site, three cages were desiccated for zero hours per week (control / 0-hour treatment), three cages were desiccated for eight hours per week (8-hour treatment) and three cages were

desiccated for twenty-four hours per week (24-hour treatment). Cages were deployed between July and December 2018.

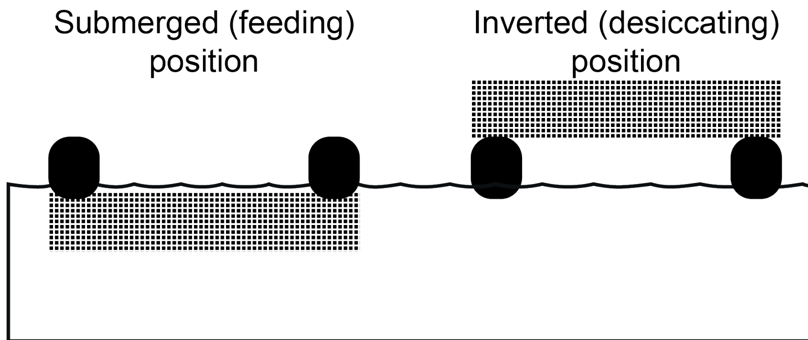

Figure 4. Flippable floating cage systems used in desiccation experiments at three Chesapeake Bay oyster farms. In their submerged (feeding) position, left, oysters and cage material float just below the water's surface, suspended by floating pontoons. In the inverted (desiccating) position, right, oysters and cage material are inverted and rest on the pontoons, remaining out of the water until the cage is re-submerged.

Biofouling assessment

Sampling for gross biofouling coverage and individual species abundance was conducted twice at each site during the four-month study: October (Period 1) and November/December (Period 2). Five oysters were randomly selected from each of the 3 replicate cages per treatment for epibiont and endobiont assessment (15 oysters per treatment x 3 treatments x 2 samplings x 3 sites; total n =270). Sampling took place without replacement. Oysters were gently rinsed, allowing only attached organisms to remain on the valves. Whole oysters were imaged (both valves) then held in ambient seawater. ImageJ image analysis software (Schneider et al., (2012); v. 1.8, U.S. National Institutes of Health, Bethesda, MD, USA) was used to ascertain the total surface area of each oyster and the surface area occupied by individual biofouling species

(Carroll et al., 2015; Newell et al., 2007). Epibiont fouling organisms were identified to the lowest taxonomic level possible (genus and species) using a stereo microscope (Olympus SZX16, Olympus Corporation, Tokyo, Japan) and visual taxonomic identification methods (Gosner & Peterson, 1982; Lippson & Lippson, 2006, Smithsonian Environmental Research Center's National Estuarine and Marine Exotic Species Information System). Oysters were rinsed, then placed into individual 16oz glass jars containing 5% ethanol and 95% artificial seawater (deionized water + Crystal Sea® Marinemix, salted to a salinity of 13) for 24 hours. Expelled worms were enumerated and discarded.

Oyster assessment

Oyster shell height, defined as the distance between the umbo and the ventral valve margin (Figure 3; Galtsoff, 1964), was recorded for fifteen oysters per treatment at each site (45 oysters x 2 samplings x 3 sites; total n=270) using Vernier calipers on the first (October) and second (November/December) samplings. Mortality was assessed for each cage by visually inspecting and tapping all oysters in each cage to determine their survival status. Dead oysters were enumerated and removed from cages.

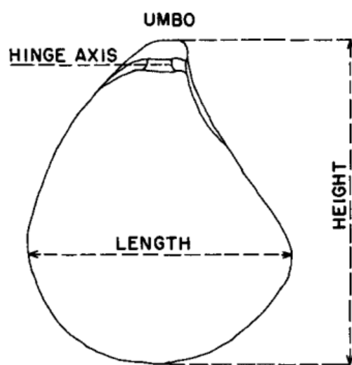

Figure 5. Shell morphology of *C. virginica*, noting the measurement of shell height (umbo to ventral valve margin). From Galtsoff, 1964.

Data analysis

Statistical analyses were conducted using RStudio (RStudio Team, 2016) to assess the effect of desiccation treatment and site on biofouling coverage, endobiont (worm) infestation, and oyster growth and mortality. Unconstrained ordination was performed on log-transformed relative abundance data using Detrended Correspondence Analysis (DCA) from the 'vegan' package (Oksanen et al., 2020) to assess similarity among the biofouling community at the three sites. To examine diversity of the biofouling community across sites, the Shannon-Weiner Index of Diversity was calculated using the 'vegan' package (Oksanen et al., 2020). Shannon-Weiner was selected as the diversity metric of interest given its inclusivity of species richness and evenness and sensitivity to small changes in diversity. Shannon-Weiner index was calculated among oysters in the control (non-desiccated) treatment at all sites according to the following formula:

$$H' = -\sum_{i=1}^{s} p_i \ln p_i$$

where H' is the diversity index, s is the total number of species present, and p_i is the relative proportion of coverage by each species i. Larger H' values indicate greater diversity.

A permutation-based ANOVA test from package 'predictmeans' (Luo et al., 2021) was used to examine independent and interactive effects of desiccation treatment and site on biofouling in a mixed-effects model, with desiccation and site serving as the fixed effects, and replicate number included as a random effect. The non-parametric, two-way factorial Scheirer-Ray-Hare test from the 'rcompanion' package (Mangiafico, 2022) was subsequently used to assess independent and interactive effects of treatment and site on the cumulative biofouling

community and worm abundances. The non-parametric, one-way factorial Kruskal-Wallis test from the 'stats' package (R Core Team, 2017) was used to assess effects of treatment on coverage by individual species at each site due to violations of the normality assumption required for parametric tests. Post-hoc comparisons of treatment effects on total fouling and coverage by individual biofouling species were performed using the Dunn (1964) multiple comparison test from the package 'FSA' (Ogle et al., 2018), and a Holm adjustment was used to account for multiple comparisons. A Kruskal-Wallis test ('stats' package, R Core Team, 2017) was used to evaluate desiccation treatment effects on abundance of worms in oysters at each site, and a Dunn multiple comparison test (Holm adjustment, 'FSA' package, Ogle et al., 2018) was subsequently used to conduct pairwise comparisons. Differences in shell height of oysters from the desiccation treatments at each site were investigated using a Kruskal-Wallis test ('stats' package, R Core Team, 2017), and significant differences were further investigated using a Dunn multiple comparison test (Holm adjustment, 'FSA' package, Ogle et al., 2018). A Scheirer-Ray-Hare test ('rcompanion' package, Mangiafico, 2022) was used to assess treatment and site effects on oyster mortality. To assess influence of environmental variables on the biofouling community present at each site, a Canonical Correspondence Analysis from the 'vegan' package (Oksanen et al., 2020) was performed on relative abundance data, using each site's mean salinity, chlorophyll a concentration (µg/l) and temperature (°C) data per period. Data visualization was carried out using ggplot2 (Wickham, 2016).

Results

Environmental conditions

Surface salinity conditions at all sites were below the 30-year long term averages (Figure 4). The mid-Atlantic region experienced record rainfall and discharge during 2018, which led to

reduced salinity in the Chesapeake Bay and its tributaries (Tarnowski, 2019). Surface salinity at Site A (obtained from MD DNR EOTB station ET4.2) ranged from 3.8 to 8.9, with an average of 5.4 and surface temperature ranged from 8.6°C to 28.3°C. Surface salinity at Site B (obtained from MD DNR EOTB station CB5.1) ranged from 5.9 to 9.3, with an average of 8.0, and surface temperature ranged from 11°C to 27.1°C. Surface salinity at Site C (obtained from MD DNR EOTB station EE3.0) ranged from 7.4 to 10.6 with an average of 8.8 and surface temperature ranged from 12.6°C to 30.8°C. See Appendix A for salinity conditions at each site during the period of investigation.

Air temperature, precipitation, relative humidity, and cloud cover data were compiled for the periods of desiccation (24-hour treatment) at each site during the investigations. Temperature during desiccation ranged from 1 to 29°C and averaged 17°C, 19°C, and 18°C at Sites A, B, and C, respectively. Total precipitation was highest at Site A with an average of 20mm of rainfall falling during desiccation periods. Precipitation totals at sites B and C were similar and averaged 6 and 5mm, respectively. Relative humidity varied from 47% to 100% and was highest at Site B with mean humidity during desiccation of 89%, compared to 67% and 78% humidity at Sites A and C, respectively. Cloud cover ranged from 0 (clear skies) to 8 (overcast) and was higher at Site B (mean cloud cover = 5) during desiccation compared to Sites A and C, both of which experienced a mean cloud cover of 2 (Figure 5). See Appendix B for raw weather conditions during the period of investigation at each site.

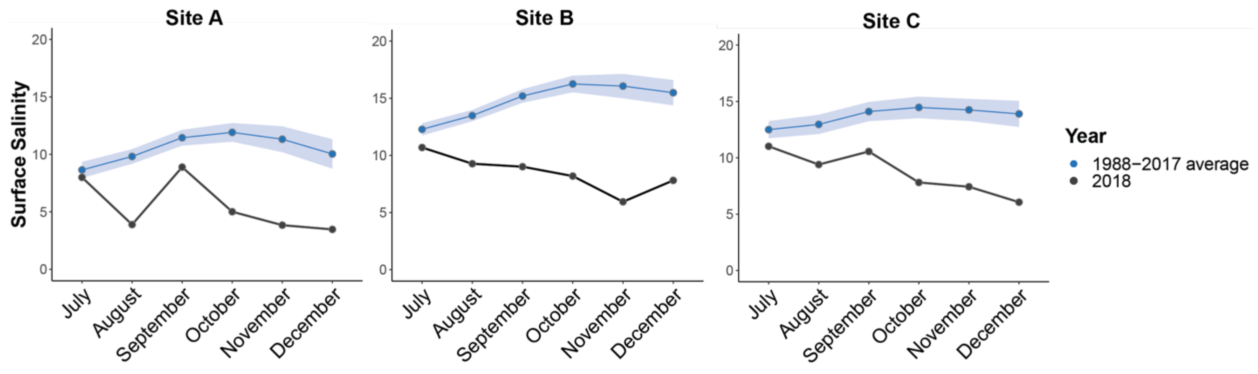

Figure 6. Surface salinity conditions at three sites where field investigations were conducted. 2018 surface salinity data are shown by the gray line and the 30-year average (1988 - 2017) is shown in blue with a blue ribbon encompassing the 95% confidence interval. Data were retrieved from Maryland Department of Natural Resources Eyes on the Bay Long-term Fixed Station Monitoring (Site A: Station ET4.2; Site B: Station CB 5.1; Site C: Station EE3.0)

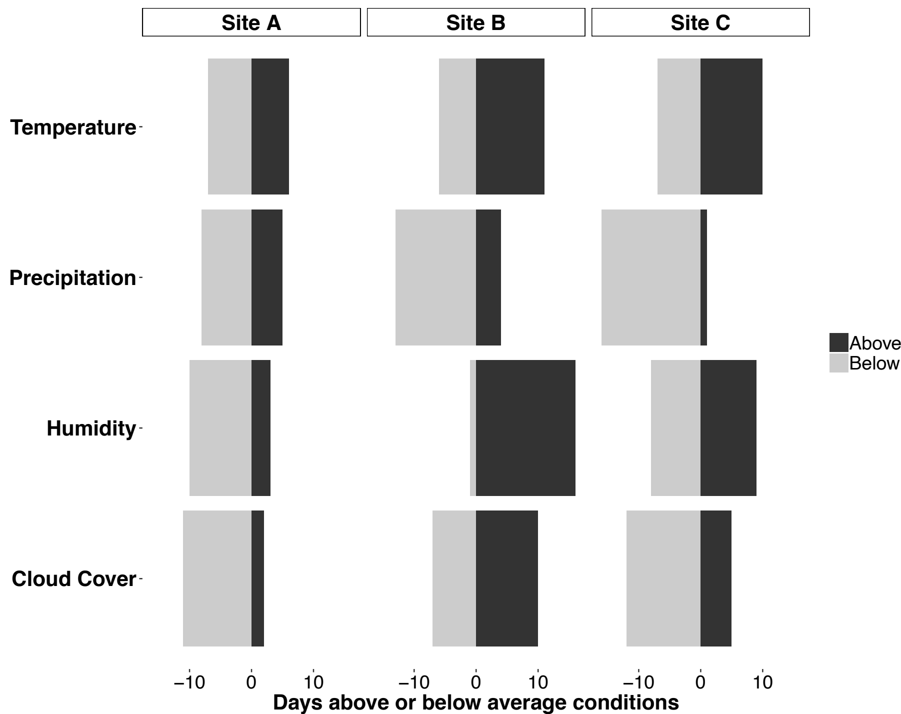

Figure 7. Number of desiccation days spent above and below average conditions at three oyster farms where desiccation treatment investigations took place. Average temperature conditions

during emersion were 18°C, average total precipitation during emersion was 9.5mm, average relative humidity during emersion was 79%, and average cloud coverage during emersion was 3. Dark bars represent the number of desiccation days spent above average conditions at each site, and light bars represent the number of desiccation days spent below average conditions at each site.

Epibiont fouling community and treatment effects

The biofouling community varied among the sites, with Sites B and C yielding more similar communities compared to Site A (Figure 6). A Canonical Correspondence Analysis relating in-water conditions to species composition and relative abundance revealed that salinity was the primary driver of differences among the sites (Figure 7). All sites yielded greater species diversity (Shannon-Weiner Index) during the first (July – October) period, and diversity decreased during the October-December period (Figure 8). Epibiont coverage at Site A was dominated by cushion moss bryozoan (*Victorella pavida)* and this site experienced the highest biofouling pressure, measured as the greatest percent coverage of the control (0-hour) treatment. Site A yielded the lowest fouling species diversity of the three sites (Shannon Weiner Index = 0.08 and 0.03 in periods 1 and 2 respectively, Figure 8). Other attached species observed on oysters at Site A were the bay barnacle (*Balanus improvisus),* freshwater hydroid *(Cordylophora caspia),* false mussel (*Mytilopsis leucophaeata),* and gutweed (*Ulva intestinalis).* Site B fouling was dominated by *U. intestinalis* and this site had the greatest diversity of fouling species according to the Shannon Weiner Index (H = 0.31 and 0.17 during the first and second samplings, respectively; Figure 8). Ancillary species present were *B. improvisus,* coffin box bryozoan (*Membranipora tenuis*)*,* lacy crust bryozoan (*Conopeum tenuissimum*)*,* rope grass (*Garveia franciscana*), and ghost anemone (*Diadumene leucolena*)*.* Site C fouling was

dominated by *B. improvisus* and had the least biofouling coverage. Fouling species diversity was similar to Site B (H = 0.21 and 0.08 in Periods 1 and 2, respectively, Figure 8). Other species documented at Site C were *G. franciscana, D. leucolena, U. intestinalis, C. tenuissimum,* and *M. tenuis.*

Desiccation interval treatment yielded varied results across the sites and species present (Figure 9, Figure 10). Mean (± SD) epibiont coverage was highest at Site A, with 98.4 (± 6), 74.0 (± 22), and 65.0 (± 30) percent coverage in the 0-, 8-, and 24-hour desiccation treatments, respectively, during the first (October) sampling. Mean fouling decreased by the second sampling to 59.2 (± 35), 6.4 (± 8), and 6.7 (± 13) percent coverage in the 0-, 8-, and 24-hour desiccation treatments, respectively. Average epibiont coverage (± SD) at Site B during the first sampling period was 19.0 (± 25), 22.6 (± 35), and 15.9 (± 27) percent coverage for the 0-, 8-, and 24-hour desiccation treatments respectively. Fouling in the 0-hour treatment was slightly reduced at Site B during the second sampling period, when mean epibiont coverage was 9.3 (± 12), 23.6 (± 26), and 15.6 (± 24) percent in the 0-, 8-, and 24-hour desiccation treatments, respectively. Site C fouling was limited and consistent between the sampling periods, with 12.2 (± 9), 0.3 (± 1), and 2.1 (± 4) percent coverage in the 0-, 8-, and 24-hour desiccation treatments, respectively during the first sampling period. During the second sampling period, mean epibiont coverage at Site C was 6.1 (± 6), 0.4 (± 1), and 0.7 (± 2) percent for the 0-, 8-, and 24-hour desiccation treatments, respectively (Figure 10).

A permutation-based ANOVA test indicated a significant effect of treatment and site on biofouling coverage with cage replicate included as a random effect ($P = 0.001$). Subsequent results of a Scheirer-Ray-Hare test revealed that biofouling coverage was significantly affected by site ($P < 0.0001$), desiccation treatment ($P < 0.0001$), and their interaction ($P < 0.0001$).

Treatment effects were consistent during both sampling periods. Post-hoc Dunn tests indicated significant differences in biofouling coverage with respect to treatment at Sites A and C ($P < 0.0001$). The 8- and 24-hour treatments were associated with significantly reduced biofouling coverage at Sites A and C during both sampling periods. No differences in fouling coverage were observed among desiccation treatments at Site B.

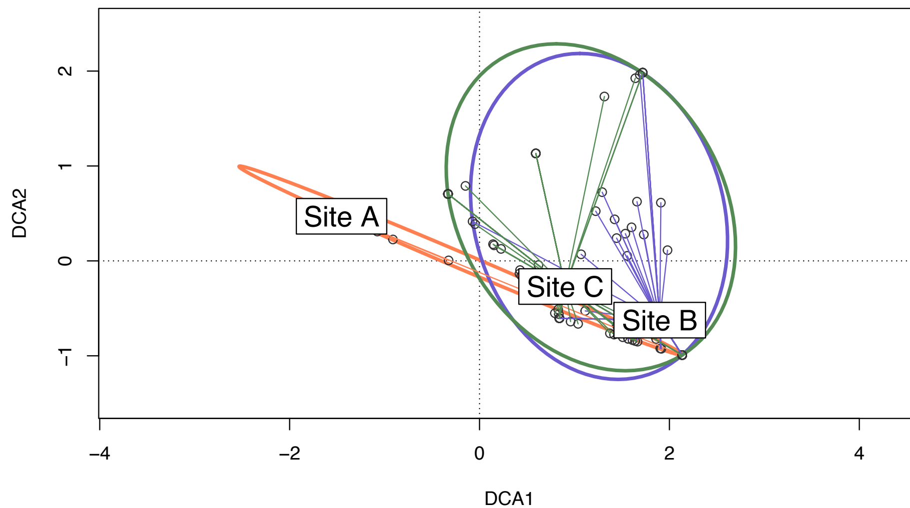

Figure 8. Detrended Correspondence Analysis (DCA) plot of the biofouling community present at three Chesapeake Bay oyster farm sites during summer and fall 2018. The orange ellipse encompasses the fouling community at Site A. The purple ellipse encompasses the fouling at Site B and the green ellipse encompasses the fouling community at Site C.

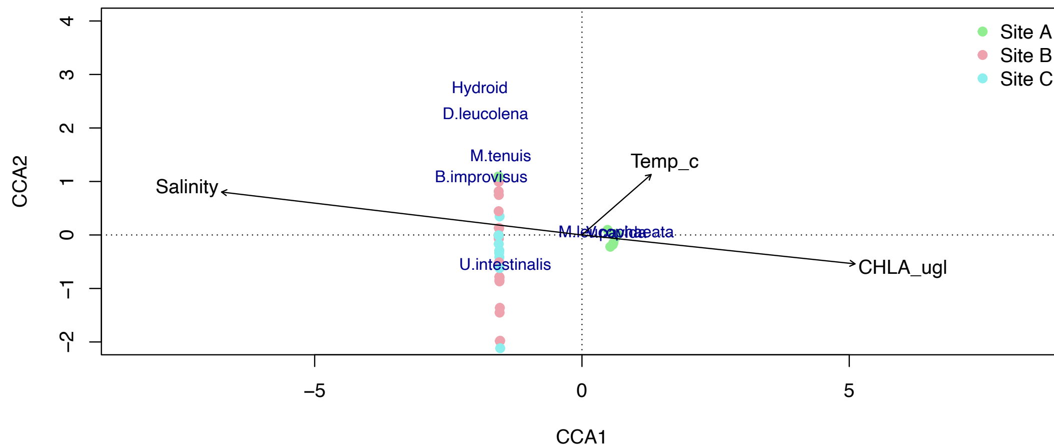

Figure 9. Canonical Correspondence Analysis (CCA) relating in-water conditions at three Chesapeake Bay oyster farms to the biofouling community composition at each. Green dots represent species data from Site A, pink dots represent species data from Site B and blue dots represent species data from Site C. Species names are listed in dark blue text. The length of the labeled black lines indicates the relative importance of that in-water parameter in describing the community composition.

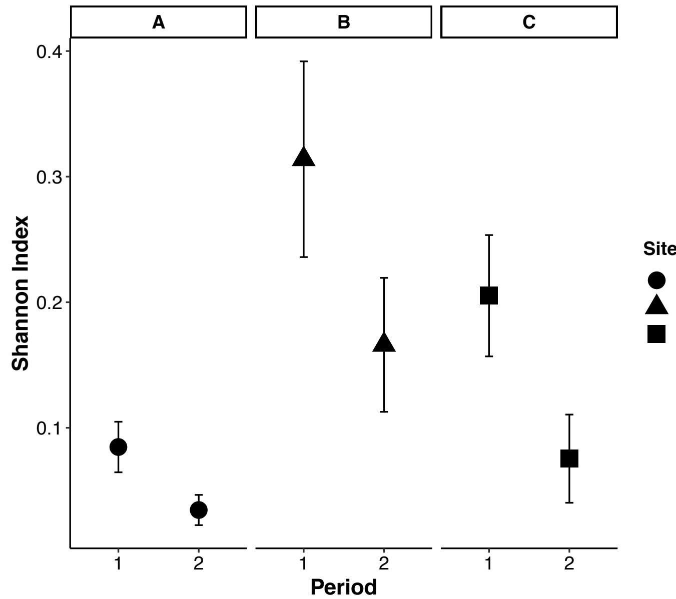

Figure 10. Mean Shannon-Weiner Index of Diversity (± SEM) for the biofouling community present on control (0-hour treatment) oysters at three Chesapeake Bay oyster farm sites (Site A: black circles, left; Site B: black triangles, center; and Site C: black squares, right) during October (Period 1) and November/December (Period 2). Higher Shannon-Weiner Index values indicate greater diversity.

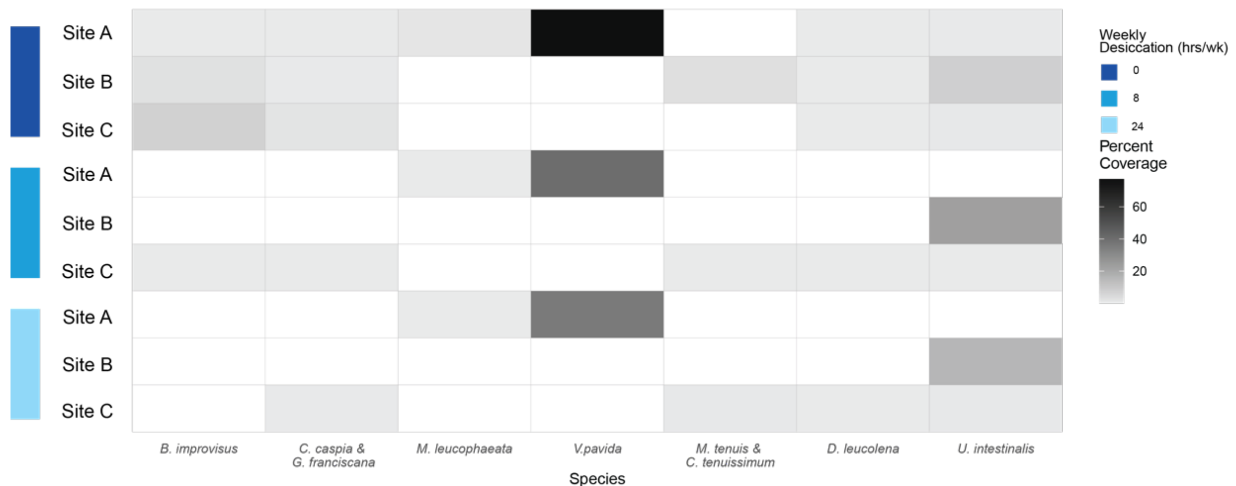

Figure 11. Heatplot of relative abundance (percent coverage) data from individual biofouling species colonizing oysters in three desiccation treatments (0, 8, 24 hours per week) at three

Chesapeake Bay oyster farms (Sites A, B, and C) in summer and fall 2018. Darker colors indicate greater average percent coverage.

