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Co-Occurring Hab Species And Phycotoxins: Interactions With Oysters

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Co-occurring HAB Species and Phycotoxins: Interactions with Oysters

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Dedicated to my Mom & Dad
for introducing me to the ocean
and encouraging me to pursue my dreams

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ABSTRACT

Harmful algal bloom (HAB) events are generally marked by the over-abundance of one particular HAB species. Co-occurrence of multiple HAB species or HAB toxins, especially at low cell or toxin concentrations, is common. While much research has been dedicated to understanding the detrimental effects of individual HAB species and toxins on human health and the environment, implications of HAB co-occurrence for seafood safety and shellfish health are poorly understood.

Oysters support economically-valuable fisheries and aquaculture worldwide, however, oysters encounter co-occurring HAB species and toxins in their environment. Some HAB species and toxins are harmful to oyster health, harming the immune system, reducing feeding rates, or causing mortalities. Additionally, oysters co-accumulate HAB toxins; some associated with human health syndromes, such as diarrhetic shellfish poisoning. To support productive and safe oyster industries, the effects of co-occurring HAB species and toxins on larval oyster health, and the bioaccumulation of multiple toxins in adult oysters in the Chesapeake Bay, were investigated.

The health and survival of larval oysters is paramount to shellfish productivity. Individual and combined effects of co-occurring HAB species and toxins were assessed using multiple series of 96-h bioassays with larval oysters; larval inactivity and mortality were measured throughout. *Karlodinium veneficum* and *Prorocentrum cordatum* are co-occurring HAB species associated with shellfish health issues. Independently, low cell concentrations of either species caused larval inactivity. Additionally, *K. veneficum* swarmed larvae and caused significant larval mortalities. The co-occurrence of *P. cordatum* did not alter the larval effects of *K. veneficum*. Separate bioassays examined co-occurring *Alexandrium catenella* and *Dinophysis acuminata*, and associated toxins: saxitoxin (STX), okadaic acid (OA), and pectenotoxin-2 (PTX2). Exposure to live *A. catenella* caused larval inactivity, while exposure to either species caused larval mortalities. Exposure to *D. acuminata* lysate or PTX2 also caused larval mortalities, with *A. catenella* lysate, STX, and OA exhibiting no significant larval inactivity or mortalities. Larval effects during lysate or toxin co-exposure were driven by *D. acuminata* lysate or PTX2, respectively. In both bioassays, the observed larval effects of co-exposure were driven by one HAB species or toxin.

To inform seafood safety management, baseline HAB toxin data from Chesapeake Bay adult oysters were collected over two years. Azaspiracids (AZA1, AZA2), domoic acid (DA), OA, dinophysistoxin-1 (DTX1), PTX2, karlotoxins (KmTx1-1, KmTx1-3), goniodomin A (GDA), and microcystins (MC-RR, MC-YR) were detected in oysters. Regulated toxins were well below seafood safety limits, however, the presence of hepatotoxic, freshwater MCs in estuarine oysters reflects an urgent need for regulation of these toxins in seafood. Co-accumulation of toxins was common. Furthermore, solid phase adsorption toxin tracking devices (SPATTs) were co-deployed with oysters to assess additional methods of toxin monitoring. SPATTs provided additional toxin data that complemented, but could not replace oyster toxin data.

As HAB species ranges shift and the need for sustainable shellfish aquaculture increases, so too does the need for understanding combined effects of HABs on shellfish, and the potential for toxin co-accumulation within shellfish. Regional and species-specific studies like these can inform and enhance HAB monitoring, mitigation, and management strategies.

Co-occurring HAB Species and Phycotoxins: Interactions with Oysters

INTRODUCTION

Harmful algal blooms (HABs) consist of certain species of phytoplankton that are associated with causing harm to aquatic and/or terrestrial life. HAB events tend to be seasonal, region-specific, and ephemeral. Some of the most famous HAB species are associated with serious human health syndromes like paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), or amnesic shellfish poisoning (ASP). These syndromes are caused by human consumption of filter-feeding shellfish that have bioaccumulated potent toxins produced by the harmful algae (Shumway 1990).

In most regions that experience HAB events, there are periods of time when more than one HAB species is present, potentially simultaneously exposing shellfish to multiple HAB toxins. The HAB species *Dinophysis acuminata* and *Alexandrium catenella*, which produce DSP and PSP toxins respectively, co-occur in Long Island Sound and Nauset Marsh on Cape Cod in the late Spring months (Hattenrath-Lehmann et al. 2013, Brosnahan et al. unpublished). This can lead to co-accumulation of HAB toxins in shellfish, as has recently been seen with DSP and PSP toxins (Hattenrath-Lehmann et al. 2018). Additionally, *Pseudo-nitzschia* spp., associated with ASP, and *D. acuminata* co-occur from February to April in the northern reaches of the Chesapeake Bay (Thessen and Stoecker 2008, Marshall and Egerton 2009).

HAB events are not only an important concern for human health, some can also negatively impact marine life, including shellfish. Laboratory studies have demonstrated that some HAB species are detrimental to the health of commercial bivalve species, e.g., the eastern oyster (*Crassostrea virginica*), and hard clam (*Mercenaria mercenaria*, Place et al. 2008, May et al. 2010, Gaillard et al. 2020). Even *A. catenella* and *D. acuminata* can affect the shellfish immune system (reviewed in Lassudrie et al. 2020). While adult

shellfish can exhibit avoidance behavior to temporarily avoid interacting with HAB species and their toxins (Ray and Aldrich 1966, Sievers 1969, May et al. 2010, Basti et al. 2011), early life stages are less capable of exhibiting avoidance behavior, as larval shellfish must swim to feed and respire. In laboratory studies, the Chesapeake Bay HAB species: *Karlodinium veneficum*, *Margalefidinium polykrikoides* (prev. *Cochlodinium*), and *Alexandrium monilatum*, have been shown to increase mortality to *C. virginica* early life stages (Gobler et al. 2008, Stoecker et al. 2008), and *Prorocentrum cordatum* (prev. *P. minimum*) caused 100% mortality in a study with juvenile *C. virginica* (Luckenbach et al. 1993). In the northern Chesapeake Bay, *K. veneficum* and *P. cordatum* co-occur in the late spring, early summer months, around the same time of year that *C. virginica* spawns (Glibert et al. 2007), increasing the likelihood of HAB co-exposure during a sensitive developmental life stage, and possibly having implications for recruitment. Currently, Virginia leads the East Coast in eastern oyster production (Hudson 2019), with oyster larvae often spawned and raised in oyster hatcheries. A healthy, reliable supply of oyster larvae is required for successful oyster fisheries and aquaculture. Additionally, high demand in the U.S. for oysters on the half shell has left room in the market for additional growth in this industry (Botta et al. 2020). Concerning both seafood safety and shellfish health, exposure of shellfish to HABs or HAB toxins could have important implications for sustainability of the shellfish industry.

Little research (*in vivo* or *in vitro*) has been done on the effects of co-occurring HAB species or toxins on either seafood safety or shellfish health (reviewed in Alarcan et al. 2018). Co-exposure studies, like multi-stressor, co-infection, and combined effects studies, attempt to assess the combined impact of multiple variables on an organism or

system. One of the goals of these types of studies is to more accurately reflect the complexities found in nature. Traditional studies generally try to single out one variable and measure the system's response to changes in that variable in the [hopeful] absence of changes in other variables. While this method is convenient and often essential to understanding causality, this traditional method fails to show how multiple variables may interact to induce a new outcome that could not have been predicted based on studying these variables independently. Two variables may have additive, synergistic, or antagonistic effects (Chou and Talalay 1984). If exposure to multiple HAB species or toxins leads to a synergistic effect, current modes of HAB management (i.e., closing shellfish harvest or changing hatchery spawning seasons based on individual toxin limits in edible shellfish meat or HAB cell concentrations in the water column) may not be adequate to protect human health during co-occurrence (Brooks et al. 2016).

To better appreciate the effects of common, co-occurring HAB species and toxins on larval oysters, a series of bioassays was completed to determine the individual and combined effects of two sets of different HAB species. A field study was used to determine the spatiotemporal distribution of phycotoxins in adult oysters and the prevalence of phycotoxin co-accumulation. Additional methods for collecting phycotoxin data were assessed in relation to oyster phycotoxin accumulation.

Effects of co-occurring HAB species on larval oysters

Many HAB species produce toxins that have direct deleterious effects on the feeding behavior and survival of invertebrate larvae. These toxins are diverse both in chemical form and mode of action, making interactions with grazers species-specific (Turner et al. 1998). Understanding how HAB species may impact larval oyster survival

will provide a better understanding of the environmental impacts of these natural events, and may provide insight into which HAB cells and toxins should be prioritized for management and mitigation.

Larval oysters encounter and interact with HAB toxins in either particulate form, i.e., intracellular to the HAB cells or sorbed onto other particulate matter, or in dissolved form, i.e., extracellular to the HAB cells (**Fig. 1**). Shellfish can uptake some HAB toxins from both particulate and dissolved forms, generally through their digestive glands, or gills and other superficial tissues, respectively (Jauffrais et al. 2013, Gibble et al. 2016). Routes of exposure to HAB toxins also vary by shellfish life stage, due in part to the fact that earlier life stages may be too small to consume larger HAB species; i.e., intracellular HAB toxins are not accessible to shellfish that are too small to consume the HAB cells. The bioavailability of these toxins to the shellfish is affected by the persistence of those toxins in the media over time, the physical and chemical properties of the toxin (i.e., size and hydrophobicity), and interactions those toxins undergo with other chemicals or particulate matter present in the media.

When investigating the possible impacts of HAB species on shellfish health, in addition to considering routes of exposure and bioavailability of HAB toxins, behavior of the HAB cell should be considered. Many HAB cells release extracellular bioactive compounds that deter grazers (Adolf et al. 2007, Place et al. 2008, Tillmann et al. 2008, Waggett et al. 2008). It is possible that the presence of larval oysters alters the suite of extracellular bioactive compounds released into the media by the HAB cells (Castrec et al. 2018, 2020). The use of a variety of bioassay HAB treatments (i.e., live cell, lysate, pure toxin) may help elucidate the mechanisms of HAB toxicity. Previous studies have

inferred the effects of HAB toxins by comparing the effects of toxigenic and non-toxigenic strains of the same HAB species (Castrec et al. 2020, Lassudrie et al. 2020). While costly, directly testing pure HAB toxins rather than comparing HAB cell strains, has the advantage of eliminating a number of important confounding factors in a bioassay. These factors include physical interactions between the HAB cells and the larval oysters, indirect effects on the larval oysters through alteration of water chemistry by respiring HAB cells, and the effects of other bioactive compounds produced and released by the HAB cells (**Fig. 1**). Furthermore, a HAB lysate treatment provides additional information to help interpret observations from HAB live cell and pure toxin treatments. By killing the HAB cells through lysis, the lysate treatment removes any physical interactions between HAB cells and larval oysters or co-administered HAB species, as well as any production of bioactive compounds by the HAB cells in response to a grazer or competitor. In the preparation of HAB lysate, the bioavailability of intracellular HAB toxins and bioactive compounds is changed. Intracellular toxins become extracellular as they are released into the media during lysing, potentially making them more accessible to the larval oysters. Additionally, the lysing process creates cell debris with ample surface area for hydrophobic HAB toxins or bioactive compounds to interact, further altering the bioavailability of these compounds.

Two sets of co-occurring HAB species were investigated for their toxicities to larval oysters. First, HAB species traditionally associated with negative impacts on shellfish health and small enough to be consumed by oyster larvae, *K. veneficum* and *P. cordatum,* and second, HAB species traditionally associated with negative implications for seafood safety and too big to be consumed by oyster larvae, *A. catenella* (associated

with PSP) and *D. acuminata* (associated with DSP). Traditional, 96-h static toxicity bioassays in well-plates were used to assess the impacts of these HABs on larval oysters, both independently and in co-exposures, using various live cell, lysate, and pure toxin treatments. These bioassays allowed for a simplified exposure design that assessed acute toxicity in a worst-case scenario with as few variables as possible, e.g., the larval oysters were not offered a non-toxic food source. The HAB toxins and bioactive compounds present in these bioassays have various modes of action, target molecules, and resulting symptoms; because of this, the universal endpoints: larval mortality and inactivity, were used to assess larval oyster health impacts of these HAB species.

Monitoring phycotoxins in oysters in the Chesapeake Bay

Given the co-occurrence of HAB species and phycotoxins in the Chesapeake Bay (Thessen and Stoecker 2008, Marshall and Egerton 2009, Wolny et al. 2020, Onofrio et al. 2021), it is not unreasonable to assume that shellfish co-accumulate phycotoxins, having implications for seafood safety and shellfish health. To-date, there have been no studies on phycotoxin spatiotemporal distribution in shellfish in the Chesapeake Bay. In fact, phycotoxin data for shellfish in the Bay is sparse. Given the presence of HAB species in the Bay that are generally associated with serious human illnesses, and despite the fact that illness associated with Virginia shellfish has not been reported to date (K. Skiles, Virginia Department of Health, pers. comm.), it is worthwhile to collect baseline phycotoxin data for the region.

Okadaic acid (OA) accumulates in shellfish tissue to cause DSP in humans, which can lead to gastrointestinal distress, diarrhea, nausea, and vomiting (reviewed in Landsberg 2002). While OA has primarily been studied in other shellfish species,

including *C. gigas, C. brasiliana,* and *Perna perna* (Mafra et al. 2015), it has also been detected in *C. virginica* tissue in the Gulf of Mexico (Dickey et al. 1992). OA toxicokinetics have been studied in mussels (*Mytilus edulis)* and other oyster species (*Ostrea edulis,* Lindegarth et al. 2009; *C. gigas* and *C. brasiliana,* Mafra et al. 2015). In general, oysters tend to accumulate less OA than mussels, and detoxify OA more slowly than mussels (Mafra et al. 2015). Given the presence of *D. acuminata* and OA in the Chesapeake and mid-Atlantic coastal bays (Marshall and Egerton 2009, Onofrio et al. 2021), local oysters should be tested for OA to assess the potential risk for DSP in the region.