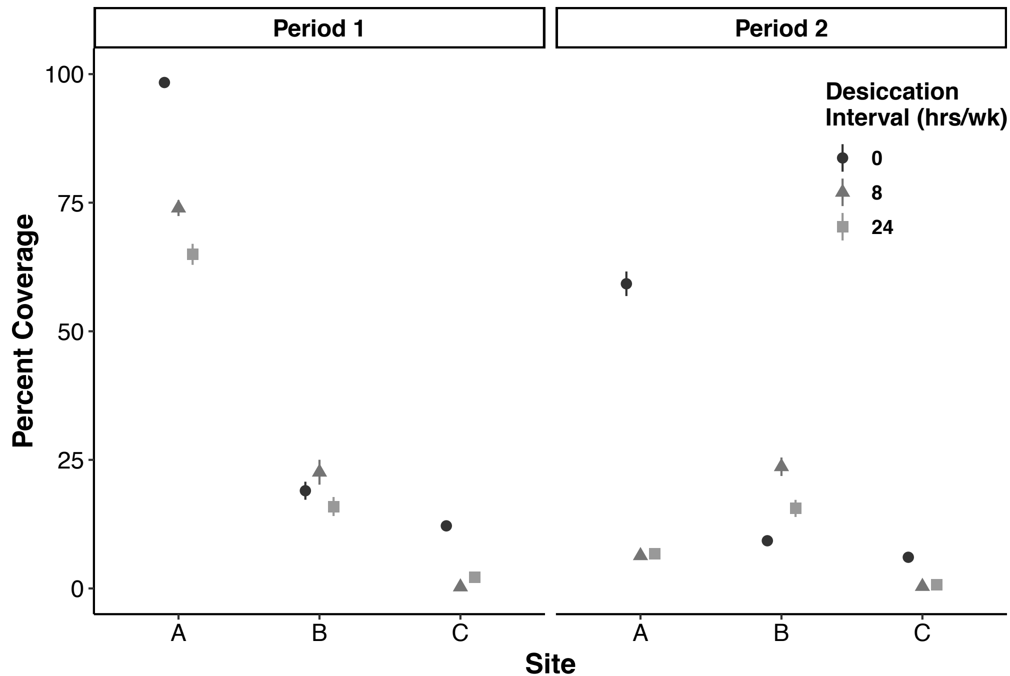

Figure 12. Mean (± SEM) total epibiont coverage of *C. virginica* oysters in three weekly desiccation intervals (0, 8, and 24 hours of consecutive desiccation per week) at three Chesapeake Bay oyster farms during the first (Period 1, left) and second (Period 2, right) sampling periods in October and November/December 2018. Solid dots represent species coverage in the non-desiccated (0-hour) treatment. Triangles represent species coverage in the 8-hour treatment, and squares represent species coverage in the 24-hour treatment.

Species-level effects

Valve coverage by all epibiotic species observed was significantly affected by desiccation treatment (all Kruskal-Wallis $P < 0.05$), with the exception of *U. intestinalis* which was not affected by desiccation treatment (Kruskal-Wallis $P = 0.70$). Aside from *U. intestinalis,*

fouling was significantly reduced in oysters from the 8- and 24-hour treatments compared to oysters in the 0-hour treatment. Table 1 provides resulting P-values from post-hoc Dunn tests performed on each species' relative abundance in each desiccation treatment. Differences in biofouling coverage between the 8- and 24-hour treatments were not significant (Table 2). Treatment effects on dominant epibionts (species whose colonization reached at least 5% during the period of study) were separated from secondary epibionts (species whose coverage remained less than 5% during the period of study) for the purposes of visualization in Figures 11 and 12.

Table 4. Results of post-hoc Dunn tests for desiccation treatment effects, after Kruskal-Wallis test, on individual species present at three Chesapeake Bay oyster farms in 2018 in three desiccation treatments: 0, 8 and 24 hours of desiccation per week. Relationships were analyzed and presented only for sites where a given species was present during the period of investigation. Sites where a species was present are listed in the second column. Values are adjusted p-values from the Dunn (1964) multiple comparison test corrected with the Holm adjustment to account for multiple comparisons. Significant relationships ($P < 0.05$) are noted in bold text.

		Treatment comparison		
Species	Site present	0 : 8	0 : 24	8 : 24
B. improvisus	A, B, C	**7.16e-26**	**3.74e-29**	0.79
C. caspia & G. franciscana	A, B, C	**0.00**	**0.03**	0.21
D. leucolena	A, B, C	**0.01**	**0.00**	0.34
M. leucophaeata	A	**3.56e-07**	**5.15e-07**	0.99
M tenuis &. C. tenuissimum	B, C	**7.64e-05**	**1.62e-04**	0.78
U. intestinalis	A, B, C	1	0.98	0.95

V. pavida	A	**4.29e-07**	**4.07e-09**	0.38

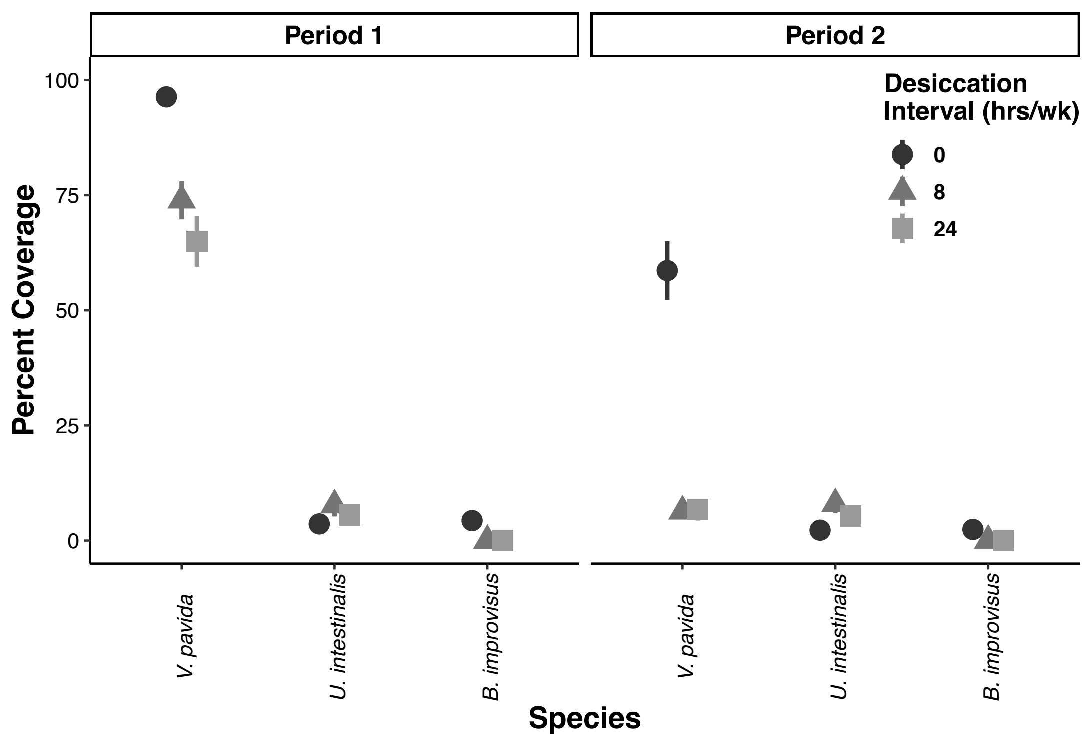

Figure 13. Mean (± SEM) coverage for dominant biofouling species in each desiccation interval treatment during Period 1 (left) and Period 2 (right). Dominant species are those species which reached over 5% total coverage at any one of the three sites during the sampling period. Species coverage data were averaged across the sites where each species was present (*V. pavida*: Site A; *U. intestinalis*: Sites A, B, C; *B. improvisus*: Sites A, B, C). Solid dots represent species coverage of oysters in the non-desiccated (0-hour) treatment. Triangles represent species coverage in the 8-hour treatment, and squares represent species coverage in the 24-hour treatment.

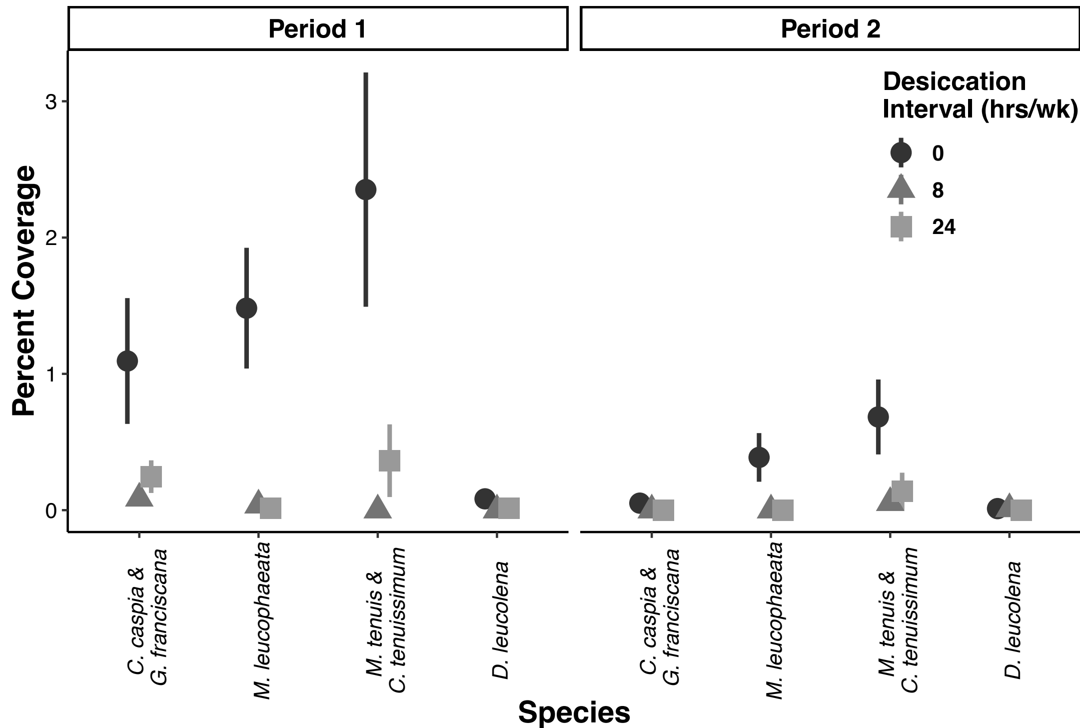

Figure 14. Mean (± SEM) coverage for secondary biofouling species in each desiccation interval treatment during Period 1 (left) and Period 2 (right). Secondary biofoulers remained below 5% coverage at all sites. Species coverage data per treatment were averaged across the sites where each species was present (*C. caspia* & *G. franciscana*: Sites A, B, C; *M. leucophaeata*: Site A; *M. tenuis* & *C. tenuissimum*: Sites B, C; *D. leucolena*: Sites A, B, C). Solid dots represent species coverage in the non-desiccated (0-hour) treatment. Triangles represent species coverage in the 8-hour treatment, and squares represent species coverage in the 24-hour treatment.

Endobiont worms

Similar to the epibiont fouling results, total worm abundances were highest at Site A during both samplings, followed by Sites B and C which yielded comparatively fewer worms per oyster. Mean (± SD) worms per oyster in the 0-hour treatment at Site A was 143.0 (± 167) during the first sampling, compared to 47.9 (± 25) and 18.5 (± 10) worms per oyster in the 8- and 24-

hour treatments, respectively. By the second sampling at Site A, the mean (± SD) number of worms per oyster fell to 46.5 (± 30), 6.5 (± 5), and 2.8 (± 4) worms per oyster in the 0, 8-, and 24-hour desiccation treatments, respectively. Site B experienced less worm infestation, with an average of 22.2 (± 12) worms per oyster in the 0-hour treatment and 0.1 (± 1) worms per oyster in the 8-hour treatment, and no worms were observed in oysters from the 24-hour treatment. The second sampling at Site B yielded similar trends as the first sampling, with 34.7 (± 14) worms per oyster in the 0-hour treatment, followed by 0.7 (± 2) and 0.1 (± 0) worms in the 8- and 24-hour desiccation treatments, respectively. Worm abundances at Site C were similar to Site B, with 29.7 (± 7), 13.5 (± 3), and 13.1 (± 8) worms per oyster in the 0-, 8-, and 24-hour desiccation treatments, respectively. During the second sampling, oysters in the 0-, 8-, and 24-hour desiccation treatments at Site C yielded 17.1 (± 9), 16.4 (± 13), and 11.3 (± 10) worms per oyster, respectively (Figure 13).

Worm abundance was significantly affected by treatment, site and their interaction (Scheirer-Ray-Hare $P < 0.05$) with reduced worm abundances in the desiccated treatments (Figure 13). Both the 8- and 24- hour desiccation treatments yielded a reduction in worms at sites A and B during both samplings (Kruskal-Wallis $P < 0.05$), although no effect of desiccation treatment was observed at Site C during the second sampling (Kruskal-Wallis $P = 0.2$; Figure 13). Pairwise comparisons revealed that differences between the 8- and 24-hour desiccation treatments were significant only at Site A during the first sampling, with worm abundances inversely related to desiccation interval (Dunn test $P = 0.01$). Otherwise, differences between the 8- and 24-hour treatments were not significant (Dunn test $P > 0.05$) and yielded similar reductions in worm abundance.

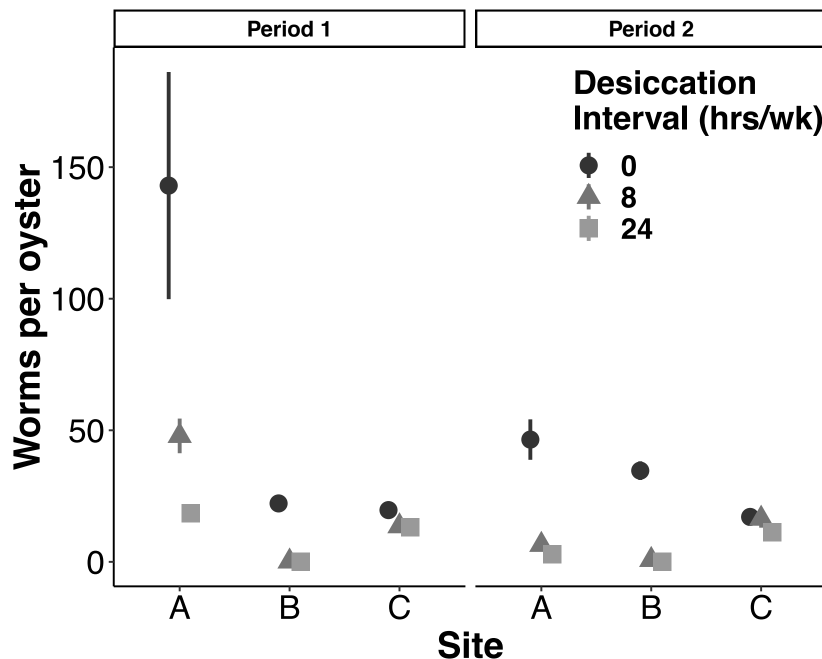

Figure 15. Mean (± SEM) number of worms per oyster in oysters desiccated for 0, 8 and 24 consecutive hours once weekly at three Chesapeake Bay oyster farm locations (Sites A, B, and C) in October (left, Period 1) and November/December (right, Period 2). Black circles represent worms in oysters desiccated for 0 hours, gray triangles represent worms in oysters desiccated for 8 hours per week, and light gray squares represent worms in oysters desiccated for 24 hours per week. Error bars represent one standard error of the mean.

Oyster performance

Oyster growth was negligible at all sites during the study period, with change in shell height averaging between 0 – 4mm for oysters in all treatments at all sites. Treatment effects on shell height were significant at Site C (Kruskal-Wallis P = 0.03), where shell height was an average of 4mm smaller in the 8-hour desiccation treatment compared to other treatments (Dunn test P = 0.04). Cumulative mortality differed across the sites during the first sampling period

only, with Site A experiencing 4% mortality, and Sites B and C experiencing 17% and 19% mortality, respectively (Table 3). New mortality during the second sampling was 2% at Sites A and B and 3% at Site C. A Scheirer-Ray-Hare test revealed differences in mortality during the first period among the sites (P = 0.00), but not the desiccation treatments (P = 0.8). During Period 2, no differences in mortality were observed with respect to treatment or site (Scheirer-Ray-Hare P = 0.7 and 1.0, respectively).

Table 5. Mortality (number of dead oysters) of oysters at three Chesapeake Bay Oyster farms desiccated for 0-, 8-, or 24-hours weekly from July - December 2018. Mortality data are provided for the July - October time period (Period 1) and the October – December time period (Period 2).

	Site A Mortality (Number dead)		Site B Mortality (Number dead)		Site C Mortality (Number dead)	
Treatment	Pd. 1	Pd.2	Pd. 1	Pd. 2	Pd. 1	Pd. 2
0	7	3	33	3	36	7
8	7	3	37	5	29	2
24	6	5	23	2	35	3

Discussion

The objective of this study was to examine the effect of desiccation interval treatment on biofouling of oysters at Chesapeake Bay oyster farms. Additionally, the study sought to understand effects of desiccation interval treatments on oyster growth and mortality, which is important for weighing the potential benefits or harms to oyster yield as a consequence of

implementing desiccation. Given the findings of previous studies (Chapman, 2019; Gamble, 2017; Kirk, 2019; Mallet et al., 2009), the 24-hour weekly desiccation treatment was expected to yield the greatest reduction in biofouling, though this fouling reduction was expected to come at the expense of growth and with elevated mortality. Findings revealed biofouling reduction (both epibionts and worms) in both the 8-hour and 24-hour treatments, with minimal differences between the treatments. Growth differences were minimal in this particular study and differential mortality was not related to treatment. The findings of this study agree with previous work on the effectiveness of a 24-hour desiccation treatment in reducing biofouling. This study adds the novel information that an 8-hour desiccation treatment confers significant biofouling reduction, although effects of both the 8- and 24- hour desiccation treatments varied across sites. In contrast to previous work, this study did not find notable divergences in growth and mortality among oysters desiccated for 8 or 24 hours weekly compared to a control treatment which remained submerged. An assessment of these results, potential explanatory factors and implications are discussed below.

Epibiont biofouling results and limitations therein

The biofouling community varied spatially (Figure 6), as did treatment effectiveness (Figure 10). The 8- and 24-hour treatments yielded less biofouling coverage at Sites A and C during both sampling periods. Sites A and C were dominated by colonial bryozoans (Site A) and solitary crustaceans (Site C). In contrast, no treatment effects were observed on epibiont fouling at Site B, where the macroalgae *U. intestinalis* colonized rapidly and reached coverage of 10-23% by the first sampling period. *Ulva intestinalis* is a resilient, rapidly colonizing, opportunistic green algae (Little et al., 1996), capable of tolerating lengthy periods of desiccation, during which the outer fronds dry but retain moisture within the interior fronds for a period of several

weeks (McAllen, 1999). Further, periods of desiccation have been linked to the release of zoospores (Li et al., 2014; Little et al., 1996), indicating the potential for regular desiccation to favor additional colonization among an otherwise submerged cage unit. In contrast, *V. pavida,* while having been documented in the intertidal (Branscomb 1976), is primarily known as a subtidal species (SERC, 2022), found submerged in shallow waters attached to pilings, stones, plants, and other substrata (Carter & Jackson, 2007) in areas with low to varying salinities characteristics of fresh and brackish waters (Carter & Jackson, 2007; Winston, 1995). *B. improvisus* is common in the subtidal and low-intertidal region of Chesapeake Bay with tidal ranges between 46-51 centimeters (Branscomb, 1976). While it is found in the intertidal zone, *B. improvisus* is most common in the low intertidal region which experiences limited emersion during low tides (Branscomb, 1976; Lippson & Lippson, 2006). The differing life histories of these common biofoulers, specifically with respect to tolerance of the intertidal, indicate the potential for different desiccation tolerances. This notion is substantiated by results of this study, whereby subtidal species were well controlled via desiccation but the species adapted to life in the intertidal (*U. intestinalis*) was not affected by the desiccation treatments. The observed species-specific treatment effects support previous findings regarding species-specific desiccation tolerance among fouling organisms (Hopkins et al., 2016). Findings indicate the value of obtaining detailed, species-level data in studies of biofouling control techniques in aquaculture settings. While a single, general purpose fouling control strategy may be desired, the biofouling species present should be considered and addressed appropriately if a farmer seeks to maximize fouling control.

Salinity conditions during the period of study were below the 30-year average at all sites due to record rainfall that inundated the region in 2018 (Tarnowski, 2019, Figure 4). Salinity is a

major driver of the species assemblages present within an estuary (Attrill, 2002; Ysebaert & Herman, 2002), and the specific species observed at each site during this period of study and the conditions they experienced may not be reflective of average or typical site conditions. Indeed, a Canonical Correspondence Analysis (CCA) indicated salinity as a major driver of the species assemblages observed among the three oyster farms sites studied (Figure 7). The effect of the rather anomalous salinity conditions observed during the study period is substantiated by the absence of a number of fouling species common to mid or upper-Chesapeake Bay oyster farms, such as *Clathria prolifera, Cliona celata, Cliona truitti, Ischadium recurvum,* and *Molgula manhattensis*. The lack of these species may be due to the historically low salinity conditions experienced during the period of study. A targeted approach to biofouling control is aided by knowledge of the species present and their tolerance to specific desiccation treatments. The lack of many common fouling species during the study makes it challenging to assess or report the effects of desiccation interval on a 'typical' fouling community in this region, although the species observed are also common in typical years. Results therefore apply to a subset of the biofouling species commonly observed on Maryland oyster farms. Thus, the results from this study about the effectiveness of biofouling control from desiccation are limited to the species present. However, overall, the results suggest that either an 8- or 24- hour weekly desiccation interval can control many common biofouling organisms in the Chesapeake Bay.

Worm infestation and desiccation treatment effects

Worm abundances in oysters were affected by desiccation treatment at all three sites, although effects varied temporally. Both the 8- and 24- hour desiccation treatments yielded fewer worms per oyster at all sites during the first sampling (October), but only at sites A and B during the second sampling (November/December). It is interesting that a significant treatment effect

was only observed at Site C during the first sampling, indicating temporal or seasonal variability in treatment response at that site. In general, results indicate that desiccation for either 8 or 24 hours weekly can be an effective strategy for limiting worm infestation. Findings confirm previous work on the effectiveness of a weekly 24-hour desiccation interval in controlling worms (Cole et al., 2020; Gamble, 2017), but adds new information regarding the effectiveness of a shorter weekly desiccation interval (8-hours). Findings are also supported by previous work into daily tidal exposures, where Littlewood et al., (1992) found a 40% daily tidal exposure (~5 hours) to be effective in reducing worm infestation. Together, these results are encouraging in pointing to desiccation as an effective strategy to control worm infestation. Given the necessity for regular (weekly) biofouling treatment to avoid worm colonization, the practicality of desiccation may make this a standout fouling control option for farmers concerned with worm infestations. This study assessed the worm species present among a subsample of oysters and found *Alitta succinea*, *Polydora cornuta*, and *Polydora websteri.* Marine worms can be morphologically cryptic and difficult to identify visually, but it should be noted that of the species present, only *P. websteri* is known to bore into oyster shells (Haigler, 1969), resulting in the problematic 'U-shaped' blister within the interior of the shell. However, the presence of *A. succinea* and *P. cornuta* also present issues for commercial oyster growers seeking to sell a product void of any additional biota.

Desiccation treatment effects on oyster growth and survival

In contrast to findings from comparable previous work, major treatment-derived differences in oyster growth and survival were absent in this study. The post-deployment mortality observed at Sites B and C was not correlated with treatment, and is instead believed to have been caused by a deployment issue or environmental conditions at those sites that was

absent from Site A. The lack of clear differences in growth among the treatments must be viewed within the context of the extremely limited growth of oysters in all treatments at all sites. During the period of investigation, average shell height in the control group at Site A actually decreased by 0.5mm, while non-desiccated oysters at Sites B and C grew an average of 0.3 and 1.4mm, respectively. As previously mentioned, salinity conditions were below average during the period of investigation. Salinity is an important factor in oyster physiology and the conditions experienced at all sites were near the lower limit for strong oyster growth (~5-9, Rybovich et al., 2016). It is therefore possible that the stress of the lower salinity environment obfuscated any treatment-derived stresses and contributed prominently to the diminutive growth experienced in oysters in all treatments. If salinity conditions at the oyster farm sites were more favorable to the oysters, treatment effects may have been observed in accordance with other studies in the literature. Low salinity is thought to be a confounding factor to the reduced growth and lack of treatment-derived differences observed in this study. Additionally, the short duration (4 months) of the study may have precluded any treatment-derived growth differences from manifesting in the data. It is possible that differences in growth may have become apparent if the study were conducted over a longer time period.

Environmental conditions may influence results

Temperature and humidity are critical factors in determining the rate of drying, with elevated humidity reducing the rate of water loss (Barati & Esfahani, 2012; Inyang et al., 2018). Although detailed information on the conditions necessary for sufficient desiccation to take place in the field for the purposes of biofouling control is lacking, one can infer that a greater degree of drying may be achieved during dry conditions lacking humidity and rainfall. Indeed, Hopkins et al., (2016) revealed elevated mortality of *Mytilus* sp. and *Ciona savigni* exposed to low humidity

conditions (66-78% relative humidity) during emersion compared to those exposed to high humidity conditions (94-97% relative humidity) during emersion. Further, the effect of temperature was dependent upon humidity. In the present study, relative humidity (RH) tended to be higher during periods of desiccation at Site B compared to the other two sites, especially during second (October – December) period (+ 24-32% RH at site B compared to Sites A and C). While we cannot say for certain that this elevated ambient humidity led to a reduction in the degree of desiccation achieved, it could be a contributing factor to the observed reduction in the effectiveness of the desiccation treatments at that site, particularly when considered within the context of Hopkins et al. (2016) findings of reduced epibiont mortality in more humid conditions. However, additional investigations would be necessary to conclusively make such a claim, especially considering the differences in dominant species at Site B (*U. intestinalis*) vs. Site A (*V. pavida*) and Site C (*B. improvisus*), and the lengthy emersion tolerance of *U. intestinalis* (McAllen, 1999).