Low abundances of *Pseudo-nitzschia* spp. and domoic acid (DA) have been reported in the Chesapeake Bay and coastal Eastern Shore of Virginia (Thessen and Stoecker 2008, Onofrio et al. 2021). DA can bioaccumulate in shellfish to cause ASP. Acute exposure to high levels of DA can lead to mortalities of aquatic birds and mammals, and serious illness in humans including: memory loss, disorientation, seizures, or death (reviewed in Landsberg 2002). Low-level chronic exposure to DA has also been shown to affect learning in mice (Lefebvre et al. 2017). DA production by *Pseudo-nitzschia* spp. has been confirmed along the West Coast and in the Gulf of Maine (Fernandes et al. 2014). The mid-Atlantic also harbors *Pseudo-nitzschia* spp., however, no human illnesses from regional shellfish or animal mortalities have yet been reported, and little is known about the relative toxicity of local strains or DA bioaccumulation in Chesapeake Bay seafood products.

Thessen and Stoecker (2008) suggested that the coastal bays of Maryland, Virginia, and Delaware may be of more concern for DA bioaccumulation in shellfish than

the Chesapeake Bay. Within the Bay, Thessen (2007) describes the "ideal vector" of DA as a species that lives in high salinity regions of the Chesapeake Bay and that feeds on phytoplankton in winter and early spring. She suggested that *M. mercenaria* or the hooked mussel (*Ischadium recurvum*) might be good vectors for accumulating DA in the Chesapeake Bay region. While no field studies have assessed DA accumulation in the Bay's shellfish, laboratory research has investigated DA uptake, biotransformation, and elimination in *C. virginica* (Mafra et al. 2009). Like with OA, DA accumulation has been found to be higher in mussels than in oysters (Mafra et al. 2010a), emphasizing the need for species-specific studies. Depuration of DA in *C. virginica* is size-dependent and can take up to two weeks (Mafra et al. 2010b). The prevalence of DA in Chesapeake Bay's local market-size shellfish has not been evaluated on a temporal or spatial scale. Such an assessment, however, is necessary to assess the risk of ASP in the region.

Adult oysters were deployed throughout the Virginia-portion of the Chesapeake Bay to assess spatiotemporal distribution and bioaccumulation of phycotoxins, and furthermore, to determine if co-accumulation of phycotoxins in oyster tissue was prevalent. This information is vital to protecting both shellfish health and seafood safety. Currently, most phycotoxin monitoring for seafood safety relies on the collection and testing of shellfish. This requires the purchase or collection of shellfish, and that the shellfish have adequate time to equilibrate to conditions at the location of deployment. In some cases, harvesting shellfish for monitoring has become challenging as sentinel shellfish populations have dwindled (Lane et al. 2010). Additionally, shellfish extractions to detect and/or quantify phycotoxins are inherently "dirty", i.e., the extraction contains other compounds that may interfere with the detection of phycotoxins (MacKenzie et al.

2004). Furthermore, shellfish can metabolize some phycotoxins, transforming them and making their detection and quantification more difficult (Hess and Nicolau 2010).

Given the cumbersome nature of directly testing shellfish tissue for phycotoxins, additional methods for collecting phycotoxin data were assessed in relation to oyster phycotoxin accumulation. This included the use of co-deployed solid phase adsorption toxin tracking devices (SPATTs). SPATT technology consists of a porous bag or disk that is filled with a resin that extracellular/dissolved phycotoxins or other compounds sorb to over the time of deployment, i.e., time-integrative sampling (MacKenzie et al. 2004, Fux et al. 2008). The sorbed compounds can be recovered off of the SPATT resin in a laboratory, for identification and quantification. Resins with various properties can be used to help target the compounds of interest, and have been used to sample a wide variety of phycotoxins (Roué et al. 2018, Onofrio et al. 2021). SPATTs have the advantage of allowing detection and quantification of phycotoxins in their parent, i.e., original, form (Fux et al. 2008).

Originally, SPATTs were created in the hopes of providing an early warning system for detecting phycotoxins before shellfish accumulated enough phycotoxin to present a seafood safety concern (MacKenzie et al. 2004), however, SPATTs have also been used to detect new phycotoxins in a region, and to monitor phycotoxin dynamics over time (Peacock et al. 2018, Roué et al. 2018, Onofrio et al. 2021). A few studies have assessed whether SPATTs can provide useful information about phycotoxins in shellfish, with mixed results. During a *Pseudo-nitzschia* bloom in California, DA was detected in SPATTs seven weeks earlier than in mussels (*Mytilus edulis*, Lane et al. 2010). Similarly, DSTs were detected in SPATTs two to three weeks earlier than in shellfish in China and

in New York (Li et al. 2016, Hattenrath-Lehmann et al. 2018). Other studies have not found SPATTs to offer any early warning, but have found them to accumulate phycotoxins similarly to shellfish. Mussels deployed near SPATTs in Ireland and Norway accumulated AZAs and DSTs at the same time as SPATTs (Fux et al. 2009, Rundberget et al. 2009). There was also a significant correlation between DSTs in SPATTs and in mussels in New York; PSTs, however, showed no such correlation (Hattenrath-Lehmann et al. 2018). In Spain, SPATTs have been documented accumulating DSTs without any associated intoxication of mussels (Pizarro et al. 2013), raising the concern that relying too heavily on SPATTs for seafood safety monitoring could result in unnecessary shellfish harvest closures. One study has attributed the observed differences in SPATTs and shellfish phycotoxin accumulation to regional differences, suggesting that region-specific assessments are vital for understanding how SPATTs can be effectively used for phycotoxin monitoring in a particular region (Hattenrath-Lehmann et al. 2018). Furthermore, due to the region-specific effects of climate change on HAB dynamics and distribution (Gobler et al. 2017, Anderson et al. 2021, Hallegraeff et al. 2021), regional baseline phycotoxin data from SPATTs and shellfish will be an invaluable tool for future HAB forecasting, modeling, and risk assessment efforts.

An additional metric explored in this study was phycotoxin in particulate organic matter (POM, > 1 µm), taken from filtered water samples collected at oyster deployment sites at specific sampling times. This metric included intracellular phycotoxins as well as phycotoxins sorbed onto particles > 1 µm. Unlike shellfish or SPATTs, POM provided a snapshot of the phycotoxins present at a point in time rather than a cumulative or time-integrative assessment of phycotoxins. Shellfish can uptake both particulate and

dissolved phycotoxins (Jauffrais et al. 2013, Gibble et al. 2016); SPATTs sample dissolved phycotoxins, and POM samples detect particulate phycotoxins. Nevertheless, SPATTs and POM samples could help improve methods for monitoring phycotoxins in shellfish.

Research Direction

With HAB species co-occurring regionally along the eastern coast of the USA, and alongside productive and successful oyster industries, a better understanding of the combined effects of HABs and toxins on different oyster life stages, and the potential for oysters to co-accumulate phycotoxins, is needed. To begin to address the impacts of combined effects of HAB species and toxins on oysters, this research assessed the impact of co-exposure of co-occurring Chesapeake Bay summer HAB species, *K. veneficum* and *P. cordatum*, and Nauset Marsh spring species, *D. acuminata* and *A. catenella* (traditionally associated with human shellfish poisonings), on early life stages of the commercially-important oyster. Phycotoxin distribution and accumulation in adult oysters in the Virginia-portion of the Chesapeake Bay were also investigated, including co-accumulation of phycotoxins. Additionally, the use of SPATTs and POM samples in phycotoxin monitoring efforts in relation to phycotoxin accumulation in oysters was investigated. This research was driven by an interest in the ecological impacts of co-occurring HAB species and toxins, particularly in how HAB toxin combined effects could impact shellfish health and seafood safety.

OBJECTIVES

1. Investigate the effects of *Karlodinium veneficum* and *Prorocentrum cordatum*, co-occurring HAB species traditionally associated with shellfish health, on larval oysters.
    a. Live cell exposures and co-exposures to *Karlodinium veneficum* and *Prorocentrum cordatum*.
2. Investigate the effects of *Alexandrium catenella* and *Dinophysis acuminata*, co-occurring HAB species traditionally associated with seafood safety, on larval oysters.
    a. Live cell exposures to *Alexandrium catenella* and *Dinophysis acuminata*.
    b. Exposures and co-exposures to *Alexandrium catenella* and/or *Dinophysis acuminata* lysate/s.
    c. Exposures and co-exposures to pure toxin/s produced by *Alexandrium catenella* and *Dinophysis acuminata*.
3. Assess bioaccumulation of phycotoxins in oysters in the Virginia-portion of the Chesapeake Bay and methods for collecting phycotoxin data.
    a. Evaluate accumulation, co-accumulation, and spatiotemporal distribution of multiple phycotoxins in oysters deployed in the Bay.
    b. Determine relationships between oyster phycotoxin data and other phycotoxin metrics, e.g., SPATTs, POM.
    c. Examine fine-scale variation in phycotoxin distribution with depth using SPATTs and POM samples.

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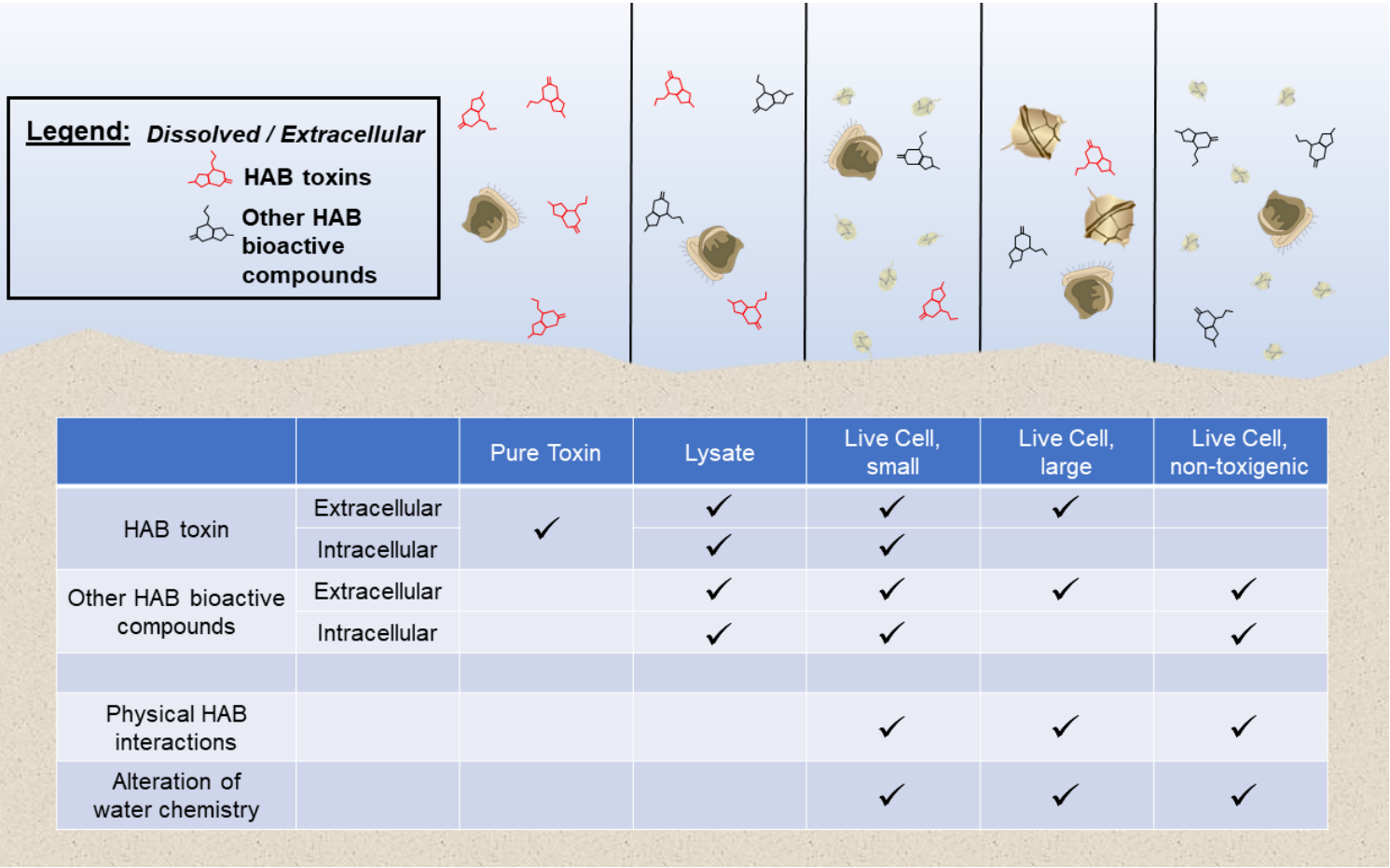

		Pure Toxin	Lysate	Live Cell, small	Live Cell, large	Live Cell, non-toxigenic
HAB toxin	Extracellular	✓	✓	✓	✓	
	Intracellular		✓	✓		
Other HAB bioactive compounds	Extracellular		✓	✓	✓	✓
	Intracellular		✓	✓		✓
Physical HAB interactions				✓	✓	✓
Alteration of water chemistry				✓	✓	✓

Figure 1
A variety of possible interactions between larval oysters and various harmful algal bloom (HAB) treatments, e.g., live cell, lysate, or pure toxin. The size of live cells determines the possible interactions between the larval oysters and the HAB species based on whether or not the cells are small enough to be consumed by the oysters. Algae and larvae art attributed to Caroline Donovan, Tracey Saxby, and Jane Thomas, Integration and Application Network (ian.umces.edu/media-library).

CHAPTER 1

Oyster hatchery breakthrough of two HABs and potential effects on larval eastern oysters (*Crassostrea virginica*)

Pease, S.K.D., Reece, K.S., O'Brien, J., Hobbs, P.L.M., Smith, J.L., 2021. Oyster hatchery breakthrough of two HABs and potential effects on larval eastern oysters (*Crassostrea virginica*). Harmful Algae 101, 101965. doi:10.1016/j.hal.2020.101965.