Implications for commercial oyster aquaculture

Oyster farmers contend with myriad biotic and abiotic factors that influence their operations. Husbandry techniques must be viewed within the context of the whole farm enterprise, with consideration given to the relative costs and benefits of adopting any new or modified approaches. Biofouling is a major concern for oyster growers and as such, farmers dedicate significant effort to fouling control. However, biofouling is not the only issue present on oyster farms and a suitable management strategy should consider the cost of the application of the process (i.e. added cost of installing new gear, labor associated with flipping cages or otherwise applying the desiccation process) against the benefits (i.e. increased market value of oysters, reduced labor associated with removing biofouling). The results of this study revealed

that desiccating once weekly for either 8 or 24 hours yielded similar biofouling reduction, and flipping cages for either 8 or 24 hours may require the same total input of labor. New information from this study provides oyster farmers with greater flexibility to apply a desiccation interval that integrates with their farm schedule. Further, although not observed within this study, the regularity with which other studies have documented reduced growth among oysters desiccated for 24 hours once weekly suggests that a shorter (perhaps 8-hour) desiccation treatment may be a safer alternative to reduce biofouling while minimizing the potential for treatment-induced oyster stress, although additional investigations are needed since growth differences were absent here. While previous studies have clearly revealed the need for a weekly frequency and this study established that either an 8- or 24-hour desiccation treatment were successful, the weather conditions during the periods of emersion may be important to conferring maximum desiccation. While frequency and interval are important, the relative humidity during emersion is another aspect that farmers may need to consider in identifying days to desiccate. In practice, this can present a challenge as the need for regularity (weekly frequency) may not integrate cleanly with the need to desiccate on days with low relative humidity. The relative importance of desiccation frequency, desiccation interval, and weather during emersion as drivers of biofouling control should be further investigated in order to recommend best practices. Given these considerations and the strong evidence for desiccation as an effective biofouling strategy, farmers must make a business decision regarding whether to adopt this process on their farms. However, results of this study have shown that desiccation for either 8 or 24 hours can be an effective management approach that controls a variety of common biofouling species in the Chesapeake Bay.

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Chapter 3: Impact of desiccation interval on biofouling and oyster performance in water-column cultured *Crassostrea virginica* in the Choptank River, MD

Abstract

The oyster aquaculture industry is growing rapidly and is a key contributor to the increasing value and volume of coastal aquaculture both nationally and internationally. While poised for continued growth, the oyster aquaculture industry is perpetually challenged by biofouling, the agglomeration of marine plants and animals on oysters and cage material, which can reduce the yield and value of oysters and increase labor costs. Desiccation, or periodic emersion, of cages and oysters is routinely used to control biofouling, yet precise information on the optimal duration of desiccation to achieve biofouling control without adding excessive stress to the oysters is lacking. Desiccation causes physiological stress for oysters and reduces growth, so an understanding of the necessary interval of desiccation is needed to optimize this biofouling control strategy. This study examined four desiccation intervals (0-, 4-, 8-, and 24-hours), applied at a weekly frequency, and monitored effects of these treatments on fouling (total epibiont biofouling coverage, individual epibiont species, endobiont worms, cage fouling) and on oyster growth, mortality, and condition index. Oysters were deployed from May through November of 2019 in the Choptank River, Maryland, during which time eight epibiont fouling species were observed. Total biofouling coverage was significantly reduced in all three desiccation treatments (4-, 8-, and 24-hours) compared to the control (0-hour treatment). However, desiccation treatments yielded variable effectiveness in the case of *Victorella pavida* and *Mytilopsis leucophaeata*. Endobiont worm abundances were reduced in all desiccation treatments compared to the control. While biofouling control was achieved with desiccation,

oysters in the 8- and 24-hour treatments grew significantly less than oysters in the 0- and 4-hour treatments. Overall, results suggest desiccating for 4, 8, or 24 hours can reduce fouling, though the diminished oyster growth in the 8- and 24- hour treatments indicates that a shorter (4-hour) treatment may be more optimal.

Introduction

Aquaculture is the fastest growing food production sector in the world (FAO, 2018). Shellfish aquaculture in particular has grown in recent years, comprising 56 percent of global marine and coastal aquaculture in 2018 (FAO, 2020). Production from shellfish aquaculture (primarily from oyster culture) has increased across the United States in recent years, including in Alabama, Maine, Maryland, Massachusetts, and Virginia (GMRI, 2016; Grice & Walton, 2020; Hudson, 2018; Massachusetts Division of Marine Fisheries, 2019; van Senten et al., 2019), and this growth is projected to continue increasing through at least 2030 at rates of 2.1 – 2.3 percent annual change per year (GMRI, 2016). Oyster aquaculture yields a multitude of benefits, contributing a low carbon source of food and providing economic, ecological, and socio-cultural benefits (Dealteris et al., 2004; GMRI, 2016; Hudson, 2018; Kellogg et al., 2018; Marenghi et al., 2009; Michaelis et al., 2020; Naylor et al., 2021; Ray et al., 2019; van Senten et al., 2019). In the US, oyster aquaculture takes two general forms: water-column aquaculture, where oysters are containerized and suspended within the water-column, and submerged-land aquaculture, where oysters are deployed, grown, and harvested directly from suitable substrate. Oysters grown in water-column leases are generally destined for the half-shell (or whole oyster) market (Parker et al., 2020) and command a higher price per oyster (GMRI, 2016; Hudson, 2018; Parker et al., 2020), but also have significantly higher labor costs to produce a clean (non-fouled), well-shaped

product that will be competitive in the increasingly diverse marketplace (Adams et al., 2011; Engle et al., 2021; Parker et al., 2020).

Biofouling, the agglomeration of marine plants and animals on cages and the shellfish themselves (Fitridge et al., 2012), presents a challenge to both market and non-market aspects of water-column oyster aquaculture. The presence of biofouling on the external valves can directly impact the market value of the shellfish (Campbell & Kelly, 2002) and indirectly, biofouling increases production costs for aquaculture operations (Adams et al., 2011). Non-market effects of biofouling are also significant and include negative impacts on the physiology of the oysters (Carroll et al., 2015; Stefaniak et al., 2005; Wargo & Ford, 1993), compromised farm infrastructure (Ramsay et al., 2008), and sub-optimal growing conditions for the crop (Gormican, 1989). Whether through direct competition for resources or a divestment of energy from somatic growth to maintenance and repair, biofouling can reduce the growth and/or meat weight of cultured oysters (Carroll et al., 2015; Claereboudt et al., 1994; Daigle & Herbinger, 2009; Fitridge & Keough, 2013; Lodeiros & Himmelman, 1996; Sievers et al., 2013) and in the case of endobionts (i.e. sponges, worms), may contribute to more brittle, fragile shells (Carroll et al., 2015). The excavated tunnels from sponges and worms increase susceptibility of oysters to predation (Buschbaum et al., 2007) and reduce market demand for the final product, evidenced by reports from shellfish buyers that shell integrity influences which oysters they purchase (GMRI, 2016; Hensey, 2020). In summary, the biotic and abiotic effects of biofouling compromise the operational efficiency and yield of commercial oyster farms through reduced oyster growth, elevated operational costs, and reduced marketability.

Given the substantial challenges biofouling poses to commercial water-column oyster aquaculture operations, considerable research has been undertaken regarding biofouling control

or mitigation strategies (reviewed in Bannister et al., 2019; Fitridge et al., 2012), and farmers employ a range of techniques to control biofouling. According to Adams et al. (2011), scrubbing shellfish to physically remove biofoulers was the most commonly applied fouling control method across the US east and west coast states surveyed, followed by gear cycling, where fouled gear is removed and replaced with clean gear. Such physical methods are labor intensive, but facilitate reliable removal of fouling organisms. Desiccation and chemical dips were tied for the next (3nd) most commonly applied fouling control methods in the areas surveyed (Adams et al., 2011). Chemical dips involve the immersion of oysters and cage material into solutions that harm or kill biofouling (e.g. acidic, alkaline, freshwater, or brine exposure), but effectiveness of this method varies over space, time, and among fouling species (e.g. Carver et al., 2010; Denny, 2008; Morse et al., 2015; Piola et al., 2010; Sharp et al., 2006) and disposal of chemical treatments may also be a challenge (Morse et al., 2015). Desiccation refers to the periodic exposure of cage materials and oysters to the air to facilitate drying that can stress or kill fouling species (Creswell & McNevin, 2009; Piola et al., 2010). Desiccation has the added benefit of being deployable in a passive manner, via tidal emersion (i.e. intertidal rack and bag, floating bag, and long-line cages; Littlewood et al., 1992; Walton et al., 2012) or in an active fashion via flipping of cages to expose oysters and biofoulers (e.g. flippable floating cages; Davis et al., 2013). The effectiveness of biofouling treatments can vary among farms or at the same farm over time, due to changing environmental conditions and variability in the biofouling communities over space and time (Littlewood et al., 1992; Marques-Silva et al., 2006; Sá et al., 2007; Sievers et al., 2014; Thomsen et al., 2007; Underwood & Anderson, 1994). Thus, it is difficult to employ a single biofouling mitigation strategy with broad-scale effectiveness, but weekly desiccation has yielded

largely consistent results across multiple sites (Chapman, 2019; Gamble, 2017; Kirk, 2019; Mallet et al., 2009).

Despite the general challenges of effective biofouling control in oyster aquaculture, desiccation holds great promise as a biofouling mitigation strategy that lends itself to commercial application and is seeing increasingly widespread use (Adams et al., 2011; Creswell & McNevin, 2009; Piola et al., 2010). In recent years, flippable floating cages (FFC) and adjustable longline systems (ALS) have entered the market as off-the-shelf growout methods, both of which facilitate regular desiccation (Davis et al., 2013; Walton et al., 2012). The desiccation process is applied by inverting FFC systems, or through raising tensioned lines of oyster cages (ALS) to a fixed position above the mean higher high water (MHHW) line, at a frequency (how often) and interval (duration of each exposure) selected by the farmer. The selection of an appropriate frequency and interval is an area of active research and interest to both the academic and oyster farming communities. Recent studies have demonstrated reduced biofouling on oysters and cages when desiccation for 24 consecutive hours was applied weekly, compared to bi-weekly, monthly, or seasonally (Chapman, 2019; Gamble, 2017; Kirk, 2019; Mallet et al., 2009). However, the necessary exposure interval or duration during weekly desiccation has received less attention to date. Weekly desiccation serves to interrupt the settlement, metamorphosis and/or early sessile life stages of biofouling species, forcing a prolonged period of emersion within 6 days of settlement. Although many biofouling species have adapted to life in the intertidal, the prolonged exposure imposed by desiccation can extend beyond a standard low tide cycle, particularly in the Chesapeake Bay region and others with limited tidal ranges (Hill, 2016). The length of time spent out of the water (desiccation interval) can also affect oyster productivity, as extended emersion can be stressful to oysters and reduce opportunities for feeding (Shumway, 1982;

Suchanek, 1978). Consistent with this expectation, reduced growth (termed a 'growth penalty') has been observed in oysters desiccated for 24-hours weekly compared to less frequent desiccation (Chapman, 2019; Gamble, 2017; Kirk, 2019), though weekly desiccation yielded the greatest and most consistent reduction in biofouling. While oyster farmers typically value non-fouled oysters, they must balance biofouling control with farm yield and efficiency, so optimization of desiccation interval is an important area of shellfish aquaculture research (Sá et al., 2007; Sievers et al., 2017).

This study investigated the response of biofouling organisms and oysters to four desiccation intervals (0-, 4-, 8-, and 24-hours), applied weekly on an experimental hoist system (Figure 1) to ensure consistent application of each interval that operated independently of tides. Biofouling community composition and relative abundance were monitored across each of these treatments, as well as impacts to oyster bio-physiological responses (growth, mortality, condition index) and farm operation (biofouling mesh occlusion and weight on cages). We hypothesized that oysters in all desiccation treatments (4-, 8-, and 24-hours) would have reduced biofouling coverage compared to the control, but that oysters in the 24-hour treatment would grow less than those desiccated for shorter weekly intervals. Results show that desiccation for 4, 8, or 24 hours per week yielded reduced biofouling coverage and worm abundance compared to the non-desiccated oysters (0-hour treatment). However, this fouling reduction came at a cost for oysters in the 8- and 24-hour treatments, which suffered a growth penalty compared to the 0- and 4-hour treatments. Overall, the results of this study shed light on the potential effectiveness of a shorter desiccation interval to control biofouling and diminish negative tradeoffs associated with desiccation as a husbandry technique.

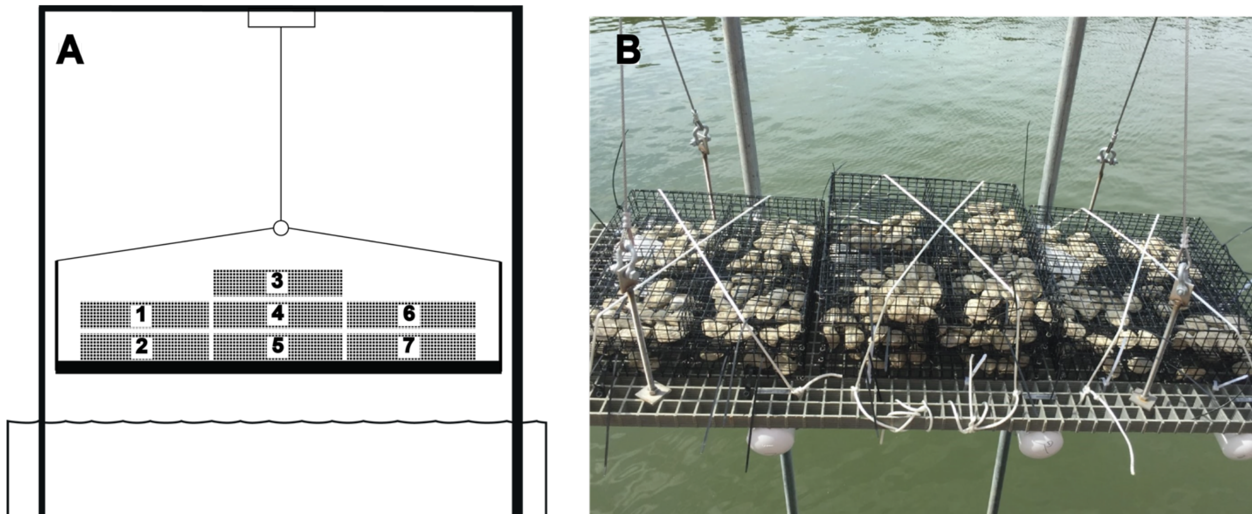

Figure 16. Desiccation hoist diagram (A, left) and operational unit (B, right), both shown in desiccating position at the study site in the Choptank River, MD. Each hoist held 7 cages, each cage consisting of 60 adult oysters. Cage positions were labeled and remained consistent. Winches provided lift capacity, lifting each apparatus above MHHW during desiccation. Cages 1, 2, 4, 5, 6, and 7 were sampled without replacement for biofouling (epibionts and endobiont worms), while cage 3 was used to track growth and mortality over time.

Methods

Site description

Experiments took place on the northwest side of a concrete pier located at the University of Maryland Center for Environmental Science Horn Point Laboratory in the Choptank River, MD, USA (38° 35' 35.556" N, 76° 7' 43.932" W, Figure 2). The site was located approximately 10 nautical miles from the confluence of the Choptank River with the Chesapeake Bay mainstem. Mean tidal range at the site is 0.5 meters (NOAA, 2022) and the 30-year mean surface salinity is 9.5; 95% CI 9.3-9.7 (Maryland Department of Natural Resources, 2021).

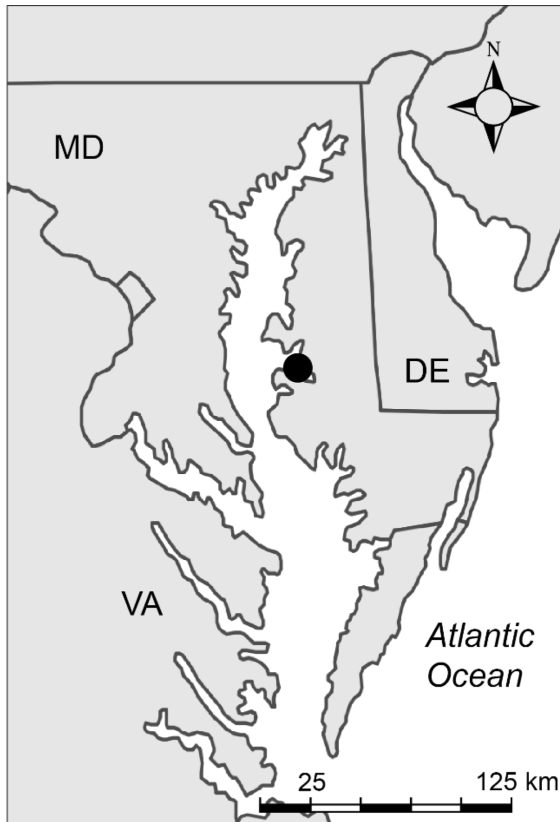

Figure 17. Location of investigations in the Choptank River, a tidal tributary of Chesapeake Bay. The study location is indicated by the black circle.

Environmental monitoring

Daily measurements of salinity and temperature were recorded using a YSI Pro 1030 handheld meter (YSI, Inc./Xylem Inc., Yellow Springs, OH, USA). See Appendix C for in-water salinity and temperature. Weather conditions during the study period were accessed using NOAA National Centers for Environmental Information Local Climatological Data (https://www.ncdc.noaa.gov/cdo-web/datatools/lcd; Station ID: WBAN:00356). Weather data are recorded every 20 minutes. Minimum, maximum and mean temperature and relative humidity during each weekly desiccation period were computed for the 24-hour treatment since

all treatments were desiccated within the 24-hour treatment's exposure window. See Appendix D for full weather conditions during emersion, including when each desiccation treatment was exposed.

Hoist construction

Four mechanized hoists were constructed (Figure 1), each consisting of a 1.8m x 0.6m Fibergrate® platform (35mm mesh) with capped polyvinyl chloride on the underside and supported by an aluminum track and steel I-beam to allow consistent application of four weekly desiccation treatments. During submerged periods, all hoists were held at the same height above the seafloor (15 centimeters above the seafloor). Each hoist held seven cages measuring 51cm x 51cm x 8cm each, and constructed of 25mm polyvinyl chloride (PVC) coated wire mesh. Each cage was affixed with four redundant squares of vinyl coated wire mesh material, aligned with the primary cage so as to not impede flow, but to allow the redundant squares to be conveniently removed to monitor cage fouling. A 12-volt battery-powered winch (WARN 12Volt DC Powered Electric Utility Winch - 2000 lb. capacity, model #92000) provided lifting power for each hoist. A control hoist experienced no desiccation (0-hour treatment) and the other hoists were desiccated for four (4-hour treatment), eight (8-hour treatment), and twenty-four (24-hour treatment) consecutive hours each week. During periods of desiccation, oysters and cage material were elevated above the mean higher high water (MHHW) line, ensuring continuous emersion during the assigned desiccation interval.

Oyster preparation and deployment

Three-year old hatchery-reared diploid oysters (*Crassostrea virginica*) were sourced from the UMCES Horn Point Laboratory Demonstration Oyster Farm in the Choptank River, MD.

Extant biofouling was removed to ensure the initial deployment was comprised of clean oysters. Epibionts were dislodged by scrubbing oysters and physically removing organisms from the valves' exterior. Oysters were then placed in a 30-minute brine dip (freshwater + Crystal Sea® Marinemix salted to 60) followed by a 24-hour desiccation under ambient lab conditions (temperature 27°C) to kill endobionts living within the oyster valves (Hooper, 2001; Morse et al., 2015). Sixty (60) adult diploid oysters were deployed to each of seven cages in a given treatment (mean ± SD shell height at deployment = 62 ± 7mm, total experiment n=1,680). Within each treatment, one cage was used to measure oyster shell height and mortality throughout the experiment (sampled with replacement; Cage 3; Figure 1), and the six other cages (six technical replicates) were sampled without replacement to monitor biofouling colonization and growth over time (Cages 1, 2, 4, 5, 6, 7; Figure 1). Oysters were deployed between May – November 2019.

Biofouling assessment

For the epibiont assessment each month, eighteen oysters per treatment (18 oysters per treatment x 4 treatments x 6 samplings; total experiment n=432) were randomly selected (three oysters each from six of seven cages) and rinsed gently to dislodge mobile organisms and sediment/detritus, allowing only attached organisms to remain on the valves. Cage position of sampled oysters was noted. Whole oysters were imaged (both valves) then held in filtered (100µm) ambient seawater to retain existing fouling while fouling quantification took place. Using ImageJ image analysis software (v. 1.8; National Institute of Health, Bethesda, MD, USA), the total surface area of each oyster was measured along with the surface area covered by individual biofouling species (Carroll et al., 2015; Newell et al., 2007). Fouling organisms were identified to the lowest taxonomic level possible (genus and species) using a stereo microscope

(Olympus SZX16) and visual taxonomic identification guides (Gosner & Peterson, 1982; Lippson & Lippson, 2006; Smithsonian Environmental Research Center's National Estuarine and Marine Exotic Species Information System). Percent coverage per species was calculated as

$$\frac{surface\ area\ of\ each\ species\ i\ (mm^2)}{total\ surface\ area\ of\ the\ valve\ (mm^2)} * 100$$

Total coverage was calculated by summing the percent coverage of all species present on a given oyster valve.

To assess endobiont fouling each month, 18 oysters per treatment (18 oysters per treatment x 4 treatments x 6 samplings; total experiment n=432) were rinsed to dislodge mobile organisms and sediment/detritus before being placed into individual 16oz glass jars containing 5% ethanol, 95% artificial seawater (deionized water + Crystal Sea® Marinemix salted to a salinity of 13) for 24 hours (modified from Morse et al., 2015). Note that these were different oysters sampled on staggered weeks from the oysters assessed for epibiont coverage. Expelled worms were enumerated and discarded.

Biofouling on cages from each treatment was assessed once (September) via quantification of mesh occlusion and weight of fouling on cages (Mallet et al., 2009; Taylor et al., 1997). Redundant cage material was sampled from three cages per treatment. Cage material was photographed and mesh occlusion was calculated using ImageJ. A clean (unfouled) piece of cage material served as a reference to compare the reduction in open pore space among deployed cages. Mesh occlusion (% obstructed) was calculated as

$$100 - \left(\left(\frac{open\ space\ in\ fouled\ mesh\ (mm^2)}{open\ space\ in\ clean\ mesh\ (mm^2)} \right) * 100 \right)$$

After imaging, each fouled piece of cage material was dried in a drying oven at 50°C for 24 hours (Taylor et al., 1997), and weighed using an Ohaus Discovery analytical electronic balance

(Model DV114C, Ohaus Corporation, Parsippany, NJ, USA). Dry weight (g) of cage fouling was calculated as

$$dry\ weight\ of\ fouled\ mesh\ (g) - dry\ weight\ of\ clean\ mesh\ (g)$$

Oyster evaluation

Shell height of all oysters (60 oysters per cage x 7 cages per treatment x 4 treatments; n=1,680), measured using Vernier calipers as the maximum distance between the umbo and the ventral valve margin (Figure 3; Galtsoff, 1964), was assessed prior to deployment and at the termination of the study. Shell height of all oysters in each treatment's non-sampled cage (cage 3; n=240) was measured monthly. Mortality in each treatment's non-sampled cage (cage 3) was recorded monthly by visually inspecting and tapping oysters to assess their survival status. Dead oysters were removed. Condition index is a metric used to assess oyster meat yield and was measured at the termination of the study period according to Lawrence & Scott (1982). Twenty (20) oysters per treatment, sampled from six of the seven cages (n=80) were scrubbed free of fouling organisms and rinsed, then allowed to dry under ambient laboratory conditions for 45-60 minutes. Whole oysters were weighed using an Ohaus Discovery analytical electronic balance (Model DV114C). Oysters were shucked and valves allowed to dry under ambient laboratory conditions for 60 minutes, then weighed. Oyster meats were dried in a drying oven at 68°C for 48 hours, then weighed (g). Internal cavity volumes were calculated by subtracting the weight of valves from the weight of the intact oyster as the density of cavity contents has been measured as 1g per cm^3 (Lawrence & Scott, 1982). Condition index was calculated according to Hopkins' formula

$$\frac{(dry\ meat\ weight\ (g) * 100)}{internal\ cavity\ volume\ (cm^3)}$$

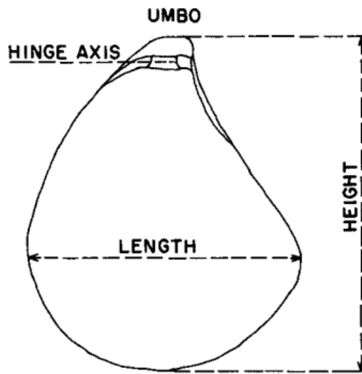

Figure 3. Shell morphology of *C. virginica*, noting the measurement of shell height (umbo to ventral valve margin). From Galtsoff, 1964.

Data analysis

All statistical analyses were conducted using RStudio (RStudio Team, 2016). A non-parametric, two-way factorial test (Scheirer-Ray-Hare) from package 'rcompanion' (Mangiafico, 2022) was used to assess independent and interactive effects of desiccation treatment and sampling date on total epibiont coverage, as the data violated the normality assumption of a two-way analysis of variance (ANOVA). Significant effects were subsequently investigated for each sampling date using the Dunn (1964) multiple comparison test from the 'FSA' package (Ogle et al., 2018) and a Holm adjustment was used to correct for multiple comparisons. The non-parametric one-way factorial Kruskal-Wallis test from the 'stats' package (R Core Team, 2017) used to assess effects of cage position on total epibiont coverage because the data were not normally distributed. A Kruskal-Wallis test ('stats' package; R Core Team, 2017) was also used to assess effects of desiccation treatment on coverage by individual species, again because these data violated the normal distribution assumption of an ANOVA. Significant interactions among desiccation treatment and coverage by individual species on each sampling date were further investigated using a Dunn (1964) multiple comparison test from the 'FSA' package (Ogle et al.,

2018) and a Holm adjustment was used to correct for multiple comparisons. A Kruskal-Wallis test ('stats' package; R Core Team, 2017) was also used to assess desiccation treatment effects on worm abundances in oysters. Significant interactions were further investigated using the Dunn (1964) multiple comparison test (Holm adjustment, 'FSA' package; Ogle et al., 2018). A one-way ANOVA from the 'rstatix' package (Kassambara, 2021) was used to investigate treatment effects on cage fouling, as well as treatment effects on oyster growth. Significant interactions among desiccation treatment and oyster growth were further investigated using Tukey's Honest Significant Difference (HSD) Tests. A one-way ANOVA ('rstatix' package; Kassambara, 2021) was also used to assess effects of desiccation interval on dry meat weight of oysters and differences were further investigated using Tukey's Honest Significant Difference (HSD) Tests. Effects of desiccation interval on condition index of oysters were investigated using a Kruskal-Wallis Test ('stats' package; R Core Team, 2017), given that data were not normally distributed. A two-dimensional density estimation plot was created to synthesize the number of worms per oyster and epibiont coverage among the desiccation treatments and results are presented as contour lines with high density contours indicating low variance and low density contours indicating higher variance. Graphic visualizations of the data were carried out using 'ggplot2' (Wickham, 2016).

Results

Environmental conditions

The early period of study (May through July) was characterized by abnormally low salinity, resulting from record rainfall and riverine discharge in 2018 (Tarnowski, 2019). Surface salinity was lowest in the early months of study, with a minimum salinity of 5.5 in May and mean of 6.1 between May and June. Salinity increased gradually in July, reaching 7.5 in late

July, then subsequently increased to a high of 14.4 in November (Figure 4). Surface salinity at the study site was below the monthly 20-year long term average until September (MD DNR, 2021). Surface temperatures during the experiment were near 20-year long term averages, which began at 21°C during the May deployment, then rose to a maximum of 30°C in July before it subsequently fell to a low of 8°C in November (Figure 4).

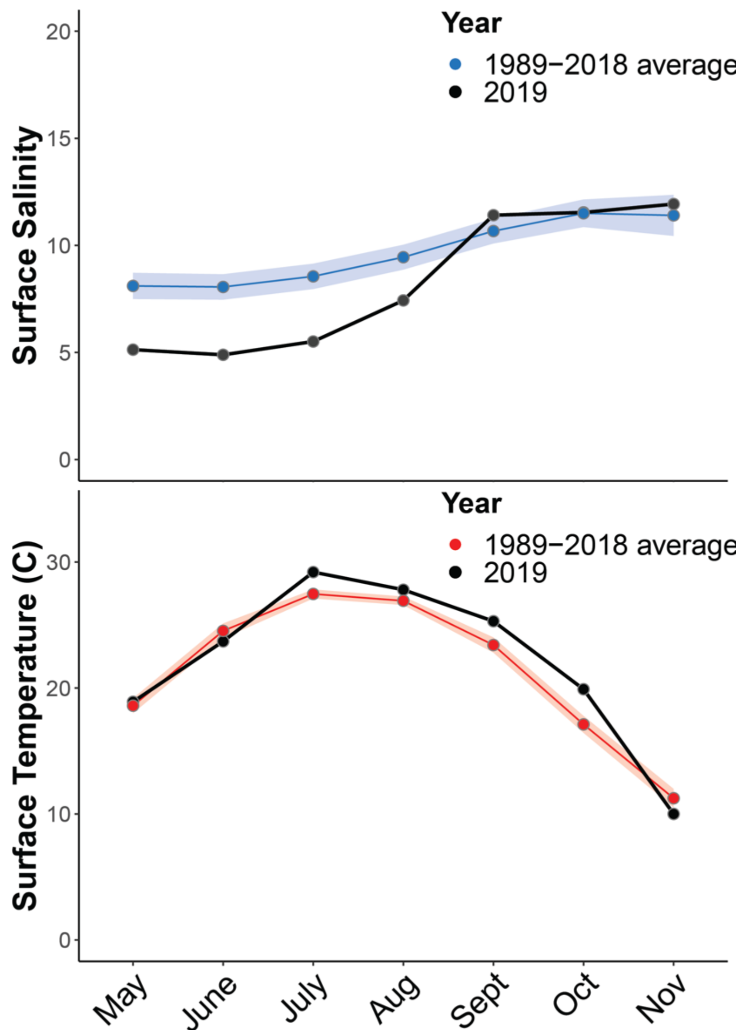

Figure 18. Surface salinity (top) and temperature in degrees Celsius (bottom) of the Choptank River at the study site. Thirty-year averages (1989 – 2018, +/- CI) are presented in the blue

(salinity, top) and red (temperature, bottom) ribbons. 2019 conditions are represented by the black lines.

Mean ambient air temperature during periods of emersion was 22°C. During the May desiccation treatments, temperatures averaged 29°C, then gradually increased to a maximum exposure temperature of 36°C in August before emersion temperature decreased to a low of 4°C in November (Figure 5). Relative humidity during periods of oyster emersion reached nearly 100% on 20 of the 26 weeks when desiccation took place. Relative humidity generally began falling after September, reaching a minimum of 27% in early November (Figure 5).

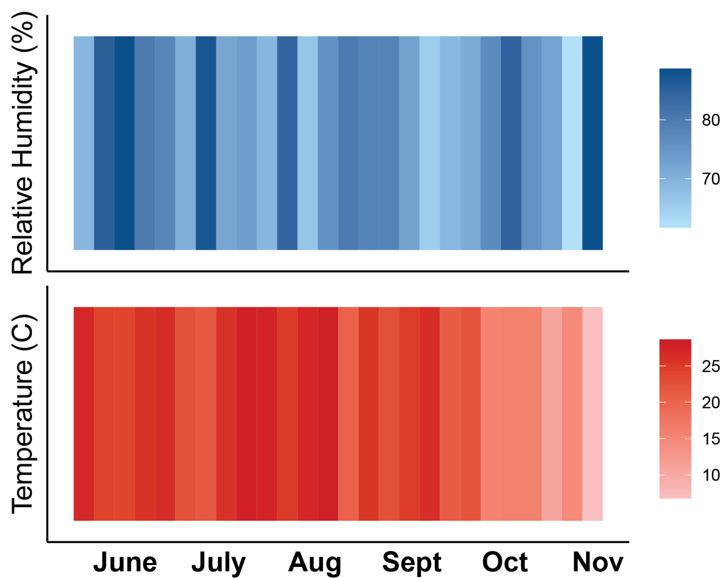

Figure 19. Mean ambient air conditions during periods of desiccation (24-hour treatment means) from May through November 2019. Relative humidity data (%) are presented on the top heatmap, with light blue indicating lower relative humidity and darker blue indicating higher relative humidity. Ambient air temperature data (C) are presented on the bottom heatmap, with light red colors indicating lower temperatures and darker red colors indicating higher temperatures.

Gross cumulative biofouling and species-level treatment effects

Biofouling community composition and relative abundance of individual species varied over the period of investigation (July through November) and total biofouling coverage peaked in late July (Figure 6). Eight sessile, attached fouling species were observed: bay barnacle (*Balanus improvisus),* lacy crust bryozoan *(Conopeum tenuissimum),* rope grass *(Garveia franciscana),* hooked mussel *(Ischadium recurvum),* coffin box bryozoan (*Membranipora tenuis),* false mussel *(Mytilopsis leucophaeata),* gutweed *(Ulva intestinalis),* and cushion moss bryozoan (*Victorella pavida)*. Biofouling coverage increased in the early part of the season to an initial peak in late-July (dominated by *V. pavida*), followed by a slight reduction in coverage until a second peak in October (dominated by *C. tenuissimum* and *M. tenuis*). The first colonizers were *B. improvisus, C. tenuissimum, M. tenuis, G. franciscana,* and *U. intestinalis*. In late July, *V. pavida* grew to be the dominant species (highest percent cover) and overset previously attached species, leading to their apparent absence in the data due to the two-dimensional limitation imposed by the epibiont quantification protocol. Due to similarities in morphology, occurrence data for *Conopeum tenuissimum* and *Membranipora tenuis* were aggregated. *Ischadium recurvum* recruited in such low abundances (N = 3 over the experiment) that it was removed from statistical analyses.

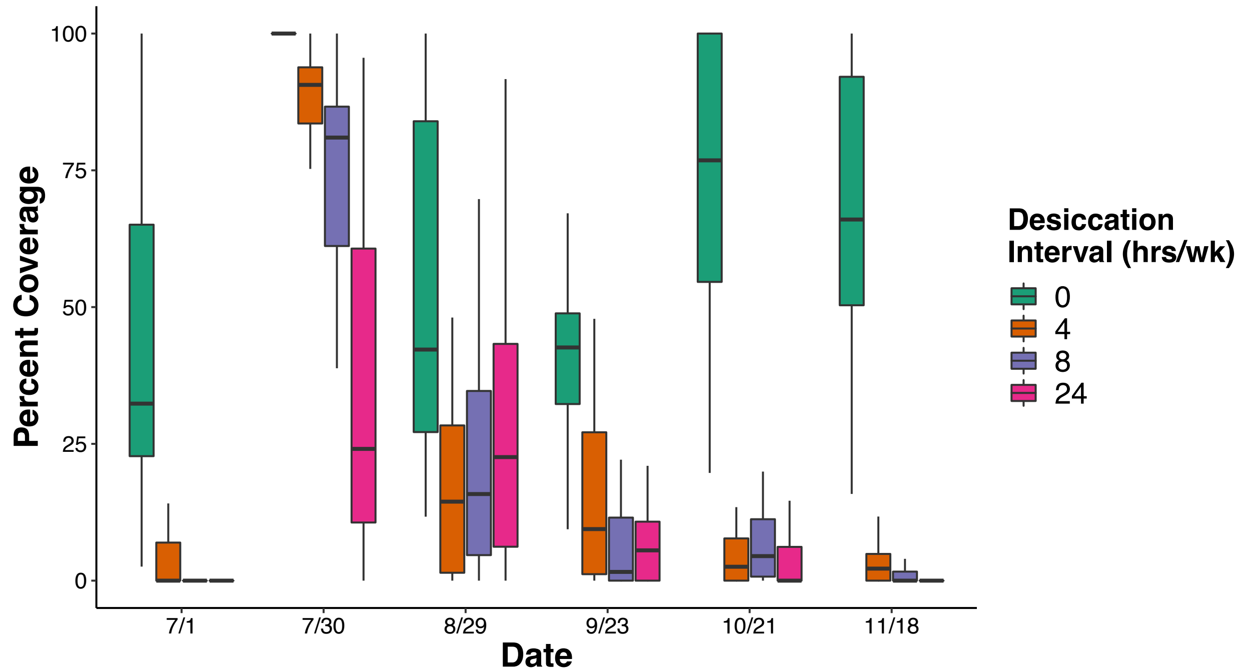

Figure 20. Box-and-whisker plot showing total biofouling coverage (%) of *C. virginica* in four desiccation treatments between July and November 2018. The green boxes represent non-desiccated oysters (0 hours per week), orange boxes represent oysters desiccated for 4 hours per week, purple boxes represent oysters desiccated for 8 hours per week, and pink boxes represent oysters desiccated for 24 hours per week. The thick line within each box represents the median, the filled boxes represent the interquartile range, and vertical lines extend to the minimum and maximum values.

Mean (total experiment) epibiont coverage of oysters in the 0-, 4-, 8-, and 24-hour desiccation treatments was 64.3, 23.7, 18.4 and 12.0 percent, respectively. Epibiont coverage varied significantly among the desiccation treatments and across time points, and there was a significant interaction between time and desiccation treatment (Scheirer-Ray-Hare test $P < 0.0001$ for the two factors and their interaction). Post-hoc pairwise Dunn tests revealed a consistent reduction of total epibiont coverage for all desiccation treatments (4-, 8-, and 24-hour)

compared to the subtidal control group (all adjusted P-values < 0.001, Table 1). On three sampling dates (8/29, 9/23, and 10/21), no differences were observed among and 4-, 8-, and 24-hour treatments, indicating epibiont fouling reduction was similar regardless of the interval of exposure (Figure 6, Table 1). When significant differences in coverage between the 4-, 8-, and 24-hour treatments were present (7/1, 7/30, 11/18), fouling coverage was inversely related to desiccation interval. The relative cage positioning of technical replicates had no effect on epibiont coverage (Kruskal-Wallis P = 0.11).

Treatment effects with respect to individual epibiont species were analyzed independently and were significant for each species (all Kruskal-Wallis P-values < 0.01). *Conopeum tenuissimum, M. tenuis, B. improvisus, G. franciscana,* and *U. intestinalis* were early colonizers and were established by the first sampling in early July (Figure 6). The colonial bryozoan *V. pavida* began colonizing in late July and overset some organisms which had previously become established, masking their presence until *V. pavida* began to recede in late summer/early fall (Figure 7). Desiccation treatment effects on individual epibiont species varied seasonally. Relative to each sampling date, all desiccation treatments aside from the control consistently reduced coverage of *B. improvisus, C. tenuissimum, M. tenuis, G. franciscana,* and *U. intestinalis*, as evidenced by post-hoc pairwise Dunn tests (adjusted P-values < 0.05). Treatment effects varied temporally on coverage of M. *leucophaeata* and *V. pavida* (Figure 6). Only the 8- and 24-hour desiccation treatments consistently reduced coverage by *M. leucophaeata* (Dunn test adjusted P-values < 0.05). Treatment effects were inconsistent for *V. pavida*, with all treatments yielding significantly reduced coverage during some, but not all sampling periods. No differences in *V. pavida* coverage were observed among treatments in the August and October samplings (Dunn test adjusted P-values > 0.05), while significant

differences (Dunn test adjusted P-values < 0.05) between the 0-hour control treatment and other desiccation treatments were observed in July (4-, 8-, and 24-hour treatments), September (8- and 24-hour treatments), and November (24-hour treatment).

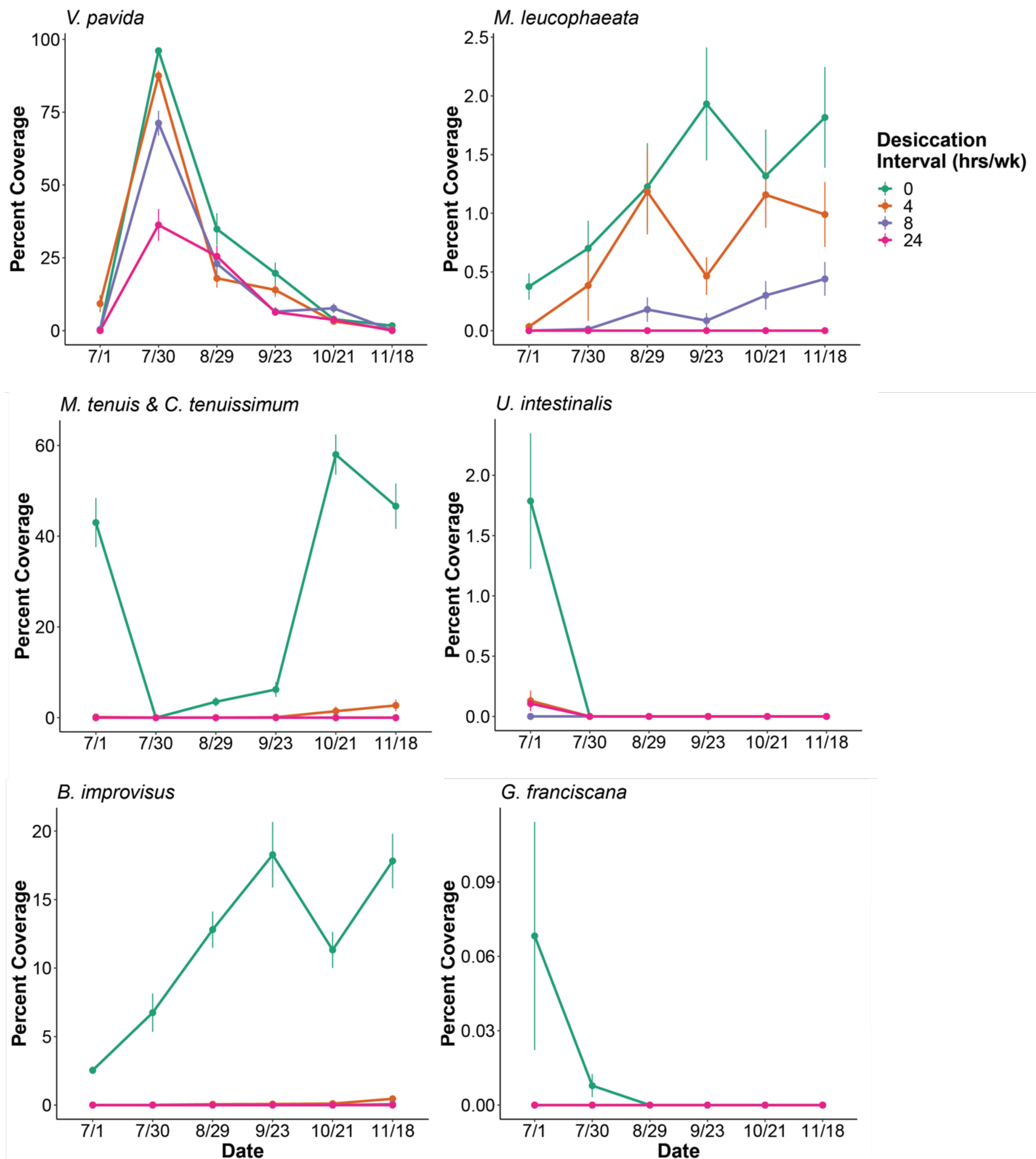

Figure 7. Mean total coverage (%, ± SEM) of individual biofouling species on *C. virginica* oysters in four weekly desiccation treatment between July and November 2019. The green line represents coverage on non-desiccated oysters (0 hours per week). Orange lines represent coverage on oysters desiccated for 4 hours per week, purple lines represent coverage on oysters desiccated for 8 hours per week, and pink lines represent coverage on oysters desiccated for 24 hours per week. Points indicate each species' group mean for the given sampling date and error bars represent one standard error of the mean. Note that the Y-axis for each plot is on a different scale.

Table 1. Results of post-hoc Dunn Tests (P-values corrected using a Holm adjustment) after Kruskal-Wallis test, on individual desiccation treatment effects on gross biofouling coverage among *C. virginica* oysters desiccated for 0, 4, 8, and 24 hours per week in the Choptank River, MD during 2019. Significant interactions (Dunn test adjusted $P < 0.05$) are noted in bold text.

	7/1/19	7/30/19	8/29/19	9/23/19	10/21/19	11/18/19
0 : 4	**8.11e-08**	**3.66e-04**	**4.16e-06**	**5.12e-07**	**7.98e-13**	**2.57e-08**
0 : 8	**5.19e-18**	**7.67e-10**	**1.18e-04**	**5.13e-13**	**4.16e-10**	**2.59e-15**
0 : 24	**3.82e-17**	**1.44e-18**	**6.55e-04**	**4.21e-12**	**2.17e-15**	**2.07e-21**
4 : 8	**3.57e-03**	**0.01**	0.92	0.09	0.72	**0.04**
4 : 24	**5.48e-03**	**1.04e-06**	0.70	0.12	0.44	**3.12e-04**
8 : 24	0.81	**0.02**	0.65	0.76	0.27	0.11

Endobiont worms

Worm abundance per oyster increased during spring and early summer, then peaked in August and gradually declined through November (Figure 8). Mean (± SD) peak worm abundances (August), were 161.8 (± 44), 101.5 (± 42), 92.8 (± 55), and 24.3 (± 16) worms per oyster in the 0-, 4-, 8-, and 24-hour treatments, respectively. Worm abundances were affected by desiccation treatment (Kruskal-Wallis $P < 0.05$). When data for the entire season were analyzed, all desiccation treatments yielded a reduction in worm infestation compared to the control (Figure 8; Dunn test adjusted P-values < 0.0001). When each sampling date was analyzed independently, the 24-hour treatment showed consistent significant reductions in worm abundances compared to the 0-hour treatment at all time points (Dunn test adjusted P-values < 0.0001), while the 8-hr treatment yielded reduced worm abundances at 5 of the 6 sampling times, and the 4-hour treatment reduced worm abundances on 3 of the 6 sampling times (Table 2). The two-dimensional density estimation plot in Figure 9 presents synthesis data on worm abundances and epibiont coverage of oysters in each of the desiccation treatments. The 24-hour treatment yielded the most consistent results, evidenced by the clustering of contour lines, in contrast to other treatments which yielded comparably greater variability.

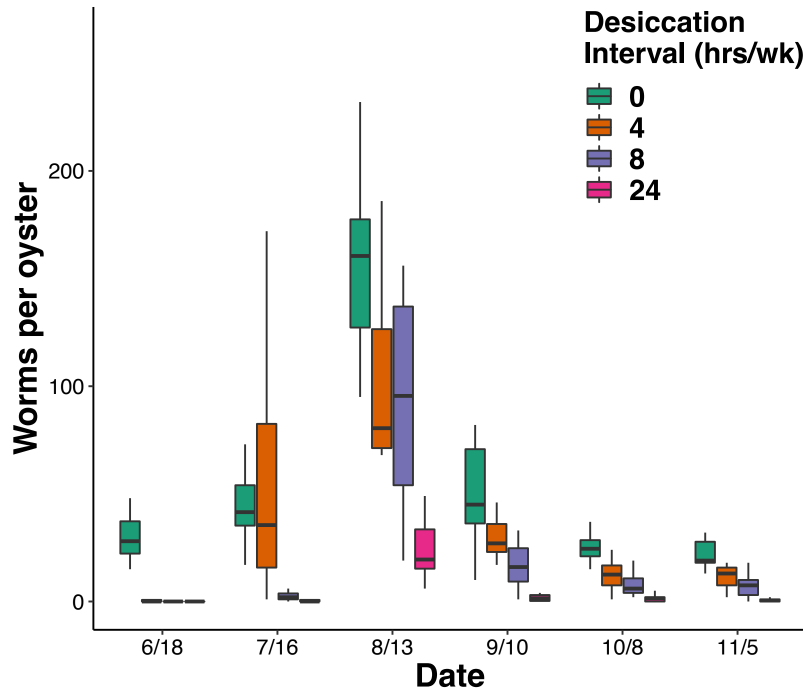

Figure 8. Boxplot showing the number of endobiont worms per oyster in oysters from four desiccation intervals in the Choptank River between June and November 2019. Green boxes represent the number of worms in oysters desiccated for 0 hours per week, orange boxes represent the number of worms in oysters desiccated for 4 hours per week, purple boxes indicate the number of worms in oysters desiccated for 8 hours per week, and pink boxes indicate the number of worms in oysters desiccated for 24 hours per week. The thick line within the colored portion of each box represents the median, the filled boxes encompass the interquartile range, and protruding longitudinal lines represent the minimum and maximum values.

Table 6. Output of post-hoc Dunn test performed after Kruskal-Wallis tests indicated significant differences in worm abundances among oysters desiccated for 0, 4, 8, or 24 hours once weekly in the Choptank River, MD in 2019. Values are adjusted P-values from the Dunn (1964) multiple

comparison test corrected with the Holm adjusted to account for multiple comparisons. Significant relationships (Dunn test adjusted P < 0.05) are noted in bold text.