Note: In-hatchery HAB screening data and analyses provided by Dr. Kimberly S. Reece

ABSTRACT

Harmful algal bloom (HAB) dinoflagellate species *Karlodinium veneficum* and *Prorocentrum cordatum* (prev. *P. minimum*) are commonly found in Chesapeake Bay during the late spring and early summer months, coinciding with the spawning season of the eastern oyster (*Crassostrea virginica*). Unexplained larval oyster mortalities at regional commercial hatcheries prompted screening of oyster hatchery water samples for these HAB species. Both HAB species were found in treated hatchery water during the oyster spawning season, sometimes exceeding bloom cell concentrations (≥ 1,000 cells/mL). To investigate the potential for these HAB species, independently or in co-exposure, to affect larval oyster mortality and activity, 96-h laboratory single and dual HAB bioassays with seven-day-old oyster larvae were performed. Treatments for the single HAB bioassay included fed and unfed controls, *K. veneficum* at 1,000; 5,000; 10,000; and 50,000 cells/mL, *P. cordatum* at 100; 5,000; 10,000; and 50,000 cells/mL. Subsequently, the 1,000 cells/mL *K. veneficum* and 50,000 cells/mL *P. cordatum* treatments were combined in a co-exposure treatment for the dual HAB bioassay. At all cell concentrations tested, *K. veneficum* swarmed oyster larvae and caused significant larval oyster mortality by 96 h ($Karlo_{1,000}$: 21 ± 5%; $Karlo_{5,000}$: 93 ± 2%; $Karlo_{10,000}$: 85 ± 3%; $Karlo_{50,000}$: 83 ± 5%, SE). In contrast, there was no significant difference in larval oyster mortality between the control treatments and any of the *P. cordatum* treatments by 96 h. By 24 h, larval oysters were significantly less active (immotile) in the presence of either HAB species as compared to control treatments (e.g., $Karlo_{1,000}$: 37.8 ± 4.1%; $Proro_{100}$: 47.3 ± 7.4%; Fed: 10.8 ± 3.2%; Unfed: 10.1 ± 4.9%, SE). In the dual HAB bioassay, larval oyster mortality associated with 1,000 cells/mL *K. veneficum* (44 ± 9%,

SE) was not changed by the addition of 50,000 cells/mL *P. cordatum* (55 ± 7%, SE), demonstrating that *K. veneficum* was primarily responsible for the observed mortality. This study demonstrated that even low cell concentrations of *K. veneficum* and *P. cordatum* are harmful to larval oysters, and could contribute to reductions in oyster hatchery production through impacts on this critical life stage.

KEYWORDS

Harmful algal bloom, *Karlodinium veneficum*, *Prorocentrum cordatum*, oyster larvae, hatchery, Chesapeake Bay, *Crassostrea virginica*, aquaculture

1. INTRODUCTION

In the late spring to early summer months, the harmful algal bloom (HAB) species *Prorocentrum cordatum* (prev. *P. minimum*, Velikova and Larsen 1999) and *Karlodinium veneficum* are common in the Chesapeake Bay (Li et al., 2000; Glibert et al., 2007; Marshall and Egerton 2009). The mixotrophic dinoflagellate *K. veneficum* (Li et al., 1999; Adolf et al., 2006a) produces karlotoxins (KmTxs), a class of bioactive compounds with hemolytic, cytolytic, ichthyotoxic, and allelopathic effects (Deeds et al., 2002; Kempton et al., 2002; Adolf et al., 2006b; Place et al., 2012; Dorantes-Aranda et al., 2015; Yang et al., 2019). This species is perhaps best known for causing finfish kills (Goshorn et al., 2004); however, it has also been shown to have harmful effects on zooplankton (Adolf et al., 2007; Waggett et al., 2008; Yang et al., 2019) and shellfish, including, blue mussels (*Mytilus edulis*, Nielsen and Strømgren 1991; Galimany et al., 2008), hard clams (*Mercenaria mercenaria*, Place et al., 2008), and some life stages of oysters (*Crassostrea virginica* and *C. ariakensis*, Glibert et al., 2007; Brownlee et al., 2008; Place et al., 2008; Stoecker et al., 2008; Lin et al., 2017). Another mixotrophic dinoflagellate known to have harmful effects on a variety of aquatic organisms, including finfish, shellfish, and zooplankton, is *P. cordatum* (reviewed in Heil et al., 2005). Exposure to *P. cordatum* has been shown to produce highly-variable effects amongst shellfish species and life stages, including juvenile bay scallops (*Argopecten irradians*) and hard clams, as well as larval, juvenile, and adult oysters (reviewed in Wikfors 2005). From their work on oyster embryos exposed to *P. cordatum* lysates and filtrates, Wikfors and Smolowitz (1995) concluded that the harmful effects of *P. cordatum* likely relied on exposure to live *P. cordatum* cells rather than a released bioactive compound.

Chesapeake Bay blooms of *K. veneficum* and *P. cordatum* spatially and temporally overlap with the spawning season of the eastern oyster (*C. virginica*; Glibert et al., 2007; Place et al., 2008). Previous research has focused on the potential effects of these HAB species on oyster wild-stock recruitment, aquaculture, and restoration (Tango et al., 2005; Glibert et al., 2007; Place et al., 2008; Stoecker et al., 2008). Little research has focused on the impacts of these HABs within the context of oyster hatcheries (Luckenbach et al., 1993). Acute exposure to these HAB species has harmful effects on many early oyster life stages (Luckenbach et al., 1993; Wikfors and Smolowitz 1995; Glibert et al., 2007; Brownlee et al., 2008; Place et al., 2008; Stoecker et al., 2008; Lin et al., 2017). All but one of these studies (Wikfors and Smolowitz 1995) focused on acute HAB exposure within 2 days post-fertilization, or after the oysters were at least 14 days old. The veliger life stage, lasting 2 days post-fertilization to 14 - 16 days, requires further investigation. During this time, larvae are free-swimming and typically feed on particles smaller than 10 μm (Fritz et al., 1984). Oyster hatchery production relies on the health and survival of this critical larval life stage.

Unexplained larval mortality events at regional oyster hatcheries have raised concerns over the possibility of *K. veneficum* and *P. cordatum* breakthrough into hatcheries (Luckenbach et al., 1993; Tango et al., 2005). “Breakthrough,” in the context of this study, is the introduction of HAB cells and/or HAB-associated toxins into hatchery water (i.e., when water treatment processes at the hatchery fail to remove or degrade HAB cells or HAB-associated toxins from the incoming source water). Furthermore, breakthrough of live *K. veneficum* has been previously reported in a finfish hatchery in the Maryland portion of the Chesapeake Bay, where it was associated with

finfish mortalities (Deeds et al., 2002). The first objective of the current study was to screen water samples from a commercial oyster hatchery in the lower Chesapeake Bay to detect and quantify any breakthrough of live *K. veneficum* and/or *P. cordatum*.

To better prepare hatchery managers to appropriately mitigate a breakthrough event of either or both of these HAB species, a better understanding of the interactions between these HAB species and larval oysters is needed. The second objective of the current study was to assess the potential harmful effects of *K. veneficum* and *P. cordatum* on oyster veliger larvae. Seven-day-old oysters were used in a series of 96-h larval oyster bioassays in the laboratory. The bioassays were designed to measure larval oyster mortality and changes in larval oyster motility during acute, static exposure to one or both of these naturally co-occurring HAB species. Treatments consisted of a range of cell concentrations, representative of cell concentrations documented in natural blooms of these HAB species and hatchery breakthrough events observed during this study. Additionally, the potential effects of *K. veneficum* on a hatchery-relevant beneficial food source, *Pavlova pinguis* were explored.

2. MATERIALS & METHODS

2.1 In-hatchery HAB screening

During the oyster spawning season, water samples were collected from inside an oyster hatchery and screened for the HAB species *K. veneficum* and *P. cordatum* to document any hatchery breakthrough. Water grab samples of 100-mL were collected inside of Oyster Seed Holdings, LLC, a commercial oyster hatchery in the lower Chesapeake Bay, Virginia, USA (37.4937, -76.3037). Hatchery staff collected water samples at-will throughout the commercial oyster spawning season (March through June). Samples were collected in 2014 and 2016, and delivered to the Virginia Institute of Marine Science (VIMS) for detection and quantification of *K. veneficum* and *P. cordatum* (see **Section 2.7**); the hatchery did not provide samples for analysis in 2015. Three types of water samples were collected from within the hatchery at various stages along the water treatment process: 1) mixed-media – incoming water that was filtered to contain only particles less than 20–30 μm using mixed-media mechanical filtration, 2) filtered water – the mixed-media water that was further filtered to contain only particles less than 1–10 μm, and 3) feed algae – incoming water that was treated with mixed-media mechanical filtration, with additional 1-μm filtration, and used to cultivate beneficial feed algae through nutrient amendment. Mixed-media and filtered water were treated for the purpose of oyster culturing, while feed algae samples were taken directly from the active algal culture bags within the hatchery. All water samples were collected in duplicate to run parallel analyses (see **Section 2.7**). Water samples were received from the hatchery in various combinations of sample type and on random dates throughout the spawning seasons; this allowed for a screening of *K. veneficum* and *P. cordatum* for the specific

dates and sample types collected. See **Section 2.7** for in-hatchery HAB cell enumeration methods.

2.2 Experimental design of bioassays

A series of 96-h, static, single and dual HAB bioassays were done in the laboratory to assess acute and combined effects of *K. veneficum* and *P. cordatum* on larval oysters (**Table 1**). Bioassays were carried out in 24-well tissue culture plates (Falcon®, Corning Inc., Corning, New York, USA), with ten replicate wells per treatment. Treatments were made by diluting algal cultures with treated hatchery water (**Table 1**). Estuarine York River water was treated by the Aquaculture Genetics and Breeding Technology Center (ABC) oyster hatchery at VIMS, using a sequence of two sand filters, a 20-µm cartridge filter, a diatomaceous earth filter, a UV sterilizer, and a 1-µm filter bag. Treated water was then sterile-filtered through a 0.2-µm Polycap 75 TC filter (GE Whatman®, Sigma- Aldrich, St. Louis, Missouri, USA) and was pre-aerated to ensure oxygen saturation. Treated water conditions were measured once at the start of each bioassay with a YSI meter equipped with pH and polarographic dissolved oxygen (DO) sensors (YSI Pro-Plus Multiparameter Instrument, Yellow Springs, Ohio, USA). Sensors were calibrated on the first day of the single HAB bioassay, 12 days before the dual HAB bioassay; pH was calibrated using a 3-point calibration with standard buffers. Initial water conditions for the single HAB bioassay were 20.0 ◦C, 8.34 mg/L DO, salinity 13.83, pH 7.92, and for the dual HAB bioassay were 19.8 ◦C, 7.71 mg/L DO, salinity 13.87, pH 7.81.

Each replicate well was loaded with 1 mL of treatment before approximately 10, actively swimming, 7-day-old, larval oysters were added. This larval density was chosen

as it was within the range of typical hatchery stocking densities for oysters of this age (4–15 larvae/ mL, Castagna et al., 1996). During the 96-h bioassays, well plates were kept in a Percival AL36L4 incubator (Percival Scientific, Perry, Iowa, USA; 19.1 ± 0.5 ◦C, 39 ± 7 μmol/m^2/s, 14:10 h light-dark cycle) with the lids on, except during daily observations of larval oyster motility, behavior, and mortality (see **Section 2.4**). No water changes were performed, no algal additions were made, and no larval oysters were removed throughout the 96 h. For all bioassays, 1-mL glass microbeaker inserts (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) were used inside of all wells to reduce chemical-surface interactions that could artificially reduce dissolved bioactive compounds in the treatments, e.g., adsorption of lipophilic toxins to plastic well plates.

In the single HAB bioassay, treatments consisted of each HAB species at a variety of cell concentrations (*K. veneficum*: 1,000; 5,000; 10,000; and 50,000 cells/mL; *P. cordatum*: 100; 5,000; 10,000; and 50,000 cells/ mL). The lowest cell concentrations were chosen to reflect the order of magnitude of 2014-2016 average cell concentrations from samples with co-occurring *K. veneficum* and *P. cordatum* from the VIMS state HAB monitoring samples (n = 261, 1,150 ± 317 cells/mL standard error [SE] and 389 ± 132 cells/mL SE, respectively). The cell concentrations tested were within the range of cell concentrations observed in natural blooms of these two species in the Chesapeake Bay (1,000 – 100,000 cells/mL for both species, Marshall and Egerton 2009). Control treatments included Unfed controls, i.e., no algae present, and Fed controls. Unfed controls were included to account for any changes in larval oyster behavior or mortality due to malnourishment. Past studies have found that oyster larvae can tolerate days to weeks without algae, and may feed off of other nutriment during these times (Kennedy

1996) such as bacteria and heterotrophic flagellates (Baldwin and Newell 1991). Fed control oysters received only 25,000 cells/mL of *P. pinguis*, a typical cell concentration used to feed hatchery oyster larvae (see **Section 2.5**).

In the dual HAB bioassay, larval oysters were co-exposed to 1,000 cells/mL *K. veneficum* and 50,000 cells/mL *P. cordatum* to assess potential combined effects of these co-occurring HAB species. These cell concentrations were selected based on results from the single HAB bioassay to maximize the potential of detecting a combined effect on larval oyster mortality within 96 h. In the single HAB bioassay, high cell concentration treatments of *K. veneficum* (>1,000 cells/mL) were determined to be too lethal for assessing combined effects over 96 h. On the other hand, larval oyster mortality was so low for *P. cordatum* treatments that the highest *P. cordatum* cell concentration treatment was used in the dual HAB bioassay. To control for variability between oyster spawns and changing water conditions, relevant controls and single HAB bioassay treatments (Fed; Unfed; $Karlo_{1,000}$; $Proro_{50,000}$) were re-run for direct comparison with the co-exposure treatment.

2.3 Feed algae study

To assess potential effects of *K. veneficum* on *P. pinguis*, *P. pinguis* cell concentration data were compared between *P. pinguis* treatments (25,000 cells/mL) with or without *K. veneficum* (1,000 cells/mL), in the absence of larval oysters, over 96 h (**Table 1**). During the dual HAB bioassay, 12 additional wells without larval oysters were made up with each of these two algal treatments. Every 24 h, the contents of three wells from each treatment were transferred into separate 1.5-mL low retention microcentrifuge tubes, fixed with 10% neutral buffered formalin (Pharmco-Aaper, Brookfield,

Connecticut, USA), and stored at 4 ◦C. At the start of the study, triplicate 1-mL samples of the initial algal treatments were collected and stored as just described. *Pavlova pinguis* was enumerated using a hemocytometer and light microscopy (Olympus CX31 or CX41, Olympus Corp., Shinjuku, Tokyo, Japan).

2.4 Larval oyster metrics

During both bioassays, the well plates were removed daily from the incubator for assessment of larval oyster mortality and behavioral observation of oysters by light microscopy (Olympus CKX53 or IX50 inverted microscopes, Olympus Corp., Shinjuku, Tokyo, Japan). To capture novel behavior of interest discovered after the start of the bioassays, still images and short videos were collected using Infinity Analyze 6.5.4 (Lumenera Corp., Ottawa, Ontario, Canada) or CellSens Standard 1.12 (Olympus Corp., Shinjuku, Tokyo, Japan) software. Daily assessment of larval oyster mortality in each well consisted of counting *dead* oyster larvae that exhibited no ciliary movement, or that had intact and empty shells; these observations were made at 40X magnification. After all observations and larval oyster mortality assessments were made at 96 h, 10% neutral buffered formalin (Pharmco-Aaper, Brookfield, Connecticut, USA) was used to fix the wells, allowing for an exact *total* larval oyster count of each well (at the start of the bioassays, wells were loaded with approximately 10 live larval oysters). Cumulative larval mortality (CLM) was calculated at each timepoint for each well using the formula: *CLM = (dead / total) * 100*. These values were used to calculate daily average CLM for each treatment (n = 10), from which the daily average *% surviving* could also be calculated, as *100 - CLM*. The daily average CLM values for each treatment were plotted over the 96 h for the single and dual HAB bioassays.

In the single HAB bioassay, larval oyster motility in each well was assessed every 24 h using a protocol designed to measure activity/ inactivity of larval shellfish species (Yan et al., 2001; Basti et al., 2015). Briefly, when the well plates were removed from the incubator, the plates were gently swirled to cause larvae to stop swimming and sink to the bottom of the wells. After five minutes, the number of non-swimming, *immotile* larvae in each well was recorded. *Immotile* larval oysters were easily distinguished from *dead* larval oysters by ciliary action, visible by light microscopy at 40X magnification. To calculate the percentage of *immotile* larvae in each well at each timepoint, while excluding *dead* larvae, the following equation was used, *% immotile = [(immotile – dead) / (total – dead)] * 100*. These values were used to calculate the average *% immotile* for each treatment at 24 and 96 h; wells with 0*% surviving* were excluded from these calculations. Sample size for average *% immotile* was 10 wells per treatment, except at 96 h for $Karlo_{5,000}$ (n = 6), $Karlo_{10,000}$ (n = 9), and $Karlo_{50,000}$ (n = 8). These lower sample sizes were due to the exclusion of wells with 0*% surviving*.

2.5 Larval oyster culturing for bioassays

Oyster larvae (*C. virginica*) were acquired from the ABC oyster hatchery at VIMS. Oysters were spawned separately for the single and dual HAB bioassays, 12 days apart. The single HAB bioassay spawn had 12 parents and the dual HAB bioassay spawn had 8 parents. For both spawns, ABC used 3-yr-old, diploid, DEBY oysters. The DEBY line of oysters is widely used at oyster hatcheries along the eastern coast of the USA (Dr. Jessica Moss Small, ABC, pers. comm.). Adult oysters were strip-spawned, all eggs were pooled and then split into a number of batches equaling the number of male oysters used in that spawn. Each male's sperm were used to fertilize one batch of eggs, and the

fertilized embryos were then re-pooled, resulting in a spawn with all possible crosses between parents. The resulting larvae were raised with aeration in 60- or 200-L barrels at 24–28 ◦C in the hatchery and were fed a daily diet of *P. pinguis* at 20,000–35,000 cells/mL. Full water changes were performed on day 2, and day 4 or 5, after the spawn. On day 7, oyster larvae were collected on a 63-μm sieve, transferred to new, treated hatchery water, and transported to the laboratory for the bioassays.

2.6 Algal culturing for bioassays and feed algae study

The ABC facility cultured *P. pinguis* using f/2 medium (Fritz Aquatics, Mesquite, Texas, USA, Guillard and Ryther 1962; Guillard 1975) using treated hatchery water. Large batch cultures were held at 20 ◦C under constant light. The average salinity of treated hatchery water during the month of the bioassays was 13.6 (Dr. Jessica Moss Small, ABC, pers. comm.).

In the laboratory, single-cell isolate, clonal cultures of the HAB species, *K. veneficum* (CCMP 1974, Stoecker et al., 2008), and *P. cordatum* (JA 98–01, Rosetta and McManus 2003), were grown in f/2-Si medium (Guillard and Ryther 1962; Guillard 1975) made with autoclaved, 0.22 μm-filtered York River seawater. The salinity of both cultures was approximately 20, as measured with a refractometer. Batch cultures were grown at 19.1 ± 0.5 ◦C (SD) under 39 ± 7 μmol/m^2/s (SD) light irradiance and a 14:10 h light-dark cycle (see **Section 2.2**). For the single HAB bioassay, the *K. veneficum* culture used to begin the bioassay was in the stationary phase (the growth phase in which the cell population has stabilized), while the *P. cordatum* culture was in stationary phase leading up to the bioassay but was diluted with new medium the day before the bioassay. For the

dual HAB bioassay, both HAB cultures were in the stationary phase at the start of the bioassay.

2.7 In-hatchery HAB cell enumeration

In 2014, *P. cordatum* was quantified in samples preserved with Lugol's solution (Carolina Biological Supply Company, Burlington, North Carolina, USA), using a 1-mL Sedgewick-Rafter slide and light microscopy (100X magnification, Olympus 1 × 51 with Olympus DP73 digital camera, Center Valley, Pennsylvania, USA). All quantification of *K. veneficum* in 2014 and 2016, as well as *P. cordatum* in 2016, was completed using quantitative real-time PCR (qPCR).

In preparation for qPCR, samples were filtered through 3-µm Isopore™ membrane filters (Millipore Corp., Darmstadt, Germany) for DNA extraction and qPCR analysis. Filters were placed in 5-mL centrifuge tubes, frozen at -20 ◦C until DNA was extracted using the QIAamp® Fast Stool Mini Kit (QIAGEN©, Germantown, Maryland, USA) according to the manufacturer's protocol with the following modifications. Instead of using only 200 µL of the lysate, the entire sample was carried through the extraction protocol. The reagent volumes were increased in subsequent steps to maintain the proper ratio of sample to reagents. A "blank" extraction (reagents only) was included with each set of samples to ensure there was no contamination. Extracted DNA samples were stored at -20 ◦C until they could be quantified using qPCR.

A previously published TaqMan® qPCR assay was used to target *P. cordatum* (Handy et al., 2008). To target *K. veneficum*, the current study used a qPCR TaqMan® assay originally developed and optimized at VIMS around 2008–2009 ([VA DEQ] 2014). This *K. veneficum* assay was chosen because it was, and remains, routinely used in

Virginia for official state monitoring of this HAB species. The *K. veneficum* primers were KvITS_242F (5′-TTCGTTGTGTAGTTGTTGACTCG-3′) and KvITS_328R (5′-TGCTGACCTAACTTCATGTCTTG-3′), and the probe was Kv_266PR (5′FAM-AGCCTGCTCCAGCTCACGACTCCT-3′TAMRA). These *K. veneficum* primers were tested for cross-reactivity against all phytoplankton species listed in Table 2 of the VA DEQ (2014) report, species all found in lower Chesapeake Bay waters. Control stocks of *K. veneficum* and *P. cordatum* were maintained in culture at VIMS. Cell counts for the control stock cultures were determined by light microscopy. DNA was extracted from a known number of cells to use as positive control material, and for generating standard curves through serial dilution of the DNA to achieve a range of cell number equivalents. Samples from the hatchery were run against these standard curves to quantify the cells in the sample. qPCR assays were performed on 7500 Fast, QuantStudio 6, or QuantStudio 3 Real-Time PCR systems (Applied Biosystems™, ThermoFisher, Waltham, Massachusetts, USA) using the following cycling parameters: an initial denaturation step at 95 ◦C for 20 s followed by 40 cycles of 95 ◦C for 3 s to denature and 60 ◦C for 30 s to anneal and extend. All reactions were performed in duplicate with reagent concentrations for each reaction of 0.9 μM for each primer, 0.1 μM for the probe and 1X concentration of the TaqMan® Fast Advanced Master Mix (Applied Biosystems™, ThermoFisher, Waltham, Massachusetts, USA) in a 10 μL final volume. A subset of qPCR results was cross verified by light microscopy counts (100X magnification, Olympus 1 × 51 with Olympus DP73 digital camera, Center Valley,

Pennsylvania, USA) of Lugol's-preserved (Carolina Biological Supply Company, Burlington, North Carolina, USA) duplicate samples.

2.8 Data analysis and statistics

Differences in larval oyster motility *(% immotile*) between treatments were assessed at 24 and at 96 h in the single HAB bioassay using Kruskal- Wallis tests followed by post hoc analyses using Dunn tests with the Benjamini and Hochberg (1995) p-adjustment method for multiple comparisons.

Differences in mortality between treatments for the single and dual HAB bioassays were analyzed using separate, linear mixed effects models (LMMs). To account for differences between wells, wells were coded as a random (intercept) factor, nested within treatment. Time, treatment, and the interaction between time and treatment, were coded as fixed factors. LMMs allowed for a time decaying correlation structure in the data, i.e., correlations between data collected in time from the same subject declined as the sampling timepoints got further apart (Liu et al., 2010). The first order autoregressive structure was applied to these models. Mortality was arcsine transformed to improve homogeneity of variance of the proportional data (Lin and Xu 2020), which was assessed through residual plots. Models were fitted using a restricted maximum likelihood (REML) approach in R using the "nlme" package (Pinheiro et al., 2020). Least-squares means (LSM) of arcsine-transformed mortality were calculated from the LMM for each treatment within each level of time using the "emmeans" package in R (Lenth et al., 2020). Significant differences between LSM of treatments within each level of time were determined by using the Tukey-Bonferroni method for multiple comparisons (Liu et al., 2010). Briefly, Tukey's pairwise comparisons of treatments

within each level of time were calculated, and the significance level (α) was adjusted by dividing by the number of levels of time that comparisons were made for, e.g., $n = 4$, therefore $\alpha = 0.05/4 = 0.0125$.

In the feed algae study, *P. pinguis* cell concentration was modeled using a multiple linear regression (MLR) to assess potential effects of *K. veneficum* on *P. pinguis*. The model variables included time (days) and presence/absence of *K. veneficum* (0 = absence, 1 = presence). Scatterplots and correlation tests were used to examine relationships between these model variables and the response, *P. pinguis* cell concentration. Cell concentration (cells/mL) was ln-transformed to normalize the residuals (Shapiro-Wilk's: $W = 0.99$, $p = 0.95$). Collinearity of model variables, and homogeneity of variance of model residuals, were assessed to ensure assumptions of the model were met.

Statistical tests were performed in R Studio (2019) using R version 3.6.1. Tests used a significance level (α) of 0.05, unless otherwise noted.

3. RESULTS

3.1 In-hatchery HAB screening

During the 2014 and 2016 oyster spawning seasons, both *K. veneficum* and *P. cordatum* were identified and enumerated in water samples collected from within a commercial oyster hatchery (**Table 2**). Both HAB species were found in all three types of treated water in 2014, i.e., mixed-media, filtered water, and feed algae, and exceeded "bloom" cell concentrations (> 1,000 cells/mL) in the former two types. Water treated for feed algae, however, contained lower cell concentrations of both HAB species, rising above 100 cells/mL in May 2014. In 2016, measurable cell concentrations of both HAB species were again present in filtered water, but remained below "bloom" cell concentrations, ranging from 19 – 937 cells/mL *K. veneficum* and 3 – 9 cells/mL *P. cordatum*.

3.2 Single and dual HAB bioassays

3.2.1 *Karlodinium veneficum* swarming behavior

In both the single and dual HAB bioassays, *K. veneficum* was observed swarming live and dead oyster larvae (**Fig. 1**), a behavior in stark contrast to the uniform distribution of *P. cordatum* (**Fig. 2**, **Supp. Fig. 1**) and *P. pinguis* (**Videos 1** and **2**) swimming cells. This was a novel behavior of interest (see **Section 2.4**) that was captured in photos and videos as well as in the following qualitative observations. Swarming behavior of *K. veneficum* was observed during every observation timepoint in both bioassays, with more larvae swarmed with time. Swarming behavior was observed in all wells with *K. veneficum* and oyster larvae, but not all larvae within each well were swarmed, or were swarmed at the same time. Less *K. veneficum* were associated with

highly active larvae (**Videos 1** and 2), while immotile larvae (see **Section 3.2.2**) were generally swarmed. Some live oyster larvae that were actively being swarmed, exhibited hindered swimming capabilities (**Video 1**). Live *K. veneficum* were frequently observed inside of the shells of living and dead oyster larvae (**Fig. 1B**, **Video 2**). Swarms of *K. veneficum* appeared to persist around larvae until the larvae died, and the larval shells were emptied of all tissue, at which point in time the empty shells were generally abandoned within 24 h.

3.2.2 Larval oyster immotility

Immotility and survival of larval oysters was quantified at 24 and 96 h in the single HAB bioassay (**Table 3**). Larvae were significantly less motile after 24 h of exposure to *K. veneficum* or *P. cordatum* when compared to controls (Kruskal-Wallis, $\chi^2 = 77$, df = 9, $p < 0.0001$). More specifically, all *K. veneficum* treatments ($Karlo_{1,000}$: 37.8 ± 4.1%; $Karlo_{5,000}$: 70.0 ± 3.6%; $Karlo_{10,000}$: 79.3 ± 3.7%; $Karlo_{50,000}$: 94.0 ± 5.0% SE), as well as the lowest *P. cordatum* treatment ($Proro_{100}$: 47.3 ± 7.4% SE), exhibited significantly higher percentages of immotile larvae than the Fed or Unfed controls at 24 h (Fed: 10.8 ± 3.2%; Unfed: 10.1 ± 4.9% SE; Dunn, all $p < 0.05$). At 96 h, there was also a significant difference in the percentage of immotile larval oysters between treatments (Kruskal-Wallis, $\chi^2 = 68$, df = 9, $p < 0.0001$). All *K. veneficum* treatments ($Karlo_{1,000}$: 42.0 ± 6.0%; $Karlo_{5,000}$: 100%; $Karlo_{10,000}$: 100%; $Karlo_{50,000}$: 79.2 ± 11.5% SE), as well as the highest *P. cordatum* treatment ($Proro_{50,000}$: 55.3 ± 6.4% SE), exhibited significantly higher percentages of immotile larvae than the Fed or Unfed controls at 96 h (Fed: 10.8 ± 4.0%; Unfed: 5.7 ± 2.5% SE; Dunn, all $p < 0.05$).