	6/18/19	7/16/19	8/13/19	9/10/19	10/8/19	11/5/19
0 : 4	**3.15e-07**	0.72	0.09	0.12	**0.01**	**0.04**
0 : 8	**1.29e-10**	**6.64e-05**	0.08	**3.96e-04**	**5.36e-05**	**3.10e-04**
0 : 24	**1.54e-10**	**3.97e-09**	**4.14e-05**	**1.58e-10**	**1.04e-11**	**4.05e-11**
4 : 8	0.39	**3.29e-04**	0.76	**0.04**	0.14	0.11
4 : 24	0.58	**3.05e-08**	0.06	**1.59e-06**	**1.50e-04**	**3.35e-05**
8 : 24	1	0.12	0.15	**0.02**	**0.02**	**0.01**

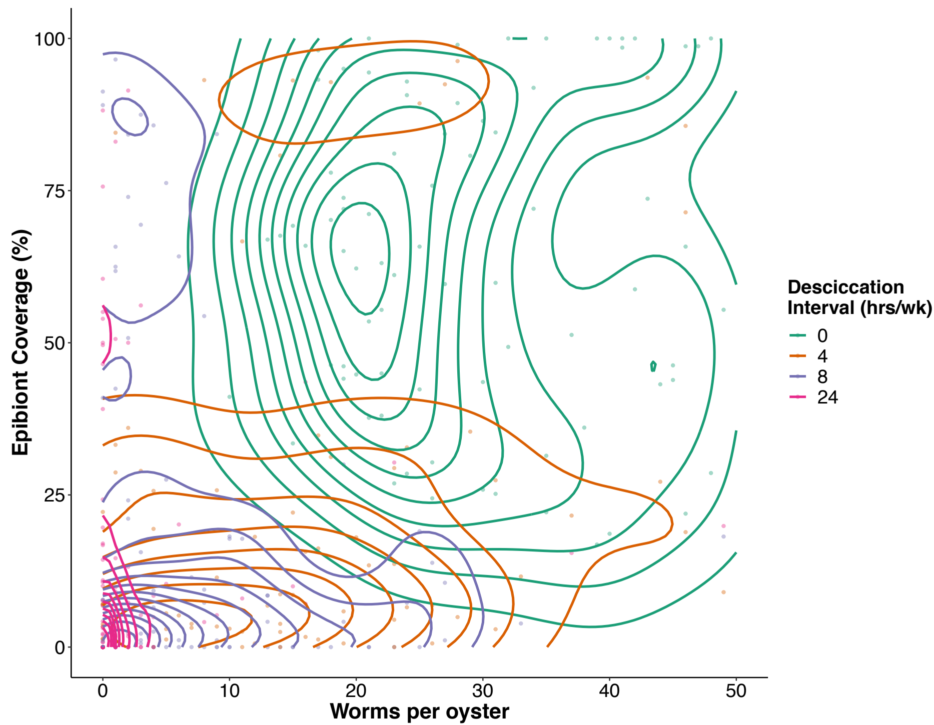

Figure 9. Two-dimensional density plot showing relationship between the total epibiont coverage (y-axis) and number of worms per oyster (x-axis) in oysters from four desiccation treatments. Spacing of contour lines indicates density of points in that particular range, where lines that are close together indicate many data points and lines farther apart indicate fewer data points. Green lines represent the 0-hour treatment, orange lines represent the 4-hour treatment, purple lines represent the 8-hour treatment, and pink lines represent the 24-hour treatment.

Cage fouling

In contrast to the trends for epibiont coverage on oyster valves, fouling on the cages was not affected by desiccation treatment. ANOVA results revealed no differences in mesh occlusion among oysters in the four desiccation treatments (ANOVA P = 0.54, Table 3). Cage positioning also had no effect on mesh occlusion (ANOVA P = 0.73). Similarly, dry weight of fouling on cages was not affected by desiccation treatment (ANOVA P = 0.82, Table 3), and cage positioning had no effect on the dry weight of fouling on the cages (ANOVA P = 0.77).

Table 3. Biofouling on cages in four desiccation treatments (0, 4, 8, and 24 hours of desiccation per week). Cage fouling was measured as percent mesh occlusion (SEM) and dry weight of cage fouling in grams (SEM) in September 2019.

Desiccation Treatment	Mesh occlusion (%) (SE)	Fouling dry weight (g) (SE)
0	29.1 (9.1)	1.9 (0.7)
4	29.9 (4.9)	1.4 (0.3)
8	36.9 (9.7)	2.4 (0.8)
24	20.2 (6.6)	2.0 (0.6)

Oyster performance

Oyster growth was impacted by desiccation treatment and growth rates varied temporally and among the desiccation treatments. Oyster growth was affected by desiccation treatment (ANOVA $P < 0.0001$) and was reduced in the 8- and 24-hour treatments compared to the 0- and 4-hour treatments (Figure 10). During the experiment (May – November), mean total growth was 16.7, 17.1, 12.0, and 7.3 mm in the 0-, 4-, 8-, and 24-hour treatment groups, respectively and oyster growth rates during this period were 0.6, 0.7, 0.5, and 0.3mm/week in the 0, 4, 8, and 24-hour treatments, respectively. Growth rates were lower in the early period of study (May through July) than in the August through November time period. Growth rate of oysters in the 0-hour treatment was 0.3mm/week between May and July, and growth rates in the 4-, 8-, and 24-hour treatments were 0.5, 0.3, and 0mm per week, respectively, during this same period. All growth rates increased during the August to November period (0.9, 0.8, 0.6, and 0.4mm/week in the 0-, 4-, 8-, and 24-hour treatments, respectively). At the termination of the study, oysters in the 0- and 4-hour treatments did not differ in size (Tukey HSD $P = 0.53$), but oysters in the 8- and 24-hour treatments were significantly smaller than oysters in the 0-hour and 4-hour treatments (Tukey HSD $P < 0.01$ for all pairwise comparisons). Oysters in the 4-hour treatment were significantly larger (shell height) than oysters in the 8- and 24-hour treatments starting in June and this difference was consistently observed at all subsequent sampling dates through the end of the experiment (Tukey HSD $P < 0.05$ on each sampling date). Shell height of oysters in the 4-hour treatment was significantly greater than in the 0-hour treatment in July (Tukey HSD $P < 0.05$), but this difference was not observed at any other sampling date. Oysters in the 0-hour treatment attained significantly greater shell height than oysters in the 24-hour treatment beginning in August and persisting through the end of the study (all Tukey HSD $P < 0.0001$), but

did not surpass shell height of the 8-hour treatment until the October and November sampling dates (both comparisons Tukey HSD P < 0.05). Dry meat weight at the termination of the study was significantly affected by desiccation interval (ANOVA P = 0.03, Table 4), with the 4-hour treatment yielding significantly greater meat weight than the 24-hour treatment (Tukey HSD P = 0.02), although dry weight was not significantly different among other treatments. Condition index of oysters did not vary among the desiccation treatments (Kruskal-Wallis P = 0.30, Table 4) but was highest in the 8-hour treatment (mean CI (± SD) = 16.3 (± 2) compared to 14.8 (± 3), 15.6 (± 2) and 15.5 (± 2) in the 0-, 4-, and 24-hour treatments, respectively). Oyster mortality between May – November was low and similar among treatments, with 6, 1, 10 and 8 oysters dead in the 0-, 4-, 8-, and 24-hour treatments, respectively.

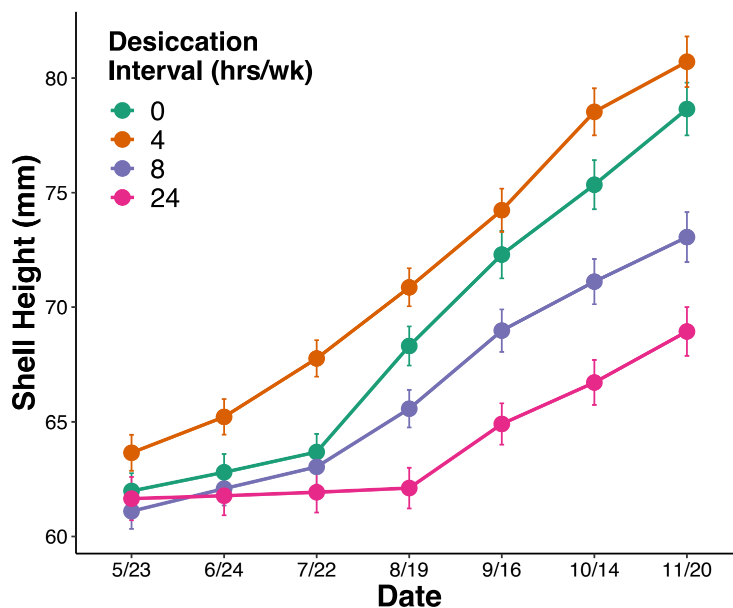

Figure 10. Shell height (measured from the umbo to ventral valve margin) of oysters in four desiccation treatments in the Choptank River, MD from May through November 2019. The green line denotes non-desiccated oysters (0 hours desiccation per week). The orange line

represents oysters desiccated for 4 hours per week, the purple line represents oysters desiccated for 8 hours per week, and the pink line represents oysters desiccated for 24 hours per week. Error bars represent one standard error of the mean.

Table 7. Mean (SEM where applicable) values of oyster shell height (mm), dry meat weight (g), condition index, and cumulative mortality (number dead) of oysters in four desiccation treatments in the Choptank River, MD at the culmination of the study period in November 2019.

Treatment	Height (mm)	Dry Meat Wt. (g)	Condition Index	Cumulative Mortality (number dead)
0	78.6 (1.2)	5.0 (0.2)	14.8 (0.7)	6
4	80.7 (1.1)	5.6 (0.2)	15.6 (0.5)	1
8	73.1 (1.1)	5.2 (0.2)	16.3 (0.5)	10
24	68.9 (1.1)	4.8 (0.1)	15.5 (0.5)	8

Discussion

This study examined the effect of four weekly desiccation intervals on oyster biofouling, cage fouling, and oyster responses (growth, mortality, and condition index) between May - November in the Chesapeake Bay. The 8-and 24-hour treatments were anticipated to confer the greatest biofouling control but were also expected to reduce growth compared to the 0- and 4-hour treatments (e.g. Chapman, 2019; Gamble, 2017; Kirk, 2019). Results of this study revealed that either a 4-, 8-, or 24-hour desiccation treatment led to reduced biofouling coverage compared to the 0-hour treatment (average reduction of 63%, 72%, and 81%, respectively), although treatment effects varied temporally and species-specific effects were evident among the 4- and 8-hour treatments. Oyster growth was also affected by desiccation interval, with the 4-hour treatment showing the highest growth and the 8- and 24-hour treatments showing

significantly less growth than either the 0- or 4-hour treatment. These findings confirm previous work on the effectiveness of a 24-hour desiccation treatment, along with its associated growth penalty (Chapman, 2019; Gamble, 2017; Kirk, 2019). The present study contributes new information regarding the effectiveness of shorter (4- and 8-hour) desiccation treatments and associated impacts to oyster growth and survival. Overall, the shorter desiccation treatments, particularly the 4-hour treatment, appear to be effective at controlling biofouling without sacrificing oyster growth, making it a potentially useful biofouling control treatment for oyster growers. Below, the main findings, potential tradeoffs of desiccation control, and the significance of these results to the oyster industry at large are discussed in greater detail.

Effects of desiccation on biofouling

Total epibiont coverage and worm abundances were both affected by desiccation treatment, although the longer (24-hour) treatment yielded the most consistent reduction in the case of endobiont worms, compared to the 4- and 8-hour treatments which exhibited temporally variable effects. Gross epibiont fouling was reduced in all desiccation treatments at all time points relative to the control, which agrees with previous research into the effectiveness of a 24-hour weekly desiccation (Chapman, 2019; Gamble, 2017; Kirk, 2019) and contributes new information regarding the suitability of shorter (4- and 8-hour) desiccation intervals for biofouling control. All of the desiccation treatments generally conferred a reduction in endobiont worm abundance, but not on all sampling dates. Only the 24-hour treatment yielded a significant reduction in worm abundances at all time points relative to the control, in contrast to the 8-hour treatment that showed significant reductions at 5 of 6 sampling dates and the 4-hour treatment that showed reduction at 3 of 6 sampling dates (Table 2). Further, the variance of fouling (both epibiont and endobiont worm) data within the 24-hour treatment was much lower than other

treatments (Figure 9), indicating more consistent (less variable) treatment effectiveness of the 24-hour treatment. Endobiont worms and select epibiont species may require an 8- or 24-hour treatment for consistent fouling control, especially during periods of heavy colonization pressure. Indeed, previous research has shown species-specific tolerances to desiccation under varying ambient weather conditions (Guareschi & Wood, 2020; Hopkins et al., 2016; Meichssner et al., 2020). Based on the results of this experiment, a 4-hour weekly desiccation may not be long enough to kill some biofoulers, especially if conditions during emersion are humid (Hopkins et al., 2016). The specific species that have colonized may also play a role in the variable effectiveness of the 4-hour treatment, as some species can retain water and serve to insulate themselves and adjacent biofoulers from completely desiccating (McAllen, 1999). Daily tidal exposure of ~5 hours reduced fouling accumulation consistently in Littlewood et al., (1992), but the less frequent (weekly) 4-hour treatment used here may not be sufficient for reliable control of all fouling organisms.

In contrast to the oyster biofouling results and in a departure from previous findings (e.g. Gamble, 2017; Mallet et al., 2009), desiccation interval did not affect biofouling accumulation on the cage material and all cages yielded minimal biofouling on the cage material. One possible explanation for this is the cage material used. Previous studies that identified differences in fouling accumulation on gear deployed oysters in high density polyethylene (HDPE) bags (Chapman, 2019; Gamble, 2017; Kirk, 2019; Mallet et al., 2009), whereas the present study used new PVC-coated wire mesh, and differences in biofilm formation on these two compounds may be the cause of the observed difference (Rożej et al., 2015). The composition of the cage material may impact formation of biofilms, the assemblages of microbial cells which adhere to a surface and form an extracellular polymeric substance matrix (de Carvalho, 2018; Pandey et al., 2014),

and are a crucial first step in the establishment of biofouling communities (de Carvalho, 2018; Qian et al., 2007). Many factors influence the establishment of biofilms, including material composition, surface roughness, and the amount of time the material has been submerged in water (Bhagwat et al., 2021; de Carvalho, 2018; Yadav et al., 2017). Previous research has identified that biofilms establish in significantly greater abundance (number of bacterial cells) on HPDE compared to PVC (Rożej et al., 2015). In the water, PVC releases compounds that can be more toxic to bacteria than compounds released from other plastics such as HDPE (Rożej et al., 2015). Thus, it is possible that differences in gear material used in this study influenced the biofilm formation, reducing subsequent biofouling colonization on all cages and preventing any treatment-derived differences in fouling accumulation on cages from developing.

Effects of desiccation on individual epibiont species

While desiccation treatments had strong and consistent effects on gross biofouling, treatment effects on individual species varied. The specific biofouling species that colonize an aquaculture operation in a given year are governed by complex processes, affected by biotic and abiotic forces (Dayton, 1971; Lubchenco & Menge, 1978; Menge, 1995; Satumanatpan & Keough, 2001; Underwood, 2000; Ysebaert & Herman, 2002). Succession after colonization can result in initial colonizers being out-competed for space or resources by other, later colonizers, leading to changes in relative abundance over time (e.g. Menge, 1995; Paine, 1970). This temporal change in biofouling species (succession) was also observed here. Early colonizers (*C. tenuissimum, G. franciscana, M. tenuis,* and *U. intestinalis)* were present by the first sampling date, but were later overset by *V. pavida* during the warm summer months before the relative abundance of *V. pavida* declined towards the latter portion of the summer and early fall. This is a typical pattern, observed previously in the Chesapeake Bay (Branscomb, 1976). While certain

species (*C. tenuissimum* and *M. tenuis)* survived the overset and were also observed later in the season, others (*U. intestinalis* and *G. franciscana*) were not observed again after being overset. The dominant oversetting fauna was *V. pavida*, a colonial bryozoan which colonizes rapidly, is well-suited to a range of low to brackish salinities (Carter & Jackson, 2007; Winston, 1995), and is a major spatial competitor among the epibiont community in Chesapeake Bay (Branscomb, 1976). While *V. pavida* is known as a subtidal species (SERC, 2022), it has been documented in high abundance in the intertidal where it can form thick mats that retain moisture during periods of emersion (Branscomb, 1976). This tolerance of the intertidal may be the reason why *V. pavida* was inconsistently affected by desiccation treatment here. In the case of another intertidal species, *M. leucophaeata*, desiccation for 4 hours weekly yielded less consistent treatment effects compared with the longer, 8- and 24-hour, treatments. *Mytiloposis leucophaeata* is a bivalve mollusk in the mussel family, well adapted to life in the intertidal zone (Souza et al., 2005). Given this tolerance of the intertidal, it is not surprising that this species was not consistently affected by a 4-hour desiccation interval. In contrast, *B. improvisus, C. tenuissimum, M. tenuis, G. franciscana* and *U. intestinalis* were consistently controlled by the 4-, 8-, and 24-hour treatments in the present study. Life history strategies and the biology of these species are important considerations in their tolerance to desiccation and thus presence as biofoulers, as they are all common in the subtidal or low intertidal of the Chesapeake Bay (SERC, 2022). However, abundance and competition among species may also be a factor in their fouling success. In other settings, *U. intestinalis* has thrived in the upper intertidal with outer fronds drying and providing cover for inner fronds to retain moisture (McAllen, 1999). If *U. intestinalis* had achieved greater relative abundance prior to colonization by *V. pavida* in July, *U. intestinalis* may have been able to survive desiccation, consistent with previous observations (McAllen, 1999). Within a given

group of organisms (e.g. barnacles, class Thecostraca) other species present in different regions or at different times may exhibit different desiccation tolerance as well. Indeed, another barnacle species in the Chesapeake Bay, *Chthamalus fragilis*, is common in the upper intertidal (Lippson & Lippson, 2006), is more tolerant of dry conditions, and may not be well controlled by weekly desiccation. Overall, these findings demonstrate the value of understanding the specific species that comprise a site's biofouling community in defining an appropriate husbandry technique for biofouling control.

Effects of desiccation on oyster growth

Reduced growth was observed in the 8- and 24-hour desiccation treatments relative to the control (0-hour) and 4-hour treatments, indicating a significant growth penalty for longer desiccation treatments. This finding is consistent with previous results from studies examining 24-hour weekly desiccation intervals (Chapman, 2019; Gamble, 2017; Kirk, 2019), although in the current study, the severity of the reduction in growth rate varied over time (Figure 10). Periods of aerial exposure are stressful to marine invertebrates, inducing large swings in temperature, a shift to anaerobic metabolism, and periodic limitations to feeding (Newell, 1973). Such stresses lead to reduced metabolic rates (Shumway, 1982) and a reduction in glycogen reserves which must be built up again upon re-submergence (de Zwaan & Wijsman, 1976; de Zwaan & Zandee, 1972). It is possible that the prolonged periods of emersion applied here (8- and 24-hour desiccation treatments) resulted in stresses to the oysters that reduced energy available for somatic growth. However, the period of study, particularly the early months of the experiment (May to July), was characterized by an additional stress, low salinity, which was near levels where oyster growth has been found to slow or cease (~5; Rybovich et al., 2016; Figure 4). Indeed, growth rates of all oysters were lower in the May-July (lower salinity) period compared

to the August-November period, but growth differences among treatments persisted between August and November when more favorable salinity conditions returned. The timing of the increased salinity was concomitant with peak water temperatures (Figure 4), and the elevated temperature may have also contributed to the increase in growth rates that began in August, as both salinity and temperature are major drivers of oyster growth (Lowe et al., 2017; Rybovich et al., 2016). Reproduction is another stressful process for oysters, requiring a large investment of energy and resulting in considerable reduction in oyster weight (Gabbott, 1975). The period of slower growth (May-July) was also at a time of year when oyster gonads develop and spawning occurs in the region (Mann et al., 2014). Thus, it is possible that gametogenesis acted as another compounding stressor to the oysters, contributing to the extremely slow growth observed during the early period of study. While difficult to distinguish the impact of environmental and biological stressors acting on the oysters, it is possible that the combination of salinity stress and reproductive investment compounded the stress of the desiccation treatments, yielding the negligible or lack of growth observed in the 8- and 24-hour treatments during the early part of the experiment. In summary, environmental stochasticity and ontogenetic or seasonal shifts in energetic demands of oysters may interact to form an added stress condition and elevate the stress of desiccation, contributing to reduced growth associated with longer periods of desiccation. Future investigations into how oyster growth, energy storage, gametogenesis, and cellular stress responses are affected by desiccation would aid in understanding oyster responses to periods of desiccation.

Conclusions and implications of results for oyster farmers

Oyster aquaculture operations take on a multitude of forms, exist in a diverse range of locations, and can experience temporally variable environmental conditions that influence oyster

growth and intensity of biofouling. Biofouling is a major concern for many oyster farmers because it increases labor costs (Adams et al., 2011) and can reduce yield (Stefaniak et al., 2005; Wargo & Ford, 1993), but desiccation can be an effective husbandry technique to control biofouling (Adams et al., 2011; Chapman, 2019; Gamble, 2017; Kirk, 2019; Mallet et al., 2009). In this study, application of a 4-hour weekly desiccation treatment reduced fouling without sacrificing growth. However, different fouling species were more or less resistant to the 4-hour treatment and desiccation effectiveness varied over time, so it is possible that temporarily increasing desiccation interval (to 8- or 24-hours) may be warranted during periods of peak fouling. While the 8- and 24-hour desiccation treatments in this study yielded more consistent biofouling reduction, the oyster growth penalty observed in these treatments may make them unsuitable for some growers, although individual farmer preferences regarding fouling control and growth will influence their decision. Of course, biofouling is not the only challenge posed to commercial oyster growers and they must select management strategies (i.e. gear types, fouling control protocols, processing schedules, etc.) that enhance farm efficiency without adding to labor costs or other farm inputs unnecessarily. Possessing the necessary infrastructure and labor are important factors for oyster farmers when considering desiccation as a biofouling control strategy. Farms that utilize flippable floating cage (FFC) or adjustable longline system (ALS) culture methods can regularly desiccate oysters and cages. However, desiccation is unlikely to be an effective fouling control strategy for farms that employ gear types not suited to desiccation (i.e. subtidal bottom cages), as these would require a substantial addition of labor to apply a desiccation treatment. Labor is already one of the largest expenses on an oyster farm and additional labor costs must be carefully considered (Engle et al., 2021). It is critical that husbandry techniques be feasible and beneficial to farms (Piola et al., 2010), so the decision to

incorporate desiccation as a management strategy will require an evaluation of available gear and infrastructure, location, cost inputs, and relative benefits. Still, based on this and previous studies, desiccation is generally a reliable and effective biofouling control technique and farmers must assess their individual farm preferences regarding growth and biofouling in deciding on a desiccation interval. Given the promising results of the effectiveness of desiccation as a biofouling control strategy, more water-column farms in the region may seek to adopt this as a control strategy in the future.

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Chapter 4: Assessing physiological and molecular responses of *Crassostrea virginica* to weekly desiccation treatments

Abstract

Biofouling poses a major challenge to the growing US oyster aquaculture industry and periodic desiccation (emersion) is a popular husbandry technique used on farms to control biofouling. However, desiccation is also associated with reduced oyster growth and elevated mortality, and the duration of desiccation can influence the magnitude of oyster stress response. A clearer understanding of the physiological responses of oysters to desiccation will help to refine desiccation as a husbandry technique. In this study, I measured growth, filtering rates, gametogenic stage, glycogen concentration, and the expression of heat shock proteins among oysters desiccated for 0, 4, 8, or 24 hours per week over a three-month period (July – September) in the Choptank River, MD. Oyster filtering was reduced immediately after re-submergence in the 8- and 24-hour treatments during the August sampling, but the filtering reduction did not persist after a 4-day re-submergence period or in September. Glycogen content and gonad development demonstrated an expected inverse relationship in July where oysters in the 0-hour treatment had more developed gonad and reduced glycogen, and oysters in the 4-, 8-, and 24-hour treatments had less developed gonad and more glycogen, but these relationships were not present in August. Oysters' expression of heat shock proteins lacked clear treatment effects but trends suggested greater expression during August compared to July in the 0- and 4-hour treatments, while oysters in the 8- and 24-hour treatments mounted a low expression of inducible HSPs during both the July and August samplings. Overall, the shorter desiccation interval (4-hour treatment) resulted in less stress to oysters, although the stress response of oysters in the

longer desiccation treatments (8- and 24-hour) varied over time. Based on these results, desiccation for 4 hours weekly may be a suitable husbandry tactic with little likelihood of elevated oyster stress.

Introduction

Shellfish aquaculture is a rapidly growing industry, comprising 56 percent of coastal and marine aquaculture production (live weight) globally in 2018 (FAO, 2020). Shellfish aquaculture provides a low-carbon source of food for a growing population, economic stimulation, and ecological benefits, and thus is considered one of the most sustainable and important sectors of coastal aquaculture (Dealteris et al., 2004; GMRI, 2016; Hudson, 2018; Kellogg et al., 2018; Marenghi et al., 2009; Naylor et al., 2021; Ray et al., 2019; van Senten et al., 2019). Across the US, many states have reported significant growth in their oyster aquaculture industries in recent years (GMRI, 2016; Grice & Walton, 2020; Hudson, 2018; Massachusetts Division of Marine Fisheries, 2019), and continued growth of oyster aquaculture represents an key component of domestic aquaculture production.