3.2.3 Larval oyster mortality

In the single HAB bioassay, Fed and Unfed controls exhibited low mortality by 96 h (Fed: 1 ± 1% standard error [SE]; Unfed: 0%; n = 10 wells per treatment). The $Karlo_{1,000}$ treatment exhibited moderate mortality by 96 h ($Karlo_{1,000}$: 21 ± 5% SE), and the higher cell concentration *K. veneficum* treatments exhibited high mortality by 96 h ($Karlo_{5,000}$: 93 ± 2%; $Karlo_{10,000}$: 85 ± 3%; $Karlo_{50,000}$: 83 ± 5% SE; **Fig. 3A**). Conversely, oyster larvae exposed to all *P. cordatum* treatments exhibited low mortality throughout the single HAB bioassay (**Fig. 3B**).

Within each timepoint (24, 48, 72, 96 h) in the single HAB bioassay, Fed and Unfed controls and all *P. cordatum* treatments showed no significant difference in mortality (Tukey-Bonferroni: all $p > 0.0125$, **Table 4**). At 24 h, $Karlo_{10,000}$ and $Karlo_{50,000}$ treatments had significantly higher mortality than all other treatments (Tukey-Bonferroni: all $p < 0.0125$). At 48, 72, and 96 h, the three highest *K. veneficum* treatments ($Karlo_{5,000}$, $Karlo_{10,000}$, and $Karlo_{50,000}$) had significantly higher mortality than all other treatments (Tukey-Bonferroni: all $p < 0.0125$). At 96 h, $Karlo_{1,000}$ had significantly higher mortality than the Fed and Unfed controls and *P. cordatum* treatments (Tukey-Bonferroni: all $p < 0.0125$). Well nested in treatment was a significant predictor of mortality ($\lambda_{LR} = 91$, $p < 0.0001$), explaining 0.02% of the difference in mortality after accounting for the effects of time and treatment. The interaction between time and treatment was significant in the model (LMM: $F_{27, 270} = 17$, $p < 0.0001$). Time (LMM: $F_{3, 270} = 115$, $p < 0.0001$) and treatment (LMM: $F_{9, 90} = 103$, $p < 0.0001$) were significant predictors of mortality.

In the dual HAB bioassay, Fed and Unfed controls and the $Proro_{50,000}$ treatment all exhibited low mortality by 96 h (Fed: 3 ± 2%; Unfed: 0%; $Proro_{50,000}$: 5 ± 2% SE; n = 10

wells per treatment), while the $Karlo_{1,000}$ treatment and the $Karlo_{1,000}$ X $Proro_{50,000}$ treatment both exhibited moderate mortality by 96 h ($Karlo_{1,000}$: 44 ± 9%; $Karlo_{1,000}$ X $Proro_{50,000}$: 55 ± 7% SE; **Fig. 4**).

At 24 h in the dual HAB bioassay, there was no significant difference in mortality between any of the treatments (Tukey-Bonferroni: all $p > 0.0125$, **Table 5**). At 48, 72, and 96 h, the $Karlo_{1,000}$ and $Karlo_{1,000}$ X $Proro_{50,000}$ treatments had significantly higher mortality than all other treatments (Tukey-Bonferroni: all $p < 0.0125$) with no significant difference in mortality between these two treatments (Tukey-Bonferroni: all $p > 0.0125$). Well nested in treatment was a significant predictor of mortality ($\lambda_{LR} = 123$, $p < 0.0001$), explaining 46.8% of the difference in mortality after accounting for the effects of time and treatment. The interaction between time and treatment was significant in the model (LMM: $F_{12, 135} = 12$, $p < 0.0001$). Time (LMM: $F_{3, 135} = 45$, $p < 0.0001$) and treatment (LMM: $F_{4, 45} = 24$, $p < 0.0001$) were significant predictors of mortality.

3.3 Feed algae study

There was a small, but significant negative effect of *K. veneficum* presence on *P. pinguis* cell concentration (MLR: $F_{1,26} = 4.8$, $p = 0.038$). Cell concentration over time of *P. pinguis*, with and without *K. veneficum*, was modeled by the equation: $Y_{Pav} = 10 - 0.08\ (Karlo) + 0.4\ (Time)$, where Y_{Pav} was the ln-transformed cell concentration (cells/mL) of *P. pinguis*, *Karlo* was the presence/absence of *K. veneficum* (0 = absence, 1 = presence), and *Time* was measured in days (MLR: $F_{2,26} = 308$, $p < 0.0001$, $R^2 = 0.96$). Time was also a significant predictor of *P. pinguis* cell concentration in the model (MLR: $F_{1,26} = 612$, $p < 0.0001$).

4. DISCUSSION

4.1 *Karlodinium veneficum* and oyster larvae

4.1.1 Swarming behavior of *K. veneficum*

This study provided novel documentation of larval oyster mortality resulting from predation by *K. veneficum*, and the swarming behavior of *K. veneficum* that preceded this mortality (**Figs. 1** and **2**, **Videos 1** and **2**). These observations support recent documentation of aggregations of *K. veneficum* cells around single-celled and metazoan prey items (Yang et al., 2020). The authors of that study proposed this behavior was part of a potential feeding mode, i.e., micropredation, for this mixotrophic HAB species. Swarming of metazoan organisms by dinoflagellates has been previously documented (Spero and Morée 1981; Delgado and Alcaraz 1999; Springer et al., 2002; Berge et al., 2012; Yang et al., 2020), including during laboratory exposures of larval oysters to *Pfiesteria* spp. (Springer et al., 2002; Shumway et al., 2006). Swarming behavior has also been reported in another *Karlodinium* species (Berge et al., 2008, 2012), *K. armiger*, where it was associated with the immobilization, predation, and ultimate death, of copepods (Berge et al., 2012). In the same study, a polychaete trochophore was swarmed and almost entirely consumed within 24 h. *Karlodinium armiger* can feed on its prey using a peduncle for tube feeding, or through phagotrophy (Berge et al., 2008), similar to *K. veneficum* (Yang et al., 2020). Chemotaxis towards prey items has been reported in *K. armiger* (Poulsen et al., 2011; Berge et al., 2012). Observations from the current study suggest that there was some form of chemosensory attraction of *K. veneficum* to the oyster larvae that may have facilitated the observed swarming.

While KmTxs were not quantified in this study due to limited cell biomass and the lack of a commercially-available standard, their probable involvement deserves some discussion. The strain of *K. veneficum* used in the current study (CCMP 1974) produces KmTx 1 and KmTx 3 (Brownlee et al., 2008; Stoecker et al., 2008; Adolf et al., 2009; Bachvaroff et al., 2009). Exposure to the same strain of toxin-producing *K. veneficum* caused immotility in 2-week-old oyster larvae (Glibert et al., 2007) and mortality in just-hatched larvae, while a non-toxin-producing strain of *K. veneficum* had no effect on mortality (Stoecker et al., 2008). KmTxs have also been shown to be involved in *K. veneficum* prey-capture by reducing swimming speeds and immobilizing single-celled prey (Sheng et al., 2010). Similarly, KmTxs are thought to have played a role in the observed larval oyster immotility and mortality in the current study. KmTxs are poorly-soluble, leading to the hypothesis that they must be administered within close proximity to the target (Sheng et al., 2010). This would indicate that *K. veneficum* swarming likely preceded any involvement of KmTxs in the current study, and that once swarmed, larval oysters were more likely to experience immobilization or other effects of KmTxs. The precise role of KmTxs in the interaction between larval oysters and *K. veneficum* deserves further study.

The current study demonstrated interactions between *K. veneficum* swarming behavior and larval motility. Larval swimming has been hypothesized to create microscale turbulence capable of preventing or reducing the risk of a dinoflagellate coming into close proximity with an active oyster larva (Springer et al., 2002). This small-scale turbulence surrounding swimming larvae could have interfered with *K. veneficum* swarming behavior and may help explain why some oyster larvae survived.

Furthermore, *K. veneficum* was found to preferentially feed on immotile prey (Yang et al., 2020). In the current study, immotile larvae were almost always observed with physically-associated *K. veneficum*. Once *K. veneficum* are physically-associated with a larva, in addition to the potential effects of KmTxs previously mentioned, the associated cells may increase physical drag during swimming attempts by the larva, or the cells may directly interfere with the larval swimming mechanism. Any of these outcomes further enhance the likelihood of larval immotility and the risk of being swarmed by additional *K. veneficum* cells. As the *K. veneficum* exposure duration or dose (cell concentration) increases, larval oysters experience more close encounters with individual *K. veneficum* cells. When considered in conjunction with previous studies, the current study suggests that without sustained, close proximity between *K. veneficum* and the larval oysters, the effect of *K. veneficum* on the oysters may be minimized. The authors propose that larval immotility ultimately enhances the opportunity for *K. veneficum* to initiate, or advance, an attack on the larvae, initiating a positive feedback loop that facilitates swarming and increases the harmful impact of this HAB species on larval oysters.

4.1.2 *Karlodinium veneficum* and larval oyster mortality

Exposure to *K. veneficum* caused significant larval oyster mortality at all cell concentrations tested, representing cell concentrations observed in hatchery breakthrough (1,000 cells/mL treatment; **Table 2**) and Chesapeake Bay bloom events (5,000 – 50,000 cells/mL treatments; Marshall and Egerton 2009). This supports previous research that has shown other early life stages of the eastern oyster (*C. virginica*) to be vulnerable to harmful effects of *K. veneficum*. Past studies have found that oyster larvae exposed to *K. veneficum* during the first two days of life exhibited deformities (Glibert et al., 2007) and

elevated mortality compared to control larvae (Glibert et al., 2007; Stoecker et al., 2008; Lin et al., 2017). Additionally, Glibert and colleagues (2007) found that 14-day-old oyster larvae (pediveligers) stopped swimming after 72 h of exposure to *K. veneficum*, an effect they hypothesized would lead to larval oyster mortality. The current study provided new information on the effects of *K. veneficum* exposure between these two previously-studied oyster developmental stages, using 7-day-old oyster larvae (veligers). Together, these findings suggest that hatchery breakthrough of *K. veneficum* can lead to significant larval oyster mortality inside of an oyster hatchery. In addition, the mortality associated with the higher *K. veneficum* cell concentrations tested in the current study (5,000 – 50,000 cells/mL), is considered ecologically significant and demonstrates severe consequences from acute exposure of oyster larvae to this HAB species (**Fig. 3**).

The Fed and Unfed controls showed no significant larval oyster mortality over 96 h, demonstrating the habitable conditions of this bioassay design. The static nature of these bioassays, however, may have led to declining water quality and subsequent larval stress. Water quality, therefore, cannot be ruled out as a possible confounding, and unmeasured, factor in the mortality metric. These mortality results, however, are highly applicable to static hatchery conditions where larvae are kept in tanks without water flow, moribund or dead larvae are not removed, and the water is changed out every few days. Water quality issues may exacerbate effects of *K. veneficum* exposure, although more research into combined effects of co-stressors in a hatchery setting is needed.

This is the first study to test the combined effects of these naturally co-occurring HAB species. During the dual HAB bioassay, there was no change in larval oyster mortality when *K. veneficum* was alone or with *P. cordatum*, signifying that *K. veneficum*

drove the observed larval oyster mortality in the co-exposure (Fig. 4). The results of the dual HAB bioassay also demonstrated that larval oyster mortalities were not explained by high cell biomass or particulate concentrations, i.e., the *K. veneficum* with *P. cordatum* treatment had the highest cell biomass and particulate concentration of all treatments tested.

4.2 *Prorocentrum cordatum* and oyster larvae

Exposure to *P. cordatum* was occasionally associated with larval immotility (Table 3), indicating that interactions with *P. cordatum* are disruptive to larval oyster behavior. Significant immotility compared to larval oyster controls was observed in the exposures as early as 24 h, even with *P. cordatum* cell concentrations as low as 100 cells/mL. Reduced motility during exposure to *P. cordatum* has previously been reported in juvenile bay scallops (Li et al., 2012) and in pediveliger Suminoe oysters, *C. ariakensis* (Glibert et al., 2007). The latter study also reported reduced swimming speeds in pediveliger eastern oysters, *C. virginica*, exposed to *P. cordatum*. Reduction in motility of larval oysters may result in reduced grazing, which could in turn reduce larval growth, and ultimately, survival.

While some previous studies have shown *P. cordatum* to cause mortality in early life stages of oysters (Luckenbach et al., 1993; Wikfors and Smolowitz 1995; Glibert et al., 2007), the current study, and a similar study with just-hatched larvae (Stoecker et al., 2008) found no significant effect of *P. cordatum* on larval oyster mortality. The lack of mortality in these studies may be due to insufficient *P. cordatum* exposure duration or dose. Moreover, it could be related to the physiological states of *P. cordatum* culture used (Li et al., 2012). *Prorocentrum cordatum* cultures in decline have been found to produce

more harmful effects than culture in other growth phases (Grzebyk et al., 1997; Li et al., 2012), such as stationary phase, as was used in the current study. Regardless, the observed larval immotility indicated a low-level, harmful effect of *P. cordatum* exposure that would have been missed if bioassay metrics had been limited to larval oyster mortality.

4.3 Implications for oyster hatcheries

This is the first documentation of breakthrough of live cells of *K. veneficum* and *P. cordatum* into an oyster hatchery (**Table 2**). Breakthrough occurred during oyster spawning season, a time of year when these two HAB species co-occur in the Chesapeake Bay (Glibert et al., 2007). Cells of *K. veneficum* (length 9–18 μm, Ballantine 1956) and *P. cordatum* (length 20 μm, Faust 1974) survived mechanical filtration with nominal pore sizes of 1–30 μm, demonstrating that filtration of incoming water, even down to 1 μm, may not be sufficient to keep these HAB species out of hatcheries. HAB cell concentrations inside the hatchery occasionally reached low-bloom cell concentrations (≥ 1,000 cells/mL). In laboratory bioassays, treatments of similar cell concentrations caused significant larval oyster immotility (by *P. cordatum* and *K. veneficum*) and significant larval oyster mortality (by *K. veneficum*). The bioassays in this study used the popular DEBY oysters and future research should explore whether different genetic lines of eastern oyster (*C. virginica*) respond differently to *K. veneficum* or *P. cordatum*.

The current study demonstrated that proximal, acute exposure to *K. veneficum* is a major concern for larval oyster health. Larval oyster mortality was preceded by *K. veneficum* actively swarming the larval oysters. Close proximity may be avoided or

minimized when oyster larvae are healthy and actively swimming, when water conditions are turbulent, or, in the case of oyster hatcheries, when frequent, full water changes are performed. Based on the results from the single and dual HAB bioassays, daily full water changes would be advised to minimize the risk of larval oyster mortality due to static exposure to *K. veneficum* during a breakthrough event. At a fish hatchery, potassium permanganate was successfully used to mitigate an incidence of *K. veneficum* breakthrough without any ill effects (Deeds et al., 2002). Testing would be needed to determine the appropriateness of this approach for an oyster hatchery.