While growth of the US oyster aquaculture industry has been a bright spot for domestic aquaculture, oyster farms also face significant challenges, one of which is from biofouling, the agglomeration of marine plants and animals on oysters and farm infrastructure. Biofouling presents a challenge to aquaculturists by reducing suitability of growing conditions (Gormican, 1989; Yukihira et al., 1998), increasing weight on gear (Ramsay et al., 2008), and reducing growth and/or condition of oysters (Stefaniak et al., 2005; Wargo & Ford, 1993). Biofouling can also lead to devaluation of shellfish based on the amount of fouling visible on the shells, resulting in economic losses (Campbell & Kelly, 2002). Desiccation, the periodic aerial exposure of cages and oysters, is a commonly applied and effective means to control biofouling (Adams et

al., 2011; Gamble, 2017; Hopkins et al., 2016; Kirk, 2019; Mallet et al., 2009), and can be applied at commercial scales using growout systems such as flippable floating cages (FFC) and adjustable longline systems (ALS) (Davis, 2013; Walton et al., 2013). However, reductions in growth (termed a 'growth penalty'; Chapman, 2019; Gamble, 2017; Kirk, 2019) and elevated mortality (Bodenstein et al., 2021; Chapman, 2019) have been observed in oysters desiccated for 24 hours once weekly compared to oysters desiccated less frequently, particularly when combined with other common farm stressors, such as tumbling (Bodenstein et al., 2021). Understanding how oyster stress and energy metabolism are affected by desiccation interval and frequency is important for maximizing yield (growth and survival) while minimizing biofouling which can reduce farm revenues.

Somatic growth and survival in oysters are affected by a number of key behaviors and biological pathways, including feeding, energy storage and use, reproduction, and stress responses, each of which can also be affected by periodic desiccation. Effects of desiccation on these pathways is an important consideration in understanding the reduced growth and elevated mortality that have been observed in desiccated oysters previously (Bodenstein et al., 2021; Chapman, 2019; Gamble, 2017; Kirk, 2019). As filter-feeding bivalves, oysters can only access food when submerged in an environment with suitable food available and desiccation stress can affect the onset of feeding upon re-submergence (Ellington, 1983; Widdows & Schick, 1985). Despite some studies which have revealed elevated bivalve growth with desiccation (Bishop & Peterson, 2006; La Peyre et al., 2017; Littlewood et al., 1992), comprehensive evidence of compensatory feeding upon re-submergence is lacking (Bayne et al., 1988; Griffiths & Buffenstein, 1981; Widdows & Shick, 1985), so understanding the effects of desiccation on oyster feeding is necessary. Glycogen is another important component of oyster energetics, as it

is the primary energy storage reserve in bivalves and is drawn upon during periods of stress and gametogenesis (Berthelin et al., 2000; Greenberg, 1977; Patrick et al., 2006). During aerial exposure, bivalves insulate themselves from their surrounding environment via valve closure (Gainey & Shumway, 1988; Porter & Breitburg, 2016), during which, the oyster may shift to anaerobic metabolism which is associated with a reduction in glycogen reserves (de Zwaan & Wijsman, 1976; de Zwaan & Zandee, 1972; Lombardi et al., 2013; Meng et al., 2018). Glycogen reserves are critical in the mobilization of energy for gametogenesis (Ruiz et al., 1992), which can seasonally represent the single greatest energetic expenditure of *Crassostrea virginica* (Dame, 1976), reducing energy available for somatic growth. Finally, insulation from stressful or inhospitable conditions via the expression of heat shock proteins (HSPs) is another relevant process that should be evaluated in understanding the response of oysters to periodic desiccation. Heat shock proteins are well conserved stress proteins and are ubiquitous components of cells, allowing organisms to protect cells from damage brought on by environmental stressors, including thermal stress and desiccation stress (Casas & La Peyre, 2020; Kregel, 2002; Roberts et al., 1997). Periods of emersion can elicit an elevated expression of HSP70 in bivalves, used to repair damaged proteins or eliminate denatured proteins, although this expression is energetically expensive (Fabbri et al., 2008; Feder & Hofmann, 1999). In summary, oyster growth and survival are affected by multiple important biological and physiological processes and an examination of how desiccation affects these processes is important in understanding the reduced growth and elevated mortality observed in desiccated oysters.

The objective of this study was to examine the molecular and physiological stress responses of *C. virginica* oysters to weekly desiccation intervals in an aquaculture setting in the Chesapeake Bay, MD. Gametogenic stage, glycogen content, filtering rates, and expression of

HSP70 of oysters desiccated for 0, 4, 8, and 24 hours weekly were assessed as indicators of oyster stress with the goal of understanding what mechanism(s) contribute to a growth penalty and elevated mortality under desiccation (Bodenstein et al., 2021; Chapman, 2019; Gamble, 2017; Kirk, 2019). The timing of investigations (summer) and use of diploid oysters necessitated an assessment of gametogenic stage as spawning can seasonally represent the single greatest energy expenditure for *C. virginica* (Dame, 1976). We hypothesized that filtering rates post-desiccation would be negatively affected by desiccation interval, with the 8- and 24-hour treatments taking a longer period of re-submergence to open and begin filtering based on the findings of Widdows and Shick (1985). Given the potential drawdown in glycogen reserves during periods of anaerobic respiration (de Zwaan & Wijsman, 1976; de Zwaan & Zandee, 1972), glycogen content was anticipated to be inversely related to desiccation interval. Heat shock protein expression was expected to be positively correlated with desiccation interval given previous findings of elevated HSP70 expression in upper intertidal bivalves compared to subtidal counterparts (Roberts et al., 1997). Understanding oyster responses during and after desiccation is important to refine the use of desiccation as a biofouling control strategy for oysters in aquaculture settings, allowing oyster farmers to achieve elevated product quality (low biofouling) without sacrificing growth or survivorship.

Methods

Oyster preparation and deployment

Four-year-old hatchery reared diploid oysters (*C. virginica*) were sourced from the University of Maryland Center for Environmental Science Horn Point Laboratory Demonstration Oyster Farm in the Choptank River, MD (38° 35' 35.556" N, 76° 7' 43.932" W). Previously established biofouling was physically removed prior to the start of the investigations to ensure

clean oysters at deployment. Shell height, measured as the maximum distance between the umbo and the ventral valve margin (Galtsoff, 1964) was recorded for all oysters immediately prior to deployment using Vernier calipers (shell height at deployment = 91.2 ± 10mm). Forty (40) oysters were deployed to each of eight cages (n=320), constructed of 25mm polyvinyl chloride (PVC) coated wire mesh and measuring 40cm x 25cm x 20cm. Cages were affixed at a consistent height above the seafloor (15cm) and were randomly assigned to one of four desiccation treatments (two replicate cages per treatment). A control treatment received no weekly desiccation (0-hour treatment). The other desiccation interval treatments (consecutive hours) were as follows: four hours per week (4-hour treatment), eight hours per week (8-hour treatment), and twenty-four hours per week (24-hour treatment). Oysters were deployed from June - September 2020.

Environmental monitoring

Daily measurements of salinity and temperature were recorded using a YSI Pro 1030 handheld meter (YSI, Inc./Xylem Inc., Yellow Springs, OH, USA). Weather conditions during the study period were accessed using NOAA National Centers for Environmental Information Local Climatological Data (https://www.ncdc.noaa.gov/cdo-web/datatools/lcd; Station ID: WBAN:00356). Weather data are recorded every 20 minutes. Mean air temperature during each period of emersion was computed for the 24-hour treatment since all treatments were desiccated within the 24-hour treatment's exposure window.

Oyster evaluation

Oyster growth and mortality were assessed weekly. Oyster shell height, measured as the maximum distance between the umbo and ventral valve margin (Galtsoff, 1964), was measured

using Vernier calipers for all oysters in each treatment. Mortality was assessed by tapping oysters to determine their survival status. Dead oysters were removed from cages.

Assessment of filtering and phytoplankton removal

Oyster filtering was measured three times during the study period according to McCarty et al. (*in prep;* see below for description of methods). Filtering was measured at two different times immediately after a desiccation treatment (August 5 and September 2), and once after a 4-day re-submergence period (August 9). Seventy-five one-liter beakers were prepared on each day of filtering trials with artificial seawater using deionized freshwater and Crystal Sea® MarineMix, salted to match the ambient salinity of the Choptank River on each day (salinity of 11 on August 5 and 9, salinity of 10.1 on September 2). Water temperatures in the beakers were 24.7, 24.8, and 23.4°C on August 5, 9 and September 2, respectively. Beakers were gently aerated using low pressure air. Just prior to the addition of oysters, aliquots of hatchery-reared *Isochrysis* spp. were added to each beaker at a consistent density on each day of filtering trials. Manual phytoplankton cell counts (cells/mL) were conducted in triplicate across a dilution series (6 dilutions) of *Isochrysis* spp. on each day using a Hausser Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA, USA). Cell counts were related to *in-vivo* fluorescence of the same dilution series, measured using a calibrated FluoroSense™ Handheld Fluorometer (model #2860-000-C, Turner Designs, San Jose, CA, USA). An ordinary least squares regression through the origin from the RStudio package 'stats' (RStudio Team, 2016) was used to relate manual phytoplankton cell counts (cells/mL) to *in-vivo* fluorescence (µg/L). A standard curve relating the two was created on each day that filtering trials took place.

For each filtering trial, fifteen oysters were randomly selected from each treatment at the end of their assigned desiccation interval (n=60 per filtering trial). Oysters were scrubbed to

remove additional organisms living on the oyster valves, then labeled. Individual clean oysters were immediately submerged into prepared beakers (artificial seawater and *Isochrysis* spp.). Blank replicates (n=15, similarly sized oyster shell only) were included to account for natural algal settlement or other fluctuations in fluorescence during the trials (used to calculate 'α' in the equation below). Chlorophyll concentration in each beaker was measured in duplicate every 30 minutes for four hours on August 5 and 9, and until filtering had diminished or phytoplankton was depleted on September 2. When chlorophyll was measured, oyster gape status (open or closed) was also noted. At the culmination of the monitoring period, presence of feces and/or pseudofeces was noted in each beaker and assigned a qualitative score (0-3). After the August 5 filtering trials finished, labeled oysters were returned to the Choptank River where they remained submerged for four days. After the four-day re-submergence period, new beakers of artificial seawater were prepared and a consistent concentration of *Isochrysis* spp. was added to each beaker. Cell counts of a dilution series (6 dilutions) were again performed, relating fluorescence (µg/L) to cells/mL. The same fifteen labeled oysters per treatment were added to the beakers and change in chlorophyll concentration was measured every 30 minutes for 4 hours using aforementioned methods, then oysters were measured (shell height), shucked, and meats dried in a drying oven at 68°C for 72 hours. Dry oysters were weighed using an Ohaus Discovery analytical electronic balance (Model DV114C, Ohaus Corporation, Parsippany, NJ, USA). Post-desiccation filtering was again monitored until phytoplankton was depleted on September 2 using methods described above, and oysters were measured, shucked, and dry meat weight recorded after the investigations.

Mass-corrected filtering rates per oyster were calculated according to Coughlan (1969)

$$m = \frac{\frac{M}{n}\left\{\frac{(\log_e C_0 - \log_e C_t) - \alpha}{t}\right\}}{w}$$

where, 'm' is the filtering rate, 'M' is the volume of solution, 'n' is the number of oysters in the beaker, 'C_0' is the chlorophyll concentration at time zero (when the oyster was placed in the beaker), 'C_t' is the chlorophyll concentration after the time interval 't', 'α' is the natural settlement rate of phytoplankton cells in the beakers (obtained using the blank replicate beakers consisting of shell only), and 'w' is the dry weight of the oyster tissue (g). This approach measures phytoplankton removed from the water column of the beaker, but does not assess ingestion and oysters were not depurated prior to filtering trials. Some phytoplankton may have been sorted to the gut and some may have been deposited as pseudofeces, but the method employed does not allow an assessment of this. Filtering rates were measured from the time when each oyster was placed into the beaker (C_0) until phytoplankton was depleted in the beaker as filtering rate would appear to cease when phytoplankton had become depleted (C_t). The interval of time that passed prior to each oyster removing 50% of phytoplankton in the beaker was also computed.

Assessment of gametogenic stage

Gametogenic stage was assessed twice during summer 2020, once during the period when gametogenic ripening was expected to take place (July) and once after spawning was expected to have begun according to conditions associated with natural spawning in the region (August, Kennedy & Krantz, 1982; Mann et al., 2014). Assessment took place at the end of each desiccation interval. Fifteen oysters per treatment (n=60 oysters per sampling x 2 samplings; total n = 120) were randomly selected and carefully shucked. Gametogenic stage was assessed using a 0-3 scale according to Kennedy and Krantz (1982), whereby a score of 0 indicated no

visible gonad, 1 indicated early development (follicles expanding and branching), 2 indicated later development (three dimensional distended follicles and reduction in vesicular tissue), and a score of 3 indicated spawning had taken place and gonad was regressing.

Tissue sample preparation

Tissue samples were collected at two time periods in summer 2020. Gill, adductor, and mantle tissue were excised from fifteen (15) oysters per treatment (15 oysters per treatment x 4 treatments x 2 sampling dates; n=120 oysters) immediately after the assigned desiccation interval had been reached. A stainless-steel scalpel, scissors, and forceps were used to excise tissues and instruments were flamed with ethanol between samples. Tissue samples were labeled and stored at -80°C in 2mL microcentrifuge tubes until they were used for their requisite assay. Gill, mantle and adductor tissue were used in glycogen content assays and only gill tissue was used in heat shock protein assays.

Glycogen content

Glycogen content of three tissue types (gill, mantle, adductor) was measured according to the anthrone method (Baturo et al., 1995; McFarland et al., 2016). Tissues were individually lyophilized (freeze-dried) and pulverized. Freeze-dried tissue (25 mg per tissue type) was then diluted (1:40 w/v) in a homogenizing phosphate buffer (50mM phosphate buffer w/ 1mM EDTA, 0.5mM PMSF). Samples were diluted with 30% KOH and boiled to extract glycogen. EtOH and Na_2SO_4 were added to purify the extracted glycogen and the samples were again boiled. Tubes were centrifuged at 2,000g for 20 minutes using an Eppendorf centrifuge (Model 5810 R, Eppendorf America, Enfield, CT, USA) and the resulting precipitate dried overnight in a drying oven at 60°C. Dried precipitate was resuspended in reverse osmosis filtered freshwater and

anthrone reagent (0.15%) was added to facilitate color development. Samples were incubated at 90°C for 20 minutes, then transferred in triplicate into a 96 well plate. Absorbances were read at 620nm using a BioTek® Synergy LX™ Multi-Mode Microplate Reader (BioTek® Instruments, Inc., Winooski, Vermont, USA). Average optical densities of the triplicate samples were computed. Absorbance (optical density units) and glycogen concentration (mg/mL) were related by creating a standard curve using a serial dilution series (0 – 5.0 mg/mL; 7 dilutions) created from glycogen stock (Glycogen type II from oyster; Sigma-Aldrich, Inc., St. Louis, MO, USA). Each serial dilution sample was measured in triplicate and plotted against corresponding optical density absorbance values to yield a linear regression curve and equation relating absorbance units and glycogen concentration for each plate prepared. Glycogen content (milligrams glycogen per gram of oyster tissue) of gill, mantle and adductor muscle tissues were averaged to provide a single glycogen content value for each oyster.

Heat shock protein expression

Heat shock protein (HSP70) expression was measured using an enzyme-linked immunosorbent assay (ELISA) according to LaPeyre et al. (2014) as described below. Total protein concentration and HSP70 expression were each quantified. Gill tissue was pulverized in a steel grinder with liquid nitrogen over a bed of dry ice. Ground tissue was added to a bicarbonate homogenization buffer (0.1 M $NaHCO_3$, 1% NP-40, protease inhibitor cocktail from CalBiochem (San Diego, CA, USA)) at a concentration of 0.5g/mL. Samples were centrifuged at 16,000g for 10 minutes at 4°C, and the resulting supernatant again centrifuged at 16,000g for 30 minutes at 4°C using an Eppendorf centrifuge (Model 5424 R). Total protein concentration of gill lysate was measured using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL, USA). Gill lysate samples were diluted to a concentration of 2 mg/mL with

bicarbonate coating buffer (0.1 M $NaHCO_3$), then diluted 1:1 (v/v) with reducing buffer (4 mM SDS in TBS, 5% ß-Mercaptoethanol) and boiled for 3 minutes. Samples were diluted 1:200 in bicarbonate coating buffer (0.1 M $NaHCO_3$) and transferred in triplicate to a 96 well plate. Plates were covered and incubated overnight at 4°C. Plates were washed with TTBS, then blocked with 1% BSA in TBS and incubated for 60 minutes at room temperature. Plates were washed with TTBS and the primary HSP antibody (HSP70 monoclonal antibody 3A3, Invitrogen, Waltham, MA USA) was added to each well. Plates were incubated for 90 minutes then washed with TTBS and the secondary antibody (Rabbit Anti-Mouse IgG Antibody, Alkaline Phosphatase conjugate, Sigma-Aldrich, Inc., St. Louis, MO, USA) was added to each well. Plates were incubated for 90 minutes then washed with TTBS and finally washed with TBS. PNPP was added to each well and plates incubated for 30 minutes at room temperature in the dark. Absorbances were read at 405nm using a BioTek® Synergy LX™ Multi-Mode Microplate Reader. Average optical density units of the triplicate samples were computed. Absorbance (optical density units) and HSP70 (ng/mL) were related by creating a standard curve using a serial dilution series (0 – 100 ng/mL; 7 dilutions) created from HSP70 standard (Heat Shock Protein 70 human, Sigma-Aldrich, Inc., St. Louis, MO, USA). Units are expressed as nanograms of HSP70 per milligram of total protein in the oyster gill tissue.

Data analysis

Statistical analyses were conducted in RStudio (RStudio Team, 2016) and data visualization was carried out using ggplot2 (Wickham, 2016). Treatment effects on shell height and dry weight were each investigated using a one-way analysis of variance (ANOVA) from the 'stats' package (R Core Team, 2017) and significant differences were further investigated using Tukey's Honest Significant Difference (HSD) Tests. The non-parametric one-way factorial

Kruskal-Wallis test from the 'stats' package (R Core Team, 2017) was used to investigate treatment effects on mortality, given that the data did not meet the normality assumptions required for parametric tests. A Kruskal-Wallis test ('stats' package; R Core Team, 2017) was also used to assess the effect of desiccation treatment on filtering rates on the three dates of filtering trials, and significant differences were further investigated using the Dunn (1964) multiple comparison test (Holm adjustment) from the package 'FSA' (Ogle et al., 2018) with a Holm adjustment applied to correct for multiple comparisons. The effect of desiccation treatment on gonad score was also assessed using a Kruskal-Wallis test, again due to violations of the normality assumption required for parametric tests, and significant differences were further investigated using the Dunn (1964) multiple comparison test (Holm adjustment, 'FSA' package; Ogle et al., 2018). An aligned rank-transformed analysis of variance (ART ANOVA) from the 'ARTool' package (Kay et al., 2021) was used to investigate treatment effects on glycogen content. Significant interactions were again investigated using Dunn's (1964) multiple comparison test with a Holm adjustment used to account for multiple comparisons. A two-way analysis of variance (ANOVA) from the 'stats' package (R Core Team, 2017) was used to examine effects of treatment, sampling month, and their interaction on expression of HSP70. Differences in HSP70 expression within each treatment between the two sampling dates were investigated using student's t-tests from the 'stats' package (R Core Team, 2017).

Results

Environmental conditions

Environmental conditions (riverine and air) during the experiment were typical for the study location. Surface salinity ranged from 9.3-11.4, with an average of 10.7. Surface temperature ranged from 22.7°C to 30.1°C and averaged 27.7°C. Air temperatures during

periods of emersion ranged from 17.8°C – 32.8°C and averaged 25.9°C. Air temperatures during exposure prior to filtering investigations averaged 26°C and 27.2°C on August 5 and September 2, respectively. Mean air temperatures during exposure prior to gonad assessments and collection of tissue for glycogen concentration and heat shock protein expression quantification were 27.8°C and 25.3°C on July 29 and August 26, respectively (Figure 1).

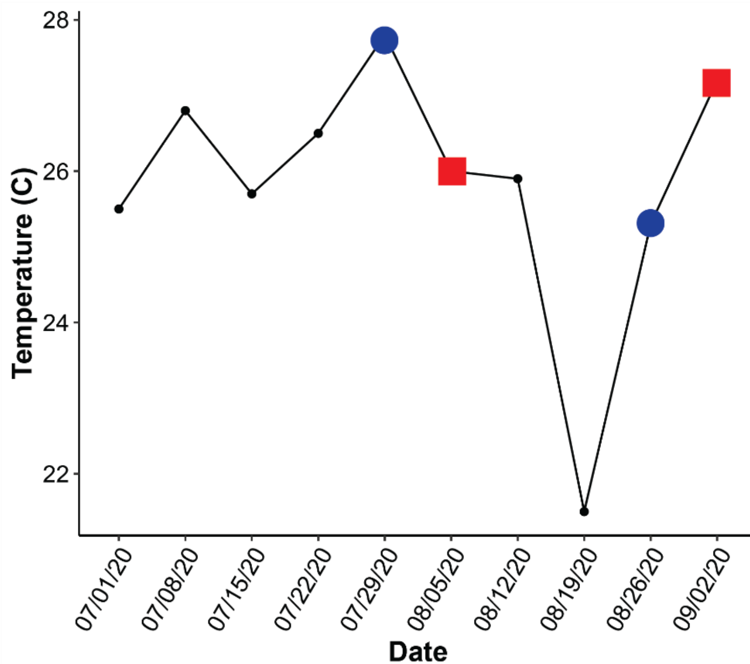

Figure 21. Air temperatures (mean C) during periods of emersion for oysters exposed for 0, 4, 8, and 24 hours weekly. Air temperatures during emersion prior to filtering investigations are noted using red squares, while temperatures during emersion prior to gonad assessments and collection of tissue for glycogen concentration assessment and heat shock protein expression quantification are noted using blue circles.

Oyster performance

Oyster growth (shell height) was relatively slow, and oysters in the 0-, 4-, and 8-hour treatments each grew an average of 2mm during the 4-month period. Mean shell height of oysters in the 24-hour treatment, however, indicated negative growth, as the mean shell height shrank by 1.4mm. Shell height was significantly affected by treatment (ANOVA P < 0.0000, Table 1), with oysters in the 24-hour treatment yielding less growth than oysters in the other treatments (Tukey HSD P-values < 0.05). Dry weight was also affected by treatment (ANOVA P = 0.00, Table 1), and mean (± SD) dry weight (g) of oysters in the 0-, 4-, 8-, and 24-hour treatments was 1.8 (± 0.7), 1.7 (± 0.4), 1.6 (± 0.6), and 1.6 (± 0.5), respectively. Post-hoc pairwise comparisons revealed reduced dry weight of oysters in the 8-hour (Tukey HSD P = 0.00) and 24-hour (Tukey HSD P = 0.03) treatments compared to the 0-hour treatment. Mortality was elevated in the 24-hour treatment, with a mean (± SD) of 7 (± 4.2) dead oysters per cage, compared to 1.5 (± 2.1), 2.5 (± 0.7), and 0.5 (± 0.7) dead oysters per cage in the 0-, 4-, and 8-hour treatments but this trend was not confirmed statistically and no differences in cumulative mortality were identified among the treatments (Kruskal-Wallis P = 0.16, Table 1).

Table 8. Mean (SD) oyster performance indicators (shell height, dry weight and mortality) for oysters desiccated for 0, 4, 8, and 24 hours weekly between July and September 2020.

	Shell height (mm)	Dry weight (g)	Mean mortality (number dead)
0	91.2 (10.0)	1.8 (0.7)	1.5 (2)
4	93.7 (9.2)	1.7 (0.4)	2.5 (0.7)
8	95.4 (9.6)	1.6 (0.6)	0.5 (0.7)
24	86.4 (10.3)	1.6 (0.5)	7 (4.2)

Filtering behavior and rates

Oysters desiccated for 8 and 24 hours weekly were delayed in the onset of filtering and had significantly reduced filtering rates post-desiccation when compared to the 0-hour treatment on August 5 (Kruskal-Wallis P = 0.00). In the August 5 post-desiccation filtering rate trials, mean (± SD) filtering rate of oysters in the 8- and 24-hour treatments was 9.5 (± 13) and 16.1 (± 16) cells hr^{-1} g^{-1}, respectively, despite gaping notably wider than oysters in the 0- and 4-hour treatments which filtered 280.3 (± 223) and 185.7 (± 192) cells hr^{-1} g^{-1} , respectively (Figure 2). Oysters in the 8- and 24-hour treatments failed to reduce phytoplankton concentration in their beakers by 50% (mean) during the first filtering trial. In contrast, oysters in the 0- and 4- hour treatments filtered 50% of the phytoplankton in their beakers within 1.5-2.5 hours. No significant differences in oyster filtering rates between treatments were present after a 4-day re-submergence period in August (Kruskal-Wallis P = 0.15), though oysters in the 24-hour treatment filtered at a slightly elevated (though statistically insignificant) mean rate of 225.1 cells hr^{-1} g^{-1} (± 144), compared to 137.2 (± 98), 134.3 (± 89), and 128.6 (± 108) cells hr^{-1} g^{-1} in the 0-, 4-, and 8-hour treatments. Oysters in all treatments depleted phytoplankton concentrations by 50% within 2.5-3.5 hours of submergence in the beakers during this post re-submergence filtering trial. Significant differences in oyster filtering rates were not present in the September post-desiccation filtering trials (Kruskal-Wallis P = 0.06), though oysters in the 24-hour treatment filtered at a slightly reduced rate of 97.8 (± 97) cells hr^{-1} g^{-1} compared to mean (SD) filtering rates in the 0-, 4-, and 8-hour treatments of 166.8 (± 210), 204.6 (± 136), and 325.5 (± 284) cells hr^{-1} g^{-1}, respectively. Oysters in the 24-hour treatment again failed to filter 50% of available phytoplankton during the September post-desiccation filtering trial, whereas oysters in the 0-, 4-, and 8- hour treatments filtered 50% of the phytoplankton in their beakers in 2.5, 3.5,

and 2 hours, respectively. All oysters were open during the filtering trials and both feces and pseudofeces were produced by oysters in all treatments.