Furthermore, both *K. veneficum* and *P. cordatum* were found in feed algae cultured at the hatchery, making feed algae an additional potential source of HABs to hatchery oysters. This study also showed that *K. veneficum* could negatively impact the cell concentration of a common hatchery feed alga (*P. pinguis*). A reduction in hatchery feed algae during a *K. veneficum* breakthrough event could represent another stressor on hatchery oysters, and poses additional challenges for hatchery aquaculturists.

This study documented HAB cell breakthrough into an oyster hatchery, harmful effects of two common Chesapeake Bay HAB species on the vulnerable veliger life stage of the eastern oyster (*C. virginica*), as well as potential negative effects of *K. veneficum* on hatchery feed algae. Evidence of oyster hatchery HAB breakthrough of low-bloom cell concentrations, and damage to larval oysters exposed to these HAB species, validate regional concerns and suggest that these HAB species could have contributed to past unexplained larval oyster mortalities at regional hatcheries. The authors recommend that hatcheries continue to monitor treated water and feed algae for HAB breakthrough, especially when water will be used with early oyster life stages. The harmful effects of

these two HAB species on larval oysters can likely be mitigated through a combination of monitoring and frequent full water changes.

SUPPLEMENTARY MATERIALS

Videos can be found in the online version of this manuscript, at doi:10.1016/j.hal.2020.101965. Other supplementary material associated with this chapter can be found in **APPENDIX I**.

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Table 1
Response variables and treatments for the single and dual HAB bioassays, and the feed algae study.

Experiment	Response variables	Treatments[a]	Algal Species	Cell Concentration (cells/mL)
Single HAB Bioassay	Larval oyster mortality	Fed (Pav)[b]	*Pavlova pinguis*	25,000
		Unfed[b]	None	0
	Behavioral observations	Karlo 1,000	*Karlodinium veneficum*	1,000
	Larval oyster immotility	Karlo 5,000		5,000
		Karlo 10,000		10,000
		Karlo 50,000		50,000
		Proro 100	*Prorocentrum cordatum*	100
		Proro 5,000		5,000
		Proro 10,000		10,000
		Proro 50,000		50,000
Dual HAB Bioassay	Larval oyster mortality	Fed (Pav)[b]	*Pavlova pinguis*	25,000
		Unfed[b]	None	0
	Behavioral observations	Karlo 1,000[b]	*Karlodinium veneficum*	1,000
		Proro 50,000[b]	*Prorocentrum cordatum*	50,000
		Karlo 1,000 X Proro 50,000	*Karlodinium veneficum*	1,000
			& *Prorocentrum cordatum*	50,000
Feed Algae Study	*P. pinguis* cell concentration	Fed (Pav)[b]	*Pavlova pinguis*	25,000
		Karlo 1,000 X Fed (Pav)	*Karlodinium veneficum*	1,000
			& Pavlova pinguis	25,000

[a] Pav = *Pavlova pinguis*, Karlo = *Karlodinium veneficum*, Proro = *Prorocentrum cordatum*, numbers represent cell concentrations in cells/mL
[b] Control treatments within each experiment

Table 2
Karlodinium veneficum and *Prorocentrum cordatum* detected in samples from a commercial oyster hatchery in the lower Chesapeake Bay, Virginia, USA.

Date		Sample Type	Cell Concentration (cells/mL) *Karlodinium veneficum*[a]		*Prorocentrum cordatum*[b]	
2014	May 12	Mixed-media	506		579	
		10 µm-filtered water	30		< 1	
		Feed algae	< 1		< 1	
	May 19	Mixed-media	60		3,630	*
		10 µm-filtered water	67		2,940	*
	May 20	10 µm-filtered water	144		192	
		Feed algae	167		716	
	May 28	Feed algae	< 1		< 1	
	June 4	Mixed-media	15		11	
		10 µm-filtered water	9		8	
		Feed algae	< 1		< 1	
	June 13	Mixed-media	1,094	*	767	
		Feed algae	0		0	
2016	March 11	10 µm-filtered water	19		7	
	March 24	10 µm-filtered water	185		3	
	April 11	1 µm-filtered water	937		9	
	April 18	1 µm-filtered water	163		6	

[a] All samples were quantified using quantitative real-time PCR

[b] 2014 samples were quantified using cell counts by light microscopy, 2016 samples were quantified using quantitative real-time PCR

*Bloom cell concentration (> 1,000 cells/mL)

Table 3
Immotility and survival of larval oysters in the single HAB bioassay.

Treatments[a]	24 h			96 h		
	Average *% surviving*	**Average *% immotile*[b]**		**Average *% surviving***	**Average *% immotile*[b]**	
Fed (Pav)	100.0 (0.0)	10.8 (3.2)		99.1 (0.9)	10.8 (4.0)	
Unfed	100.0 (0.0)	10.1 (4.9)		100.0 (0.0)	5.7 (2.5)	
Karlo 1,000	98.1 (1.3)	37.8 (4.1)	*	79.0 (4.8)	42.0 (6.0)	*
Karlo 5,000	90.9 (2.8)	70.0 (3.6)	*	6.6 (1.9)	100.0 (0.0)	*
Karlo 10,000	85.0 (3.6)	79.3 (3.7)	*	15.5 (2.8)	100.0 (0.0)	*
Karlo 50,000	78.0 (4.6)	94.0 (5.0)	*	16.9 (4.6)	79.2 (12.9)	*
Proro 100	100.0 (0.0)	47.3 (7.4)	*	96.3 (2.7)	14.2 (4.3)	
Proro 5,000	99.0 (1.0)	33.2 (5.6)		96.1 (2.2)	13.6 (4.8)	
Proro 10,000	100.0 (0.0)	17.9 (4.7)		99.0 (1.0)	8.9 (2.4)	
Proro 50,000	100.0 (0.0)	18.1 (2.6)		92.9 (3.0)	55.3 (6.4)	*

Values indicate the treatment average with standard error given in parentheses.
Sample size was n = 10 wells per treatment, except for average *% immotile* values at 96 hours for $Karlo_{5,000}$ (n = 6), $Karlo_{10,000}$ (n = 9), and $Karlo_{50,000}$ (n = 8), due to the exclusion of wells with 0 *% surviving*.
[a] Pav = *Pavlova pinguis*, Karlo = *Karlodinium veneficum*, Proro = *Prorocentrum cordatum*, numbers represent cell concentrations (cells/mL)
[b] Percentage of surviving larval oysters that were immotile
*Value significantly different from the Fed and Unfed control *% immotile* values (Dunn, $\alpha = 0.05$)

Table 4
Least-squares means of arcsine-transformed cumulative larval mortality in the single HAB bioassay.

Treatments*	24 h	48 h	72 h	96 h
Fed (Pav)	0.00 a	0.00 c	0.00 e	0.03 h
Unfed	0.00 a	0.00 c	0.00 e	0.00 h
Karlo 1,000	0.06 a	0.08 c	0.22 e	0.44 i
Karlo 5,000	0.24 a b	0.49 d	0.74 f	1.37 j
Karlo 10,000	0.35 b	0.53 d	0.99 g	1.19 j
Karlo 50,000	0.46 b	0.66 d	0.83 f g	1.20 j
Proro 100	0.00 a	0.04 c	0.07 e	0.09 h
Proro 5,000	0.03 a	0.06 c	0.08 e	0.11 h
Proro 10,000	0.00 a	0.00 c	0.03 e	0.03 h
Proro 50,000	0.00 a	0.03 c	0.06 e	0.19 h

Standard error = 0.047 for all reported least-squares means values.
Letters denote groups of treatments that were or were not significantly different from one another based on Tukey's post hoc pairwise comparisons calculated within each level of time (Tukey-Bonferroni adjusted $\alpha = 0.0125$).
*Pav = *Pavlova pinguis*, Karlo = *Karlodinium veneficum*, Proro = *Prorocentrum cordatum*, numbers represent cell concentrations (cells/mL)

Table 5
Least-squares means of arcsine-transformed cumulative larval mortality in the dual HAB bioassay.

Treatments*	**24 h**	**48 h**	**72 h**	**96 h**
Fed (Pav)	0.00 a	0.00 b	0.00 d	0.08 f
Unfed	0.00 a	0.00 b	0.00 d	0.00 f
Karlo 1,000	0.18 a	0.44 c	0.56 e	0.67 g
Proro 50,000	0.03 a	0.03 b	0.03 d	0.14 f
Karlo 1,000 X Proro 50,000	0.16 a	0.38 c	0.60 e	0.83 g

Standard error = 0.056 for all reported least-squares means values.
Letters denote groups of treatments that were or were not significantly different from one another based on Tukey's post hoc pairwise comparisons calculated within each level of time (Tukey-Bonferroni adjusted $\alpha = 0.0125$).
*Pav = *Pavlova pinguis*, Karlo = *Karlodinium veneficum*, Proro = *Prorocentrum cordatum*, numbers represent cell concentrations (cells/mL)

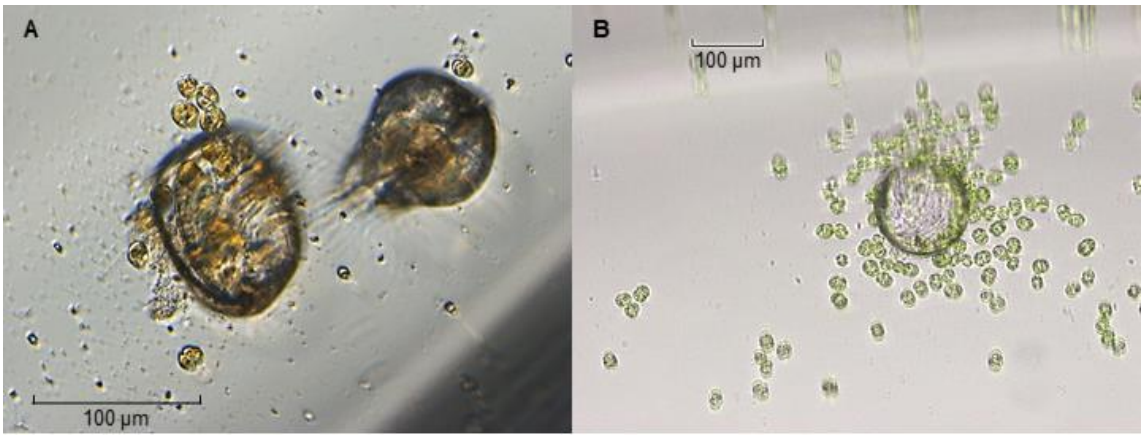

Figure 1
(A) *Karlodinium veneficum* inside of a larval oyster shell with a live oyster larva swimming in the background in a follow-up study. (B) *K. veneficum* swarming around an empty larval oyster shell during the dual HAB bioassay.

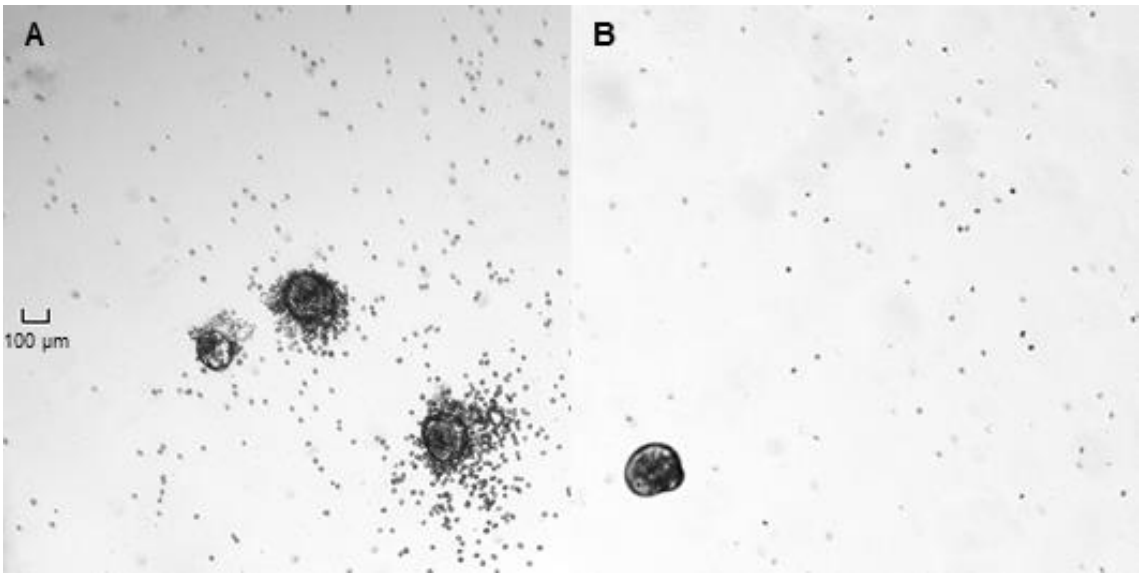

Figure 2
(A) *Karlodinium veneficum* and (B) *Prorocentrum cordatum*, both at 5,000 cells/mL, with larval oysters at 96 hours during the single HAB bioassay.

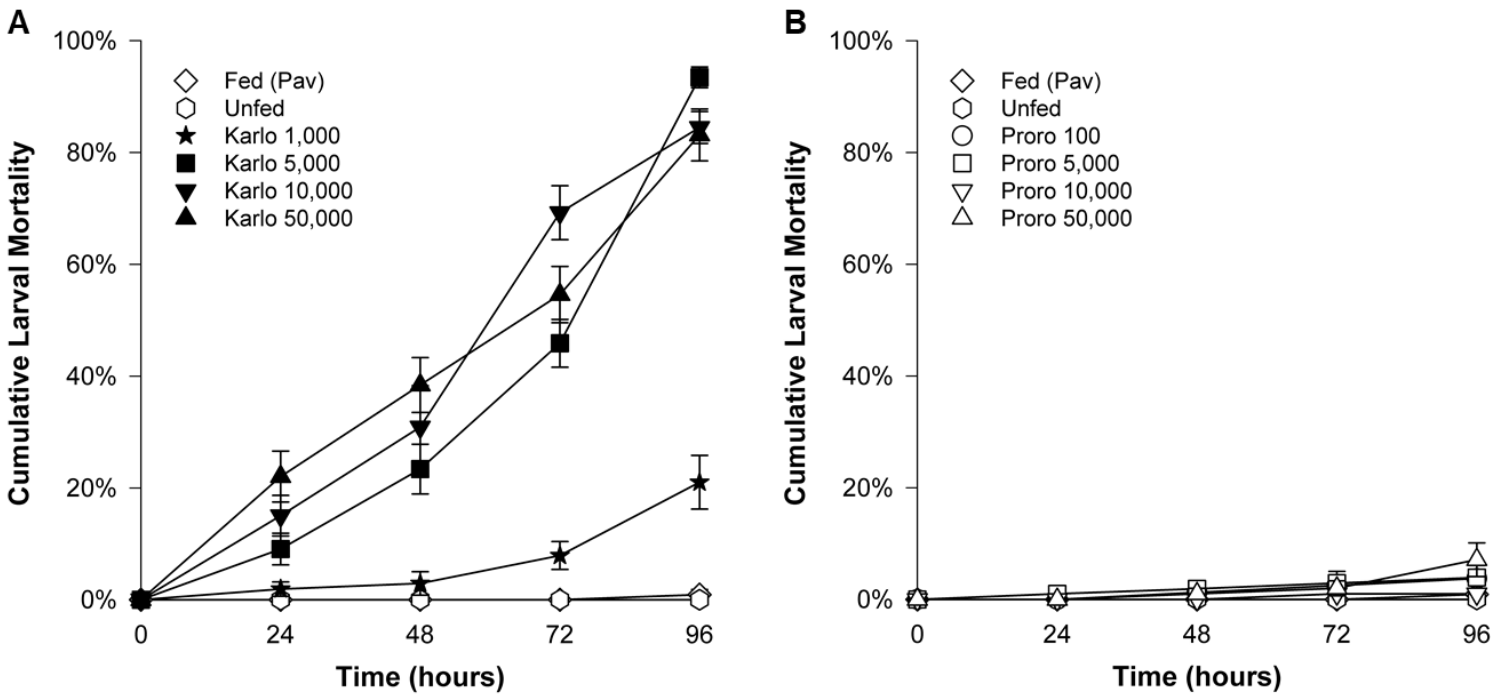

Figure 3
Cumulative larval mortality curves over time (hours) for the single HAB bioassay, when larval oysters were exposed to (A) *Karlodinium veneficum* (Karlo), or (B) *Prorocentrum cordatum* (Proro), at four different initial cell concentrations (cells/mL). Error bars show standard error (n = 10 wells per treatment). Pav = *Pavlova pinguis*.

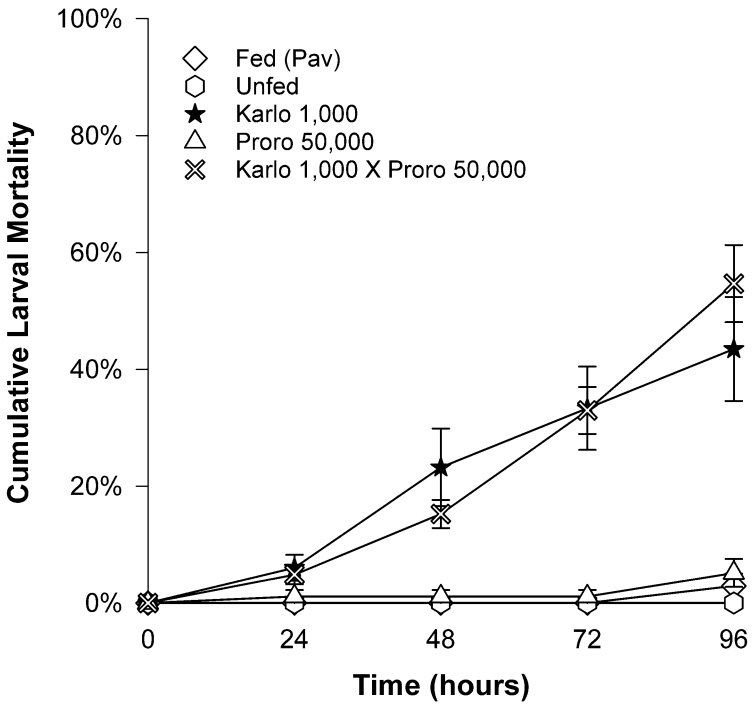

Figure 4
Cumulative larval mortality curves over time (hours) for the dual HAB bioassay, when larval oysters were exposed to co-exposure or control treatments. Error bars show standard error (n = 10 wells per treatment). Pav = *Pavlova pinguis*, Karlo = *Karlodinium veneficum*, Proro = *Prorocentrum cordatum*.

CHAPTER 2

Effects of two harmful algae, *Alexandrium catenella* and *Dinophysis acuminata*, on survival and activity of larval shellfish

ABSTRACT

Harmful algal bloom (HAB) species *Alexandrium catenella* and *Dinophysis acuminata* can co-occur, creating risk of co-exposure to aquatic life. While much study has focused on understanding the toxicity of these HAB species as they relate to human health, there is considerably less research on the effects they have on shellfish, the common vector to humans. To investigate the potential for these HAB species to affect larval eastern oyster (*Crassostrea virginica*) activity and mortality, 96-h laboratory bioassays with seven-day-old oysters were performed using live cell (10 – 1,000 cells/mL), lysate (1,000 cells/mL equiv.), and pure toxins (10,000 cells/mL equiv.). Exposure to the highest concentration of live *D. acuminata,* 1,000 cells/mL, led to significant larval mortality (21.9 ± 7.0%). While mortality was not observed after exposure to live *A. catenella*, exposure to cell concentrations (10 – 1,000 cells/mL) resulted in significant larval inactivity (>45%), i.e., larvae were alive but ceased swimming. Lysate of *D. acuminata* (1,000 cells/mL equiv.) was also toxic, causing significant larval mortality by 96 h (10.2 ± 4.0%). Exposure to high concentrations of pure saxitoxin and/or okadaic acid (10,000 cells/mL equiv.) had little effect on larval oysters, demonstrating that these toxins were not wholly responsible for the larval effects observed during live cell and lysate exposures. Pectenotoxin-2 exposure, however, was associated with complete loss (100%) of larval activity and rapid larval mortality (49.6 ± 5.8% by 48 h). Co-exposures of larvae to either multiple toxins, OA, PTX2, and/or STX, or lysate from *D. acuminata* and *A. catenella* produced similar results to exposures to only PTX2 or *D. acuminata* lysate, respectively, indicating their dominant role in the observed toxicity. Larval oysters are negatively impacted by exposure to *A. catenella* and/or *D. acuminata*, and as such, blooms of these HAB species co-occurring in

time and space with larval oysters could have consequences for oyster recruitment in the wild.

KEYWORDS

Alexandrium catenella, *Dinophysis acuminata*, *Crassostrea virginica*, saxitoxin, okadaic acid, pectenotoxin, harmful algae, oyster larvae

1. INTRODUCTION

In the northeast United States, two species of harmful algae typically associated with human health concerns have been found to co-occur: *Alexandrium catenella*, associated with paralytic shellfish poisoning (PSP) and *Dinophysis acuminata*, associated with diarrhetic shellfish poisoning (DSP; Hattenrath-Lehmann et al. 2013, Brosnahan et al. unpublished). Natural blooms of these two species in the USA are typically 1 – 100 cells/mL, occasionally reaching over 1,000 cells/mL (Crespo et al. 2011, Brosnahan et al. 2017, Brosnahan unpublished). *Alexandrium catenella* produces a suite of hydrophilic PSP toxins (PSTs), most famously – saxitoxin (STX), while *D. acuminata* produces two classes of lipophilic toxins: the pectenotoxins (PTXs) and DSP toxins (DSTs) – consisting of okadaic acid (OA), dinophysistoxins (DTXs) and their derivatives.

Beyond human safety is the possible threat that these co-occurring harmful algal blooms (HABs) and their associated toxins may pose to aquatic life. Only one study to-date has examined the combined effects of *A. catenella* and *D. acuminata* on aquatic organisms. Rountos and colleagues (2019) assessed the individual and combined effects of exposure to these harmful algal bloom (HAB) species on early life stages of estuarine fishes. Both HAB species reduced growth and swimming activity of fishes compared to fed controls, however, *A. catenella* was lethal to newly-hatched fishes, and combined treatments of *A. catenella* and *D. acuminata* expressed similar lethality, with no additive effects observed (Rountos et al. 2019).

No data are yet available regarding bivalve response to co-exposure to both HAB species; however, there are some studies on the effects of larval bivalve exposure to a single *Alexandrium* sp., a *Dinophysis* sp., or a pure toxin. Larval responses to these HABs

and toxins are species-specific and range from mortality to no observable effect. Exposure to PST-producing *A. tamarense,* reduced swimming activity and survival of larval scallops, *Chalmys farreri* (Yan et al. 2001), while exposure of this same *A. tamarense* strain to another larval scallop species, *Argopecten irradians concentricus*, reduced larval swimming activity and growth, but did not impact survival (Yan et al. 2003). Larval mussels (*Mytilus galloprovincialis*) exposed to *A. catenella* exudate (i.e., supernatant from centrifuged culture) experienced mortality (Supono et al. 2020); another species of larval mussels (*Mytilus edulis*) exposed to pure OA exhibited reduced protein phosphatase activity and reduced larval viability (De Rijcke et al. 2015). Exposure of clam larvae (*Mercenaria mercenaria*) to *A. catenella* had no effect on larval mortality (Tang and Gobler 2012). Basti and colleagues (2015) found exposure of larval pearl oysters (*Pinctada fucata martensii*) to either PST-producing *A. catenella* or non-PST-producing *A. affine*, resulted in significant larval inactivity but had no effect on larval mortality. Another larval oyster species, *Crassostrea gigas*, exhibited some aberrant or arrested development, reduced growth, reduced settling rates, and reduced survival from exposure to *A. catenella* (Mu and Li 2013), while exposure to *A. catenella* exudate also caused larval mortality (Supono et al. 2020). Larval *C. gigas* have also been exposed to PST-producing and non-PST-producing strains of *A. minutum*; both strains caused reduced feeding, reduced growth, reduced development, and reduced settlement yield of larvae, but the non-PST-producing strain also caused reduced swimming activity and malformations of the mantle in exposed larvae (Castrec et al. 2019, 2020). Additionally, unpublished data from Gaillard (2020) showed that larval *C. gigas* exposed to *D. sacculus* exhibited developmental issues, while exposure to pure PTX2 led to mortality.

In addition to co-exposures, there have been no studies of these HAB genera or associated toxins with larval oysters *C. virginica*, nor any looking into the larval bivalve effects of exposure to lysates of these HAB genera. Furthermore, the larval bivalve effects of exposure to *D. acuminata* and pure STX have not yet been explored.

The objective of this study was to assess the individual and combined effects of *A. catenella* and *D. acuminata* and their toxins on larval eastern oysters (*C. virginica*). A series of bioassays with larval oysters were designed: (1) a live cell bioassay, to determine dose-responses for larvae exposed to *A. catenella* and *D. acuminata* individually; (2) a lysate bioassay for each HAB species, administered individually and combined; and (3) a pure toxin bioassay for representative toxins from *A. catenella* (STX) and *D. acuminata* (OA, and PTX2), administered individually and combined. Oyster larvae used in the study were too small to consume *A. catenella* or *D. acuminata*, meaning that larval interactions were restricted to other physical and chemical interactions with live cells, and encounters with suspended particles, extracellular toxins, and bioactive compounds, depending on the bioassay. Pure toxins or lysate, and not live cells, were used in the co-exposures as a way to minimize HAB-to-HAB interactions as they were not a focus of this study.

2. MATERIALS & METHODS

2.1 Experimental design

A series of 96-h, static bioassays (live cell, lysate, pure toxin) were done in the laboratory to assess acute effects of *A. catenella* and *D. acuminata* and their toxins on larval oysters (**Table 1**). Bioassays were carried out in 24-well tissue culture plates (Falcon®, Corning Inc., Corning, New York, USA), with ten replicate wells per treatment. Treatments were made by diluting live algal culture (live cell bioassay), algal culture that had been lysed (lysate bioassay), or pure toxin standards (pure toxin bioassay), with treated hatchery water to reach desired concentrations of cells or chemicals. Lysate treatments were tested to assess the effects of extracellular as well as intracellular bioactive compounds and toxins associated with these HAB species alone and in combination. Additionally, the lysate bioassay facilitated assessment of whether living cells were needed to produce a toxic effect, i.e., test whether cells actively produced and released toxic compounds, and to remove any confounding effects of HAB-to-HAB interactions in the combined treatment. Pure toxin treatments were tested to assess the effects of STX, OA, and PTX2 toxins alone and in combination in the absence of cell effects or other bioactive compounds associated with these HAB species.

Each replicate well was loaded with 1 mL of treatment before approximately 10, actively swimming, 7-day-old, larval oysters were added. This larval density was within the range of typical hatchery stocking densities for oysters of this age (4-15 larvae/mL, Castagna et al. 1996). During the bioassays, well plates were kept in a Percival AL36L4 incubator (Percival Scientific, Perry, Iowa, USA; 19 ± 0.5 ºC, 38 ± 10 µmol/m^2/s, 14:10 hour light-dark cycle) with the lids on, except during daily observations of larval oyster

activity and mortality (see **Section 2.2**). Throughout the bioassay, no water changes were performed, no algal additions were made, and no larval oysters were removed. To reduce adsorption of lipophilic toxins to plastic well plates, 1-mL glass microbeaker inserts (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) were used inside of all wells.

2.1.1 Live Cell Bioassay

In the live cell bioassay, treatments consisted of each HAB species, *A. catenella* or *D. acuminata*, at the following cell concentrations: 10, 100, 500, and 1,000 cells/mL (treatment abbreviations provided in **Table 1**). Cell concentrations were selected to test acute toxicity of these HAB species to larval oysters. Control treatments included Unfed and Fed controls. Unfed controls, i.e., no algae present, were included to account for any changes in oyster activity or mortality due to malnourishment. Past studies have found that oyster larvae can tolerate days to weeks without algae, and may feed off of other nutriment during these times (Kennedy 1996) such as bacteria and heterotrophic flagellates (Baldwin and Newell 1991). Fed control oysters received 25,000 cells/mL of *Pavlova pinguis*, a typical cell concentration used to feed hatchery oyster larvae (see **Section 2.5**).