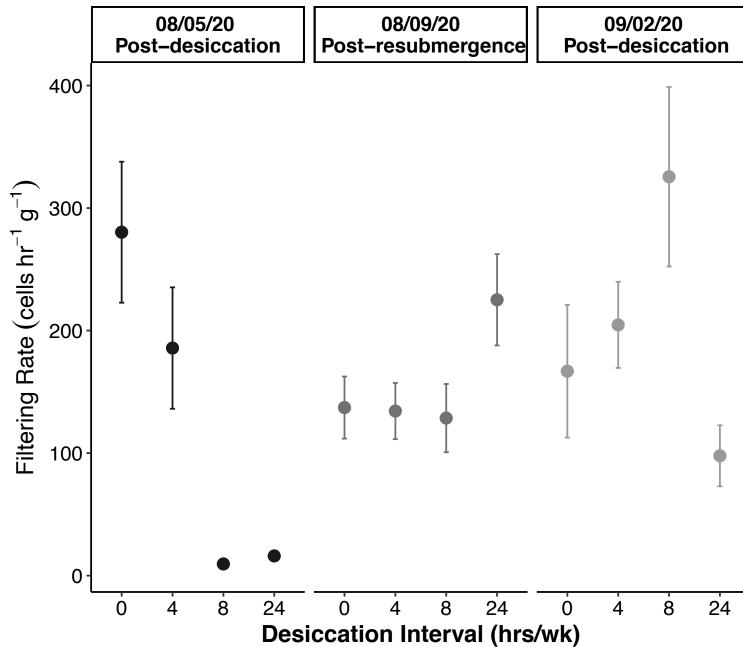

Figure 22. Mean mass-corrected filtering rates (cells hr^{-1} g^{-1}) of oysters in four desiccation treatments (0, 4, 8, and 24 hours per week). On August 5 (black points, left) and September 2 (light gray points, right), filtering rates were measured immediately after the desiccation interval. On August 9 (gray points, middle), filtering rates were measured after a 4-day re-submergence period. Error bars represent one standard error of the mean.

Gametogenic stage

Gametogenic stage investigations indicated lower gonad scores in July compared to August for all treatments (Figure 3). In July, oysters in the control (0-hour treatment) demonstrated a more developed gonad, yielding a higher gonad score compared to oysters in the 4-, 8-, or 24-hour treatments. The only treatment that showed evidence of spawning in July was

the 24-hour treatment, in which 27% of oysters were observed to have spawned. Mean gonad scores (± SD) for oysters in the 0-, 4-, 8-, and 24-hour treatments in July were 2.1 (± 0.3), 1.2 (± 0.6), 0.9 (± 0.5) and 1.1 (± 1.2), respectively. Results were more variable in August, with a large proportion of oysters from each treatment group having spawned (60%, 40%, 40% and 80% in the 0-, 4-, 8-, and 24-hour treatments, respectively). In August, gonad scores for oysters in the 0-, 4-, 8-, and 24-hour treatments were 2.3 (± 1), 1.5 (± 1.3), 1.8 (± 1.2), and 2.7 (± 0.7), respectively. Desiccation treatment affected gametogenic score in July (Kruskal-Wallis $P = 0.00$) and August (Kruskal-Wallis $P = 0.02$). Post-hoc investigations revealed that oysters in the 0-hour treatment had a higher gonad score than the 4-hour treatment (Dunn test adjusted $P = 0.03$), 8-hour treatment (Dunn test adjusted $P = 0.00$) and 24-hour treatment (Dunn test adjusted $p = 0.00$) in July. In August, only the 4- and 24-hour treatments differed significantly (Dunn test adjusted $P = 0.03$), with the 24-hour treatment yielding a higher gonad score than the 4-hour treatment due to the presence of more spawned-out oysters.

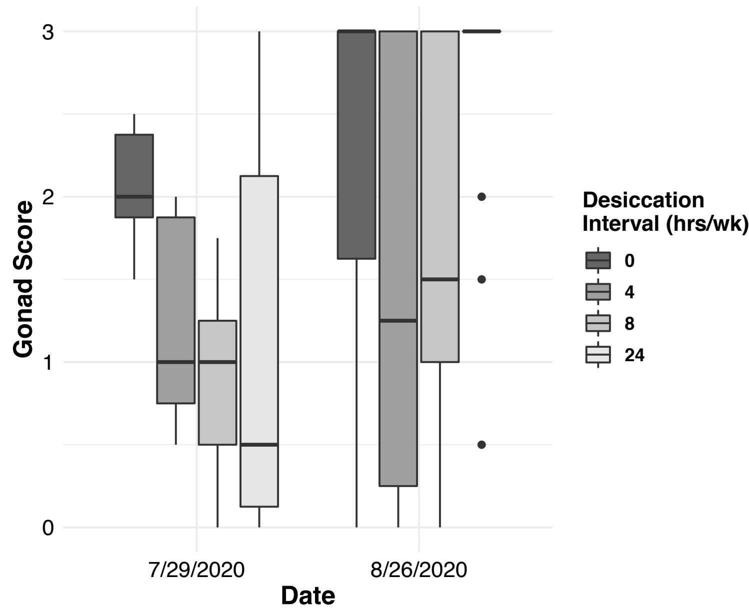

Figure 23. Box plot showing gametogenic stage / gonad score of oysters desiccated for 0, 4, 8, and 24 hours weekly between July - September 2020. The darkest boxes represent gonad score of oysters desiccated for 0 hours weekly, and progressively lighter boxes represent gonad score of oysters desiccated for 4, 8, and 24 hours weekly. Gonad assessments took place on July 29 and August 26. The thick line in the middle of each box represents the median, the filled boxes represent the interquartile range, and protruding longitudinal lines represent the minimum and maximum values. Outliers are represented by black dots.

Glycogen content

Glycogen content (milligrams glycogen per gram of dry tissue weight) of oysters was generally lower in July and increased in late August, with the exception of the 4-hour treatment which decreased in glycogen content between July and August (Figure 4). Total glycogen content was influenced by desiccation treatment (ART ANOVA $P < 0.0001$), but was not affected by sampling date (ART ANOVA $P = 0.95$). In July, mean glycogen content (± SD) for oysters in the 0-, 4-, 8-, and 24-hour treatments was 10.3 (± 18), 43.5 (± 55), 19.1 (± 21), and

23.4 (± 33) milligrams glycogen per gram of tissue, respectively. Oysters from the 0-hour treatment had significantly less glycogen than oysters in the 4-, 8-, and 24-hour treatments in July, as evidenced by pairwise comparisons (Dunn test adjusted P = 0.00, 0.02, and 0.02 comparing the 0-hour treatment with the 4-, 8-, and 24- hours treatments, respectively). In August, mean glycogen content (± SD) of oysters in the 0-, 4-, 8-, and 24-hour treatments was 15.5 (± 32), 28.0 (± 26), 27.5 (± 29), and 26.9 (± 37) milligrams glycogen per gram oyster tissue, respectively. In August, oysters in the 0-hour treatment had significantly less glycogen than oysters in the 4- and 8-hour treatments (Dunn test adjusted P = 0.00 and 0.01, respectively). No significant differences were present between the 0- and 24-hour treatments, although the 24-hour treatment data yielded higher variance. Overall, oysters that experienced any desiccation (4-, 8-, and 24-hour treatments) had more glycogen than oysters from the 0-hour treatment in July, but in August, only oysters in the 4- and 8- hour treatments had elevated glycogen content.

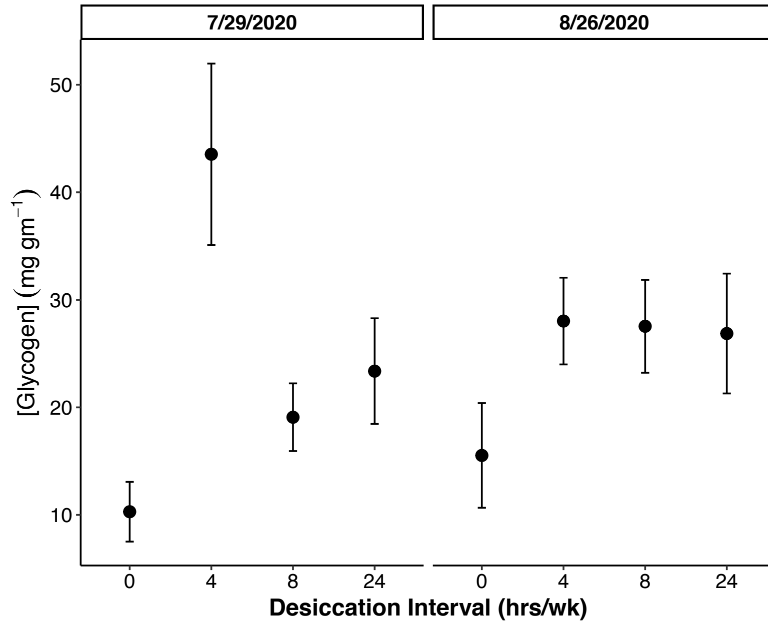

Figure 24. Mean glycogen content (in mg glycogen per g dry oyster tissue weight, ± standard error of the mean) of three tissue types (mantle, gill, adductor) from oysters desiccated for 0, 4, 8, and 24 hours weekly in July (left) and August (right) of 2020.

HSP70 expression

Heat shock protein expression (nanograms of HSP70 per milligram of total protein in oyster gill tissue) was not affected by desiccation treatment (ANOVA P = 0.14) or sampling date (ANOVA P = 0.11, Figure 5). In the July sampling, higher mean (± SD) HSP70 levels were observed in oysters from the 4-, 8-, and 24-hour treatments (HSP (ng/g) of 29.2 (± 5), 16.9 (± 9), and 37.9 (± 37) compared to the 0-hour treatment (9.7 (± 4)), but these differences were not statistically significant (ANOVA P = 0.35). In August, the 0- and 4-hour treatments yielded higher HSP70 concentrations (55.2 (± 33) and 56.9 (± 8), respectively) compared to the 8- and 24-hour treatments (10.3 (± 12) and 29.2 (± 25), respectively), but again, the differences in mean HSP expression between treatments was not confirmed statistically (ANOVA P = 0.09). Oysters

in the 4-hour treatment expressed significantly more HSP70 in August compared to July (t-test P = 0.01). Oysters in the 0-hour treatment also expressed more HSP70 in August than they did in July but this relationship was not significant (t test P = 0.14) and variance among oysters was high in August. Oysters in the 8- and 24-hour treatments yielded similar levels of HSP70 in July and August with the 24-hour treatment having the highest variance.

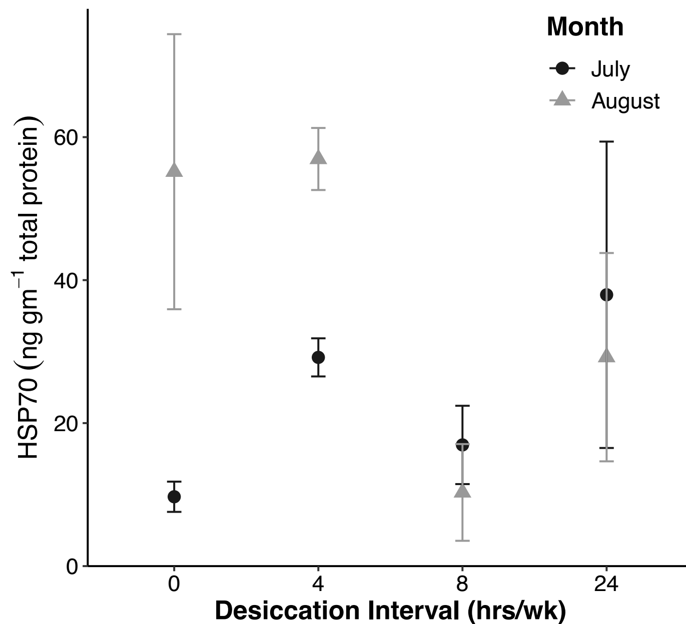

Figure 5. Mean expression (± standard error of the mean) of heat shock proteins (HSP70) in gill tissue from oysters desiccated for 0, 4, 8, and 24 hours weekly between July-September 2020. The first sampling (July) is represented by black dots and the second sampling (August) is represented by gray triangles.

Discussion

Desiccation is an effective biofouling control technique on eastern oyster farms, yet extended periods of desiccation can lead to reduced yield compared to non-desiccated oysters or

those desiccated less frequently (Bodenstein et al., 2021; Chapman, 2019; Gamble, 2017; Kirk, 2019). The objectives of this short-term (3 month) study were to better understand the physiological responses of *C. virginica* to desiccation during summer conditions in the Chesapeake Bay region, using 0, 4, 8, and 24 hours of weekly desiccation as the desiccation intervals. Gross indicators of oyster stress were measured via growth and mortality, while more detailed molecular and physiological responses were measured through quantification of gametogenic stage, filtering rates, glycogen content, and heat shock protein expression. Oysters in the 8- and 24-hour treatments were smaller (dry weight) than oysters in the 0- and 4-hour treatments as seen in previous studies (e.g. Chapman, 2019; Gamble, 2017; Kirk, 2019), but mortality was not affected by desiccation treatment. During the mid-summer (late July) samplings, oysters in the 0-hour treatment had more developed gonad and less glycogen in their tissues compared to the treatments that spent time exposed to the air. By late August, gonad scores were higher in the 24-hour treatment due to the presence of more post-spawning oysters, but this relationship was only significant when compared to the 4-hour treatment. Analyses of oyster filtering rates revealed that oysters in the 0- and 4-hour treatments filtered algae at a significantly higher rate than oysters in the 8- and 24-hour treatments after desiccation in August, but differences in filtering after 4 days of re-submergence were not observed and no differences were observed in the September filtering trial. Below, I discuss effects of desiccation on the measured molecular and physiological responses in oysters and the implications of these results for refining desiccation as a biofouling control strategy to minimize oyster stress.

Effect of desiccation on growth and mortality

Reduced growth of oysters desiccated for 24 hours weekly compared to those desiccated less frequently (i.e. biweekly, monthly) has been observed in previous studies (Chapman, 2019;

Gamble, 2017; Kirk, 2019), and a similar result was found here when frequency was held constant (weekly) and interval was manipulated. Oyster size (dry weight) was reduced by an average of 10% in the longest (8- and 24-hour) desiccation treatments compared to the 0-hour treatment, and shell height of oysters in the 24-hour treatment was reduced compared to oysters in the 0-hour treatment. While significant differences in mortality were not observed among desiccation treatments, oysters desiccated for 24 hours weekly showed the highest level of mortality, consistent with previous work (Bodenstein et al., 2021; Chapman, 2019), although the study's short duration (3 months) may have influenced results. In summary, results show that oysters desiccated for 8 and 24 hours weekly yielded less somatic growth than non-desiccated oysters but no differences were present in oysters desiccated for 4 hours weekly despite also spending less time in the water compared to oysters in the 0-hour treatment.

Post-desiccation filtering rates and treatment effects

Oyster filtering (feeding) demonstrated temporally variable responses to desiccation interval, suggesting the potential for ancillary physiological or adaptive processes to contribute to the observed differences in filtering among the desiccation treatments over time. Filtering trials revealed a short-term reduction in oyster filtering post-desiccation in the 8- and 24-hour treatments, while no differences in filtering rates were present in oysters from the 0- and 4-hour treatments post-desiccation. Oysters in the 8- and 24-hour treatments demonstrated very low filtering rates upon re-submergence in early August, despite gaping noticeably wider than oysters in the 0- and 4-hour treatments. Similarly, Frank et al., (2007) found an incongruous relationship between valve gape and clearance rate of oysters and noted that valve gape is not a reliable indicator of feeding, only accounting for 25% of variation in clearance rates. It's possible that the gaping behavior in the 8- and 24-hour treatments was due to ventilation as opposed to feeding. A

post-emersion “oxygen debt” has been reported in *Mytilus edulis*, which is repaid through ventilation (Widdows & Shick, 1985). The dramatic gaping observed among the 8- and 24-hour treatment may be an indication of ventilation and repayment of the oxygen deficit accrued during anaerobic metabolism prior to the onset of feeding (Ellington, 1983). Indeed, filtering rates were affected by desiccation treatment after desiccation on August 5, but no differences persisted after a 4-day re-submergence period, indicating that the filtering reduction in the 8- and 24-hour treatments was short lived and perhaps an acute response (i.e. ventilation to repay an oxygen debt) rather than a chronic reduction in filtering.

In contrast to the drastically reduced filtering found in the 8- and 24-hour treatments in August, no differences were found in filtering rates post-desiccation in September despite similar environmental conditions. Facultative metabolic rate depression by intertidal organisms is a plastic response which can be induced in stochastic environments under changing environmental stress (Hui et al., 2020; Storey & Storey, 2015). Prior acclimation to emersion may affect energy utilization and metabolism under desiccation stress as previous intertidal exposure has been associated with elevated ATP available during periods of emersion, indicating that acclimation may increase tolerance to desiccation and delay the onset of anaerobic metabolism (Meng et al., 2018). Exactly how long this acclimation to emersion takes is not precisely known, but hormesis, or stress-priming animals by exposing or pre-conditioning them to a stressor has been shown to enhance resilience to future stressors in a range of species, taking place with few stress exposures on limited timescales (Berry & López-Martínez, 2020). Therefore, it is possible that oysters in the 8- and 24-hour treatments began acclimating to the extended periods of emersion by September, reducing anaerobic buildup during desiccation and allowing them to resume feeding quickly upon re-submergence. This is not possible to discern concretely and the study duration

may have been too brief to allow acclimation to begin, but it is an interesting consideration for future desiccation studies. Understanding the consistency or seasonality of reduced filtering rates post-desiccation in the 8- and 24-hour treatments by conducting filtering trials on additional sampling dates would yield greater insight into the scope and magnitude of reduced filtering post-desiccation, and allow additional speculation as to its contribution towards reduced growth.

Desiccation impacts on gametogenesis and glycogen content

Gonad score and glycogen content revealed potentially interesting patterns of energy utilization and stress response between the two samplings, although the relative impact of treatment (desiccation) stress versus spawning stress is difficult to disentangle. Gonad scores in July (anticipated pre-spawning period) indicated more developed gonads in oysters from the control (0-hour) treatment compared to the 4-, 8-, and 24-hour treatments and glycogen content showed an inverse relationship to gonad score. This finding was expected given the important role glycogen plays in the mobilization of energy reserves for gametogenesis and spawning. Glycogen content in temperate *C. virginica* is typically highest prior to the onset of gametogenesis and depleted after spawning, gradually accumulating in tissues during the winter months before gametogenesis begins again the following spring (Aranda et al., 2014; Gabbott, 1975; Krishnamoorthy et al., 1979). Accumulated glycogen reserves are typically drawn from during periods of high metabolic demand, such as gametogenesis (Gabbott, 1975). Results observed here followed this expected pattern of glycogen use and gonad score in July but the variability in August indicates that other processes, including desiccation stress, also influenced gonad score and energy utilization.

Interestingly, mean gonad score was not an entirely accurate predictor of spawning and oysters in the longest (24-hour) desiccation treatment consistently spawned more than other

treatments, despite lower mean gonad scores and a presumed elevated level of stress. This suggests that emersion-stressed oysters were more likely spawn early. Previous research has similarly documented elevated gamete output (weight) among *Choromytilus meridionalis* (black mussels) in the intertidal compared to their subtidal counterparts (Griffiths, 1981). Reduced energy allocation toward growth and reduced metabolic rate during emersion were thought to facilitate an elevated reproductive expenditure among this group of intertidal mussels (Griffiths, 1981). Thus, similar to *C. meridionalis*, oysters that spent the longest time period exposed had reduced somatic growth and were the first to spawn. Findings were similar in August when most (80%) of oysters in the 24-hour treatment had spawned, again in agreement with Griffiths' (1981) findings. Prior research (Bernard et al., 2016; Hopkins, 1936; Nelson, 1928; Prytherch, 1929) has indicated that oyster (*C. virginica, C. gigas, O. lurida)* spawning is influenced by tide and that natural spawning takes place during the flood tide, confirming a relationship between spawning and tidal inundation. It is possible that some oysters in the 24-hour treatment spawned upon re-submergence (perhaps as a response to "high tide") despite having lower mean gonad scores overall, although this does not explain why oysters in the 4- and 8-hour treatments did not yield a similar response. Serotonin regulates reproduction among other physiological processes in bivalves (Alavi et al., 2017) and increases with increasing intervals of air exposure (Dong et al., 2017). Indeed, the addition of serotonin has been used to successfully induce spawning in bivalves, including *C. virginica* (Gibbons & Castagna, 1984). Although it was not measured here, it is possible that elevated serotonin in oysters from the 24-hour treatment may have contributed to more oysters spawning in that treatment compared to oysters in the other (0-, 4-, 8-hour) treatments.

In summary, oysters in desiccated treatments showed lower mean gonad scores and higher glycogen content than oysters in the 0-hour treatment in July but results were more variable in August although the longest desiccation interval (24-hour) again showed more post-spawning oysters. Thus, a consistent response of desiccation, at least for the longest interval, was increased spawning, possibly indicating preferential allocation of energy towards reproduction at the expense of somatic growth of oysters in the 24-hour treatment. Oyster energetics and spawning are complicated processes and it is likely that observed differences are the results of an interplay between spawning, glycogen utilization, and other factors, with desiccation as an added stressor potentially influencing energy allocation and use. Monitoring glycogen and gonad score on additional dates (i.e. biweekly or monthly from April through October) would perhaps have allowed additional trends to become apparent regarding energy allocation and changes with respect to gametogenic stage and treatment. It is likely that multiple factors contributed to the differences in gonad score and glycogen content observed here, but it is not possible to discern the exact cause of the observed differences.

Treatment effects on heat shock protein expression

Contrary to expectations, analyses of heat shock protein expression did not yield statistically significant results (no treatment effects) but interesting patterns of expression were observed among the treatments and among sampling dates. Upregulating heat shock proteins is energetically expensive (Fabbri et al., 2008), but confers stress tolerance to oysters (Clegg, 1998; V. G. Encomio & Chu, 2007), allowing them to protect cells from environmental stressors, repair damaged proteins or eliminate denatured proteins (Casas & La Peyre, 2020; Fabbri et al., 2008; Feder & Hofmann, 1999). In the present study, oysters in the 0- and 4-hour treatments mounted a greater expression of heat shock proteins in August compared to oysters in the 8- and 24-hour

treatments, although the high variance of HSP expression in the 0-hour treatment indicates that other processes, outside of the desiccation stress, were also acting on the oysters and influencing this response. Oysters in the 8- and 24-hour treatments may have lacked the available energy necessary for production of inducible HSPs, driving their reduced HSP70 expression and rendering them less tolerant of stressful conditions. Oyster dry weight was also reduced in the 8- and 24-hour treatments (gross stress response) and conditions were undoubtedly stressful for these cohorts given ambient temperatures during the emersion periods, yet they were unable to mount a defense via upregulated HSP70 to aid in their stress resilience. Findings are in agreement with Li et al., (2007), who found post-spawning diploid oysters subject to elevated temperatures had a reduced expression of HSPs. Similarly, Ivanina et al., (2009) found reduced HSP levels in oysters exposed to heat stress and metal pollution. Greater temporal resolution (additional sampling dates) may provide clarity on when HSPs were mobilized by oysters to protect cells, and under what conditions their expression became suppressed. However, results of this study indicate that oysters in the 0- and 4-hour treatments were able to mobilize HSP70 to aid in their stress resilience to a greater extent than oysters in the 8- and 24-hour treatments, which concurs with previous literature on HSPs and stress response. Overall, results suggest greater stress in August compared to July, as evidenced by elevated HSP70 expression in the 0- and 4-hour treatments, but the lack of response from the 8- and 24-hour treatments indicates that while HSPs can aid in stress resilience, these molecular chaperones may not be readily upregulated in all conditions, particularly during periods of high or concomitant stressors.

Conclusions and implications for oyster aquaculture

Weekly desiccation is a proven technique for biofouling control on oyster farms, but comes with a tradeoff in the form of reduced growth or elevated mortality among the desiccated

oysters (Bodenstein et al., 2021; Chapman, 2019; Gamble, 2017; Kirk, 2019). Balancing crop quantity (growth and survival) and quality (low biofouling) are important considerations for oyster farmers in evaluating new techniques or husbandry methods on their farms. Here we provide novel insight into gross and fine-scale physiological responses of oysters to desiccation stress and contribute an advanced, albeit complicated picture of desiccation-induced stress to oysters. Results herein support the existing body of research regarding the gross stress responses of *C. virginica* (reduced growth) to long (24-hour) weekly periods of desiccation (Bodenstein et al., 2021; Chapman, 2019; Gamble, 2017; Kirk, 2019), and contribute new information on oyster stress responses to shorter (4- and 8-hour) desiccation, demonstrating how oyster physiology may mitigate this stress response.

The effect of desiccation treatment on physiological pathways that ultimately influence oyster energetics and growth is difficult to isolate given the interdependent nature of many aspects of oyster physiology, and a complicated picture of oyster stress response has been revealed. Desiccation treatment affected gonad development, a major energetic draw for *C. virginica* (Dame, 1976; Gabbott, 1975; Li et al., 2007), although different gonad score and glycogen content responses between the 8- and 24-hour treatments indicate that reproductive investment at the expense of somatic growth was not the sole cause of reduced growth of oysters in both treatments. Mobilization of molecular chaperones (HSPs) to protect cells during environmental stress was not a consistent or linear response to desiccation interval, suggesting that this, too, cannot be identified as a primary energetic draw on oysters in the 8- and 24-hour treatments that led to their slower growth. However, the inability of oysters in the 8- and 24-hour treatments to upregulate these HSPs to protect cells may have compromised their resilience to desiccation. Ultimately, oysters in the 8- and 24-hour treatments spent less time in the water than

oysters in the 0- and 4-hour treatments, reducing the time available to them for feeding and aerobic respiration. Additionally, oysters in these longer treatments (8- and 24-hours) filtered less phytoplankton upon re-submergence, but the temporal limitation to the reduced filtering suggests it may not be fully responsible for the significantly reduced growth of oysters in those treatments. In contrast, oysters in the 4-hour treatment did not suffer a growth or filtering penalty and were able to mobilize HSPs to protect cells during desiccation. Oysters in the 4-hour treatment consistently had more glycogen in their tissues than oysters in the 0-hour treatment, an important consideration from both an energetics perspective and a consumer perspective since glycogen influences the texture, quality and color of oyster meat (Liu et al., 2020). Collectively, results suggest that desiccating oysters for 4 hours weekly imposed less stress on oysters than an 8- or 24-hour weekly desiccation, and did so without yielding any reduction in growth, suggesting this as a biofouling control strategy with a low likelihood of a detectable oyster stress response.

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Chapter 5: Conclusions and considerations for oyster farmers

The overall objective of my dissertation was to understand the response of biofouling organisms and oysters to weekly desiccation treatments with the intent of identifying a desiccation interval that controls biofouling without compromising oyster growth. Biofouling is a major challenge to the oyster aquaculture industry, disrupting farm productivity, reducing yield, and increasing costs. Evaluating techniques to control biofouling while also considering impacts to the oysters is important, as both impact farm operations. Such an approach is expected to enhance applicability of results in evaluating fouling control techniques (in this case, desiccation), potentially reducing a burden to farms and facilitating greater industry growth.

Major findings on desiccation as biofouling control

Desiccation tolerance varied among the species observed, and an examination of the habitat preferences of observed species suggests desiccation may be a more suitable technique to control subtidal species, while effectiveness in controlling intertidal species may be more variable, particularly for the shortest (4-hour) desiccation interval. Findings from Chapters 2 and 3 revealed that species consistently controlled by desiccation (*Balanus improvisus, Cordylophora caspia, Conopeum tenuissimum, Diadumene leucolena, Garveia franciscana,* and *Membranipora tenuis)* primarily inhabit the subtidal zone and those not consistently controlled by desiccation among the sites (*Mytilopsis leucophaeata, Ulva intestinalis,* and *Victorella pavida)* are often found in the intertidal zone (SERC, 2022). Species-specific responses to desiccation were also documented in Hopkins et al. (2016) where the species common in the intertidal (*Crassostrea gigas* and *Mytilus galloprovincialis)* demonstrated longer desiccation tolerance than subtidal species (*Ciona* spp.). While it is not possible to speculate on the

suitability of desiccation to control species not observed in the studies included in this dissertation, an evaluation of the habitats occupied by other fouling species may lend insight into their potential desiccation tolerance. Future studies of desiccation or other biofouling control techniques may consider species-level monitoring in order to suggest groupings of biofoulers based on habitat preference or other life history traits. In summary, the habitat preferences of the biofouling species present at a farm may influence the necessary interval of desiccation, and perhaps the effectiveness of desiccation altogether.

In addition to species-specific differences, site-specific differences in treatment effects were also observed, which may be a reflection of environmental or ecological pressures affecting biofouling organisms. While desiccation stress is one stress acting upon the biofoulers, other factors such as timing/chronological order of settlement and spatial competition are also important mediators of colonization (Dayton, 1971; Lubchenco and Menge, 1978; Menge, 1995). Site-specific responses of species to desiccation treatments may be influenced by order of settlement and interspecific competition, particularly evident here in the case of *U. intestinalis*. *Ulva intestinalis* was not controlled by any desiccation treatment (4-, 8-, or 24-hours) at the site where it was the dominant fouler (Chapter 2, Back Creek, Site B). However, where spatial competition was present from *V. pavida* (Chapter 3, Choptank River), *U. intestinalis* abundance remained low, perhaps keeping it from reaching a density at which it could tolerate desiccation (McAllen, 1999) and facilitating effectiveness of the desiccation treatments. These observations reflect conditions at two different locations in different years so caution should be applied in making comparisons, but it is interesting that different treatment responses were observed in conjunction with different levels of competition. Future studies may consider deploying new, clean cages and oysters periodically (i.e. monthly) to capture treatment effects under more

realistic conditions since farms tend to deploy and/or move oysters at various time points as opposed to deploying all oysters at a single time point. Understanding how desiccation treatments perform across multiple deployments (and hence varying colonization and competition pressures) could yield useful information regarding the broad-scale effectiveness of desiccation using typical farm operating procedures.

Insights into desiccation as a stressor

Desiccation reduces total biofouling, but the specific characteristics associated with desiccation (i.e. humidity, temperature, sunlight, etc.) that lead to fouler stress or death are not precisely understood. In practice, weekly desiccation serves to disrupt settlement, metamorphosis and/or growth of newly settled juvenile fouling organisms (<6 days post-set). The manipulation of desiccation interval strategically employs the stress of desiccation to biofoulers as a benefit but seeks to minimize the negative effects of this stress on the oysters. While the overall stress response is known (death or reduced growth of biofoulers), we do not yet fully understand exactly which conditions associated with desiccation lead to this gross stress response in the biofoulers. Hopkins et al. (2016) demonstrated the importance of conditions during desiccation, with biofoulers surviving in air for longer during more humid conditions. Indeed, of the three sites included in Chapter 2, the site that tended to desiccate on more humid days was the only site where desiccation did not lead to a reduction in biofouling, indicating that conditions during emersion, particularly humidity, may be an important factor. However, effects of sunlight, air temperature, wind speed, and other conditions associated with desiccation have not been quantified with respect to stresses to the biofoulers or the oysters. Such information would be helpful to growers who seek to optimize their farm management and may be considerations for future research.

Overall findings suggest that oysters desiccated for 8 and 24 hours weekly were stressed, which was evidenced by reduced growth, reduced dry weight, and the results of molecular and physiological investigations. Growth rates of oysters in the 24-hour treatment (Chapter 3) were 50% lower (0.3mm/week) than growth rates of oysters in the 0-hour treatment (0.6mm/week), a considerable growth penalty that merits diligent consideration in assessing the utility of this treatment. The molecular and physiological responses of oysters to desiccation (Chapter 4) shed light on how desiccation interval affects critical cellular, energetic, and developmental processes of *C. virginica,* although their utility in refining the desiccation practice may be of limited value. Physiological data were highly variable, as they often are, making it difficult to disentangle treatment effects versus impacts of other biological and environmental parameters. Despite the limitations of the measured physiological data towards the practical application of desiccation, interesting findings were still observed. Investigations revealed reduced filtering post-desiccation and lack of upregulation of protective molecular chaperones (HSPs) by oysters in both the 8- and 24-hour treatments, and elevated reproductive effort in the 24-hour treatment. Results indicate that a combination of factors, in addition to reduced time spent in the water for feeding, may contribute to the reduced growth observed in the 8- and 24-hour treatments. Indeed, oysters in the 4-hour treatment also spent less time in the water than oysters in the 0-hour treatment, and if no other factors were acting on the oysters, the magnitude of reduced growth should mirror the reduced time spent in the water (Gillmor, 1982). That was not the case here, evidenced by the lack of a growth penalty in oysters from the 4-hour treatment. It is likely that a combination of factors including reduced time in the water engaged in aerobic respiration and feeding, reduced filtering upon re-submergence, and general stress condition contributed to the slower growth observed in the 8- and 24-hour treatments, and perhaps the limited fouling of oysters in the 4-

hour treatment contributed to their strong growth despite the time spent out of the water. While the detailed physiological data are interesting, their use to the oyster aquaculture industry may be minimal since the gross stress response (growth and mortality) are of primary interest to farmers, not the underlying physiological responses. Future desiccation or fouling control studies may choose to omit or limit physiological measurements to those which demonstrated interesting trends (i.e. filtering rates), allowing greater replication and more focused efforts.

The oysters used in this dissertation research were naïve to the intertidal and prior acclimation to periods of emersion may influence oyster stress response (Newell & Bayne, 1973). Some studies have found enhanced growth of intertidal bivalves compared to subtidal counterparts (Bishop & Peterson, 2006; Gillmor, 1982; Littlewood et al., 1992), and other research has found that oysters acclimated to the intertidal exhibited less mortality than subtidal oysters when exposed to prolonged periods of emersion (Meng et al., 2018). Reduced growth was observed here in oysters in the 8- and 24-hour treatments (Chapters 3 and 4), and a growth penalty in oysters desiccated for 24 hours weekly has been documented previously (Chapman, 2019; Gamble, 2017; Kirk, 2019). An assessment of the potential to pre-acclimate oysters to desiccation in order to reduce a desiccation stress response (slower growth) is an interesting area to consider for future research, especially since the longest (24-hour) treatment yielded the greatest and most consistent reduction in biofouling, though the considerable growth reduction necessitates caution in applying this treatment. If pre-acclimation of oysters could enhance their tolerance of desiccation, oyster farmers could potentially benefit from the consistent fouling reduction of a 24-hour desiccation treatment and limit the reduction in growth.

Tools to aid oyster farmers in the selection of a biofouling control strategy

In developing a management plan for their farms, oyster farmers may choose to select a conservative, general purpose biofouling control strategy or to use a targeted, adaptive management approach to fouling control. Such a targeted approach would include selecting a fouling control strategy based on the species present, which requires knowledge of the species present and their susceptibility to various biofouling control techniques. Tools designed to aid oyster growers in identifying the species present on their farm and deciding on an appropriate control strategy may help. For example, Table 1 provides salinity tolerance information for common biofouling species in the Chesapeake Bay since salinity is a major driver of species assemblages in estuarine environments (Attrill, 2002; Ysebaert & Herman, 2002). Complementary visual aids (i.e. Figure 1) can help farmers in locating their farm to assess whether a given species is likely to be present in their area, allowing a process-of-elimination approach to help with species identification. Additional resources including images, description of species and proven control techniques (i.e. Gosner and Peterson, 1982; Hood et al., 2020; Lippson and Lippson, 2006, Loosanoff, 1960) can aid in species identification and selection of an appropriate fouling control strategy. However, oyster farms are dynamic environments and while biofouling control is a challenge, it is not the only challenge facing oyster farmers who must also consider other aspects of husbandry as well as marketing, sales, regulatory compliance, and other needs that arise in a commercial operation. Assessing the species present on a farm at any one time may be too time-consuming for commercial oyster growers who need to move quickly and achieve the many tasks required on a given day. For these farmers, the selection of a single, consistent biofouling control strategy may be most suitable and would require an evaluation of tradeoffs and preferences on the part of the farmer. The suitability of multiple fouling control strategies has been assessed, but it is the choice of the individual farmer to decide

whether they will apply a targeted or general fouling control strategy, as a ‘silver-bullet’ approach to fouling control remains elusive.

Table 9. Common biofouling species found on Chesapeake Bay oyster farms, along with their salinity distribution range to aid oyster farmers in identifying the species colonizing their farm.

Colloquial name	**Species**	**Salinity distribution**	**Reference**
Macroalgae	*Ectocarpus* sp.	11-30	(Lippson and Lippson, 2006)
	Gracilaria sp.	18-30	(Lippson and Lippson, 2006)
	Ulva intestinalis	11-30	(Lippson and Lippson, 2006)
	Ulva lactuca	11-30	(Lippson and Lippson, 2006)
Bryozoan	*Conopeum tenuissimum*	6-37	(Cook, 1985; Osburn, 1944)
	Membranipora tenuis	6-37	(Cook, 1985; Osburn, 1944)
	Victorella pavida	1-36	(Fofonoff et al., 2018; Lippson and Lippson, 2006)
Anemone	*Diadumene leucolena*	7-35	(Fofonoff et al., 2018)
Hydroid	*Garveia franciscana*	1-30	(Lippson and Lippson, 2006)
Tunicate	*Molgula manhattensis*	10-35	(Cory, 1967; Fofonoff et al., 2018)
Sponge	*Clathria prolifera*	15-35	(Cohen, 2011)
	Cliona celata	3-36	(Hopkins, 1962)
	Cliona truitti	14-36	(Hopkins, 1962; Old, 1941)
Barnacle	*Balanus improvisus*	3-18	(Kennedy and DiCosimo, 1983; Lippson and Lippson, 2006)
	Balanus subalbidus	1-18	(Kennedy and DiCosimo, 1983)
	Chthamalus fragilis	20-35	(Fofonoff et al., 2018; Lippson and Lippson, 2006)
	Semibalanus eburneus	20-35	(Fofonoff et al., 2018; Lippson and Lippson, 2006)

Mussel	*Ischadium recurvum*	5-35	(Allen, 1960; Fofonoff et al., 2018)
	Mytilopsis leucophaeata	1-10	(Lippson and Lippson, 2006)
Limpet	*Crepidula* sp.	20-35	(Blanchard, 2015)
Worm	*Alitta succinea*	3-35	(Fofonoff et al., 2018; Lippson and Lippson, 2006)
	Polydora cornuta	7-35	(Fofonoff et al., 2018; Lippson and Lippson, 2006)
	Polydora websteri	5-30	(Lippson and Lippson, 2006)

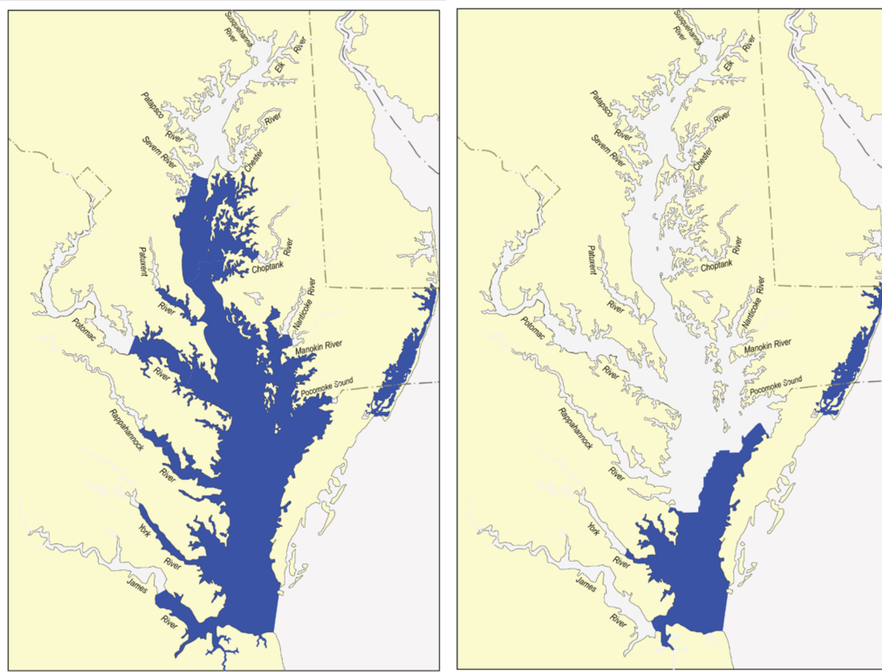

Figure 25. Example of visual aid (maps) to show oyster growers the salinity range of two common biofouling species in the Chesapeake Bay to aid in their identification of species on the farm. The maps presented show the salinity range tolerated by *Cliona* sp. (left) and *Crepidula fornicata* (right). The blue areas indicate where salinity is favorable to each species based on surface salinity data (1985 – 2018). Maps are provided to oyster growers for all common fouling

organisms in the Chesapeake Bay, along with descriptive text to help farmers identify fouling species on their farm in Hood et al., 2020.

Biofouling is one of many important aspects of farm operation to oyster growers, and farmers must evaluate the practicality of incorporating a new management technique given their preferences and infrastructure. A decision tree is a tool that can help guide an oyster farmer through a series of questions, ultimately directing the user towards a suitable end-result that reflects their preferences and operational constraints. An example of a decision tree to facilitate evaluation of the incorporation of desiccation is provided in Figure 2. Questions encourage farmers to consider their infrastructure, market, and preferences as they determine whether desiccation may be a suitable husbandry technique. The decision tree is provided to guide the decision-making process, not necessarily to make a recommendation regarding which fouling management strategy to select. It is ultimately up to the individual oyster farmer to evaluate their preferences, but a decision tree can help facilitate a thorough evaluation of important considerations.

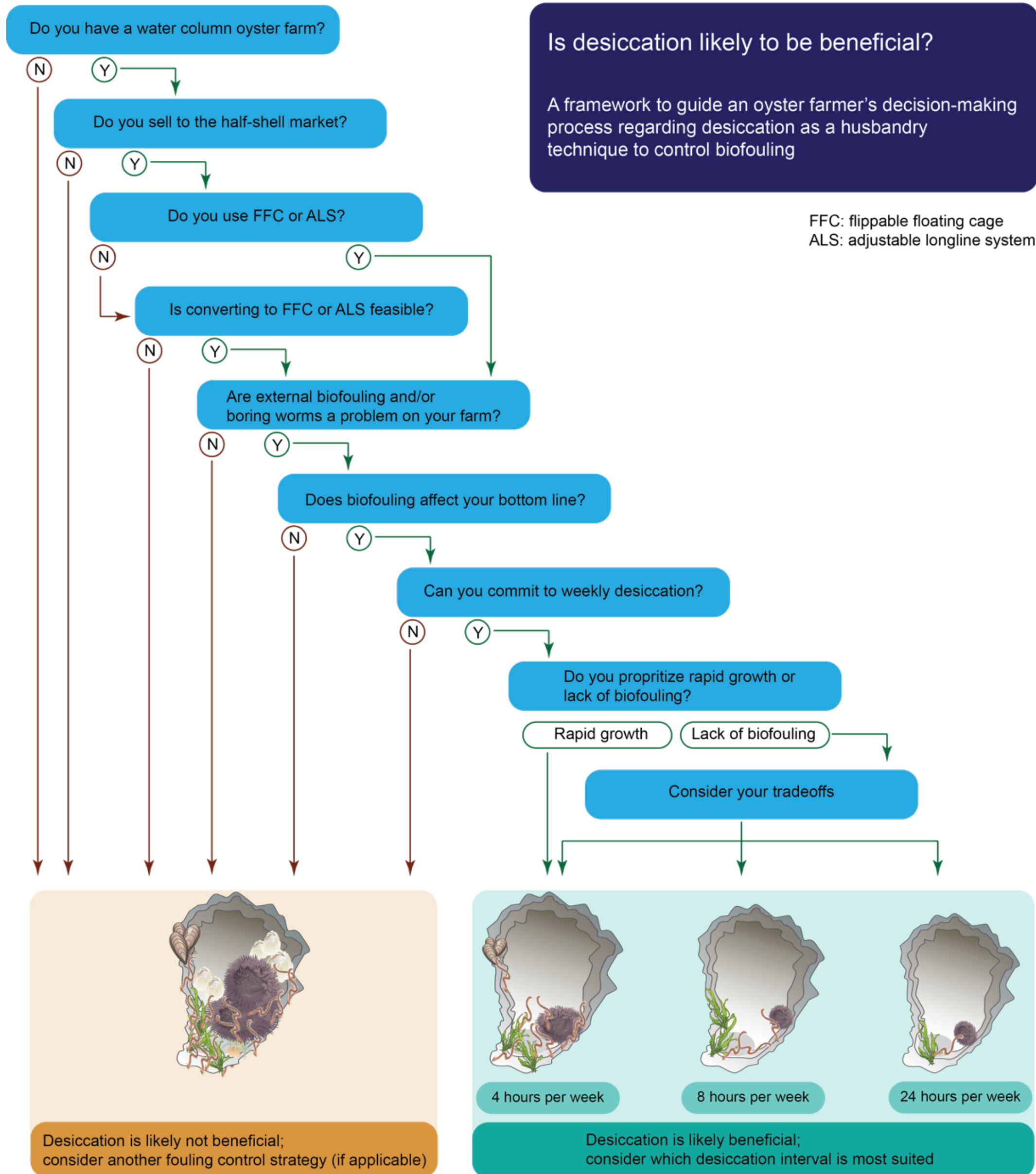

Figure 26. Example of a decision tree framework to help oyster farmers consider whether incorporating desiccation as a management strategy to control biofouling is likely to be beneficial to their operation. Note FFC stands for flippable floating cage and ALS stands for adjustable longline system. Non-desiccated oysters were, on average, 64% fouled with 55 worms per oyster. Oysters desiccated for 4 hours per week were, on average, 24% fouled and had a 44% reduction in worm abundance compared to the 0-hour treatment with no growth penalty. Oysters

desiccated for 8 hours per week were, on average, 18% fouled and had a 78% reduction in worm abundance compared to the 0-hour treatment, and had a 7% growth reduction. Oysters desiccated for 24 hours weekly averaged 12% fouling coverage and a 95% reduction in worm abundance, although they showed a 12% growth penalty compared to the 0-hour treatment.

Final conclusion

Desiccation can reduce biofouling without sacrificing growth, but the decision regarding whether to change farm practices to facilitate desiccation is more complicated and requires an evaluation of other biotic factors, abiotic factors, and preferences. Species-specific responses of the biofouling community require farmers to either adaptively manage their farms based on fouling present at the time (which may be unfeasible for many farmers), or to select a generally suitable desiccation interval with the understanding that the efficiency of this technique may vary based on the species present and relative fouling pressure. Additional research is warranted to fully assess economic impacts and treatment-derived benefits of desiccation. However, results of the studies encompassed within this dissertation indicate that desiccating oysters and cages for 4, 8, or 24 hours weekly can reduce biofouling, and desiccating for 4 hours does so without any reduction in growth or demonstrable stress response, although the 4-hour treatment was not consistently effective in controlling all species. Farmers must weigh their preferences regarding fouling control and yield, but results indicate that shorter, weekly desiccation intervals can control biofouling and this information, coupled with previous research, may enhance more widespread use of desiccation as a biofouling control strategy in commercial oyster aquaculture operations.

Appendix A

Surface salinity and temperature during the period of deployment (July – December 2018) were retrieved from Maryland Department of Natural Resources Eyes on the Bay Long-term Fixed Monitoring Stations. To access the data, click on the following link and use the details below.

https://eyesonthebay.dnr.maryland.gov/bay_cond/LongTermData.cfm

Site	Station	Parameters	Layer
A	Upper Eastern Shore: Lower Chester River ET 4.2	Temperature (°C), Salinity	Surface
B	Chesapeake Bay Mainstem: Cedar Point CB5.1	Temperature (°C), Salinity	Surface
C	Lower Eastern Shore: Fishing Bay EE3.0	Temperature (°C), Salinity	Surface

Appendix B

Weather data during periods of desiccation were retrieved from the National Oceanic and Atmospheric Administration's Local Climatological Data Set.

To access the data during the period of deployment (July – December 2018), click on the following link and use the details below. Desiccation began at 7:00am and continued until 3:00pm for the 8-hour treatment and 7:00am (following day) for the 24-hour treatment.

https://www.ncdc.noaa.gov/cdo-web/datatools/lcd

Site	Station ID	Desiccation Days
A	WBAN: 00124	Wednesdays
B	WBAN: 93720	Mondays
C	WBAN: 93720	Wednesdays

Appendix C

Daily measurements of salinity, temperature, and pH were collected at the study location in the Choptank River, MD (38° 35' 35.556" N, 76° 7' 43.932" W) using a YSI Pro1030 handheld meter. Data can be retrieved at the following link:

https://figshare.com/articles/dataset/Choptank_River_salinity_temperature_and_pH_May-November_2019/21231869

Appendix D

Weather data during periods of desiccation were retrieved from the National Oceanic and Atmospheric Administration's Local Climatological Data Set.

To access the data during the period of deployment (May – November 2019), click on the following link and navigate the portal to site WBAN: 00356 for Wednesdays, which is when desiccation intervals were applied. Desiccation began at 8:00am and continued until 12:00pm for the 4-hour treatment, 4:00pm for the 8-hour treatment, and 8:00am (following day, Thursday) for the 24-hour treatment.

https://www.ncdc.noaa.gov/cdo-web/datatools/lcd

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