Changes in HAB cell concentrations throughout the live cell bioassay were monitored using additional HAB treatment wells. Daily, triplicate wells of each treatment level of *A. catenella* and *D. acuminata* were collected, fixed with 10% neutral buffered formalin (Pharmco-Aaper, Brookfield, Connecticut, USA), and stored at 4 ºC until they could be counted. Samples were enumerated in a Sedgewick-Rafter slide using light microscopy (Olympus CX31 and CX41, Olympus Corp., Shinjuku, Tokyo, Japan). Cell

cultures (equiv. to 20,000 cells) were collected, separated into dissolved and particulate fractions by gentle centrifugation: 12 min at 3,234 x g at 4 ºC (5804R, Eppendorf, Hauppauge, New York, USA), and frozen, at -20 ºC, for toxin extraction and analysis for endogenous PSTs and DSTs.

2.1.2 Lysate Bioassay

In the lysate bioassay, *A. catenella* and *D. acuminata* cultures were lysed and diluted with hatchery water to create treatments that were equivalent to cell concentrations used in the live cell bioassay. Treatments included two *A. catenella* lysate treatments (100 and 1,000 cells/mL equiv.), one *D. acuminata* lysate treatment (1,000 cells/mL equiv.), and one lysate co-exposure treatment representing 1,000 cells/mL equiv. of both HAB species. Unfed and Fed control treatments, as described in **Section 2.1.1**, were also included.

To lyse the cultures, two days prior to the start of the bioassay, both HAB cultures were sieved through 10-µm Nitex mesh, resuspended in treated hatchery water, and enumerated. Cultures were bath sonified for 15 min at 40 kHz (M5800H, Branson, Danbury, Connecticut, USA), frozen and thawed 3X, and probe sonified on ice (Digital Sonifier-450, Branson, Danbury, Connecticut, USA) for 10-20 minutes in 20-s cycles at 40% amplitude. Cell lysis was verified using light microscopy (Olympus CX31 and CX41, Olympus Corp., Shinjuku, Tokyo, Japan). Lysate was stored at -20 ºC until ready for use in the bioassay, and a portion (equiv. to 8,825 *A. catenella* and 20,150 *D. acuminata* cells) was aliquoted for toxin extraction and analysis and frozen at -20 ºC.

2.1.3 Pure Toxin Bioassay

To assess whether toxic effects observed in live cell and lysate bioassays could be induced with exposure to dissolved toxins, oysters were exposed to high levels of purified material. Effects of saxitoxin (STX), okadaic acid (OA), and pectenotoxin-2 (PTX2) were directly tested in the current study through the use of pure toxins. For proof-of-concept, a simplified toxin profile was administered based on intracellular toxin quotas from isolates of *A. catenella* (Salt Pond isolate: 2.7 pg STX/cell) and *D. acuminata* (DATC03: 0.54 pg OA/cell, 17.9 pg PTX2/cell) from the Nauset Marsh System, MA, USA. Final concentrations of toxins were made to represent 10,000 cells/mL equivalents for each HAB species. Certified toxin reference materials purchased from the National Research Council Canada (NRC CRM-STX-f, NRC CRM-OA-d, NRC CRM-PTX2-b) were used in making the treatment levels: 27 ng STX/mL, 5.4 ng OA/mL, and 179 ng PTX2/mL. All three toxins were administered in a full factorial design (**Table 1**). A combined carrier control was included in the pure toxin bioassay: 4% methanol (MeOH) and 3 µM hydrochloric acid (HCl), based on the OA x PTX2 x STX treatment.

Changes in PTX2 concentration during the pure toxin bioassay were monitored using additional pure toxin treatment control wells without oysters. Triplicate well water samples were collected at the bioassay start and termination time points for toxin analysis (see **Section 2.3**).

2.2 Larval oyster metrics & well water

During 96-h bioassays, the well plates were removed daily from the incubator for assessment of larval oyster mortality and activity by light microscopy (Olympus CKX53 or IX50 inverted microscopes, Olympus Corp., Shinjuku, Tokyo, Japan). An oyster was

counted as *dead* if it exhibited no ciliary movement or had intact and empty shells; observations were made at 40X magnification. At the end of each bioassay, well plates were briefly placed in a -20 ºC freezer to cause larvae to stop swimming and fall to the bottom of the wells, allowing for a *total* larval oyster count of each well. Cumulative larval mortality (CLM) was calculated at each timepoint for each well using the formula: *CLM = (dead / total) * 100*. These values were used to calculate daily average CLM for each treatment (n = 10), from which the daily average *% surviving* could also be calculated, as *100 - CLM*. The daily average CLM for each treatment was plotted over the 96 hours for the live cell, lysate, and pure toxin bioassays.

Larval oyster activity in each well was assessed every 24 hours (Yan et al. 2001, Basti et al. 2015, Pease et al. 2021). Briefly, plates were gently swirled to cause larvae to stop swimming and sink to the bottom of the wells. Swimming naturally resumed, and after five minutes, the number of non-swimming, *inactive* larvae in each well was recorded. *Inactive* larval oysters were easily distinguished from *dead* larval oysters by ciliary action, visible by light microscopy at 40X magnification. To calculate the percentage of *inactive* larvae in each well at each timepoint, while excluding *dead* larvae, the following equation was used, *% inactive = [(inactive – dead) / (total – dead)] * 100*. These values were used to calculate the average *% inactive* at each timepoint for each treatment across 10 replicate wells.

Larval oysters were collected during the three bioassays for the quantification of DSTs and PTXs. At the end of the bioassays, larvae were pooled by treatment to reach biomass requirements for toxin analysis (7 wells pooled = approx. 70 oysters); toxin results were normalized to pg toxin/oyster. Larvae were collected on 64-µm Nitex mesh,

rinsed with hatchery water, and excess water removed via aspiration. An additional control sample of pooled oysters was collected at the start of the bioassays to assess background toxin concentrations. Three of the wells used in the pooled oyster sample for the pure PTX2 treatment had the well water removed using a pipette tip fitted with 64-µm Nitex mesh. This tip allowed for the collection of triplicate well water samples without oysters to compare with the pure PTX2 control wells described in **Section 2.1.3**. PSTs were not analyzed in larval oysters or well water due to the elevated detection limits for the quantification method for these hydrophilic toxins.

2.3 Toxin analyses

All samples collected for the quantification of PSTs or DSTs/PTX2 were separated into two groups for extraction and analysis: dissolved and particulate toxins. Samples collected for dissolved PSTs (i.e., extracellular component of the *A. catenella* culture, initial hatchery water, lysate) required no further cleanup prior to toxin analysis. Samples for dissolved DSTs and PTX2 (i.e., extracellular component of the *D. acuminata* culture, initial hatchery water, lysate, well water), however, were processed using solid phase extraction (SPE) with an Oasis HLB 60-mg cartridge (Waters, Milford, Massachusetts, USA) prior to analysis, as described in Smith et al. (2012).

For the analysis of particulate toxins, the sample collected for PSTs (i.e., intracellular component of *A. catenella* culture) was extracted as described in Armstrong et al. (2018) with the following modification: centrifugation at 3,234 x g for 12 minutes at 4 ºC (5804R, Eppendorf, Hauppauge, New York, USA). Samples collected for particulate DSTs and PTX2 (i.e., intracellular component of *D. acuminata* culture and oysters) were extracted with methanol using bath sonification for 15 min at 40 kHz

(M5800H, Branson, Danbury, Connecticut, USA), or probe sonification on ice for 1 min at 40% amplitude (Branson Digital Sonifier-450, Danbury, Connecticut, USA), respectively. Methanolic extracts for DSTs and PTX2 were centrifuged for 5 min at 3,234 x g at 4 ºC (5804R, Eppendorf, Hauppauge, New York, USA) and the pellet discarded.

All extracts were 0.22-µm syringe filtered (13-mm, Millex PVDF, Durapore) prior to the quantification of PSTs or DSTs/PTX2 by hydrophilic interaction chromatography (HILIC) tandem mass spectrometry (MS/MS; Armstrong et al. 2018, Boundy et al. 2015) or ultra-performance liquid chromatography – tandem mass spectrometry with a trapping dimension and at-column dilution (UPLC-MS/MS with trap/ACD; Onofrio et al. 2020), respectively. Alkaline hydrolysis was used to convert DST derivatives (i.e., esterified forms) in all DST/PTX2 methanolic extracts into the parent toxins OA and DTX1, following the methods of Villar-González et al. (2008). The original methanolic extracts were analyzed for both DSTs and PTX2. The hydrolyzed methanolic extracts were only analyzed for DSTs. Toxins quantified included PTX2 and DSTs: OA and DTX1, and PSTs: STX, NEO, GTX1, GTX2, GTX3, GTX4, GTX5, dcNEO, dcSTX, dcGTX2/3, C1, and C2. Triplicate standard curves, with 5 – 8 points, were run using certified reference material from NRC. All peaks with signal-to-noise ratios (S/N) below 10, or without peaks, were reported as below the detection limit (< DL); peaks with S/N > 10 but with peak areas below the average of the lowest point on the standard curve were reported as below the limit of quantitation (< LOQ).

2.4 Treated hatchery water

Treated hatchery water was used in all bioassays, including in wells, HAB resuspensions, and treatment dilutions. Water from the York River, VA was treated by

the Aquaculture Genetics and Breeding Technology Center's (ABC) oyster research hatchery at the Virginia Institute of Marine Science (VIMS) using a sequence of two sand filters, a 20-µm cartridge filter, a diatomaceous earth filter, a UV sterilizer, and a 1-µm filter bag. Treated water was then sterile-filtered through a 0.2-µm Polycarp 75 TC filter (GE Whatman®, Sigma-Aldrich, St. Louis, Missouri, USA) and was pre-aerated to ensure oxygen saturation before the start of the bioassays. Treated water conditions were measured once at the start of each bioassay with YSI meters equipped with pH and polarographic dissolved oxygen (DO) sensors (YSI Pro Plus and YSI EXO^3, YSI Incorporated, Yellow Springs, Ohio, USA). Sensors were calibrated within one week of the start of each bioassay; pH was calibrated using a 3-point calibration with standard buffers. Initial water conditions for the live cell bioassay were 20.0 º C, 8.34 mg/L DO, salinity 13.83, pH 7.92, and for the lysate and pure toxin bioassays were 20.0 º C, 8.22 mg/L DO, salinity 17.83, pH 7.73. Hatchery water from the start of each set of bioassays was collected to test for background presence of PSTs, DSTs, and PTX2.

2.5 Algal culturing for bioassays

The ABC facility cultured *P. pinguis* in batch using f/2 medium (Fritz Aquatics, Mesquite, Texas, USA, Guillard and Ryther 1962, Guillard 1975) made from hatchery water. Single-cell isolate, clonal cultures of the HAB species, *A. catenella* (N5-MP3; Sehein et al. 2016) and *D. acuminata* (DATC03; D. Anderson and M. Brosnahan, WHOI) from the Nauset Marsh System were acclimated step-wise to f/6-Si medium (Guillard and Ryther 1962, Guillard 1975) made with autoclaved, 0.22 µm-filtered seawater, salinity of 20. Batch cultures were grown at 20 ºC with a 14:10 hour light-dark cycle; light ranged from 38 ± 10 to 39 ± 7 standard deviation (SD) µmol/m^2/s between bioassays. Cultures of

D. acuminata were fed live *Mesodinium rubrum* (Park et al. 2006). Prey were removed 24 h prior to the start of the bioassay by using a 10-µm Nitex mesh; *D. acuminata* was resuspended in hatchery water.

2.6 Larval oyster culturing for bioassays

Seven-day-old, diploid oyster larvae (*C. virginica*) were acquired from the ABC hatchery for use in the bioassays. Oysters were spawned in 2018 for the live cell bioassay and in 2020 for the lysate and pure toxin bioassays. The 2018 spawn had 12 parents and the 2020 spawn had 21 parents. Diploid, 2 to 3-yr-old oysters were strip-spawned, all eggs were pooled and then split into a number of batches equaling the number of male oysters used in that spawn. Each male's sperm was used to fertilize one batch of eggs and the fertilized embryos were then re-pooled, resulting in a spawn with all possible crosses between parents. The resulting larvae were raised with aeration in 60- or 200-L barrels at 24-28 ºC in hatchery and were fed a daily diet of *P. pinguis* at 20,000-35,000 cells/mL. Full water changes were performed on day 2, and day 4 or 5, after the spawn. On day 7, oyster larvae were collected on a 63-µm sieve, transferred to new hatchery water, and transported to the laboratory for the bioassays.

2.7 Data analysis and statistics

Differences in oyster activity and mortality (see **Section 2.2**) between treatments for the bioassays were analyzed using separate, linear mixed effects models (LMMs). To account for differences between wells, wells were coded as a random (intercept) factor, nested within treatment. Time, treatment, and the interaction between time and treatment (when significant), were coded as fixed factors. LMMs allowed for a time decaying correlation structure in the data, i.e., correlations between data collected in time from the

same subject declined as the sampling timepoints got further apart (Liu et al. 2010). The first order autoregressive structure was applied to these models. CLM was arcsine transformed to improve homogeneity of variance of the proportional data (Lin and Xu 2020), which was assessed through residual plots. Models were fitted using a restricted maximum likelihood (REML) approach in R using the "nlme" package (Pinheiro et al. 2020). Least-squares means (LSM) of arcsine-transformed CLM were calculated from the LMM for each treatment within each level of time using the "emmeans" package in R (Lenth et al. 2020). Significant differences between LSM of treatments within each level of time were determined by using the Tukey-Bonferroni method for multiple comparisons (Liu et al. 2010). Briefly, Tukey's pairwise comparisons of treatments within each level of time were calculated, and the significance level (α) was adjusted by dividing by the number of levels of time that comparisons were made for, e.g., $n = 4$, therefore $\alpha = 0.05/4 = 0.0125$.

In the pure toxin bioassay, treatments containing PTX2 were only evaluated at 24 and 48 h, because of this, these treatments were not included in the pure toxin bioassay LMM. To assess treatment effects at 24 and 48 h, separate Kruskal-Wallis tests for each timepoint were used with all of the pure toxin bioassay treatments. If the Kruskal-Wallis test identified a significant difference between treatments, these were explored using post-hoc Dunn tests with Benjamini-Hochberg adjusted p-values. The same set of tests were used to assess differences between PTX2 concentrations in the 0 h and 48 h toxin well water samples for the PTX2 treatments with and without oysters.

Statistical tests were performed in R Studio (2019) using R version 3.6.1. Tests used a significance level (α) of 0.05, unless otherwise noted.

3. RESULTS

3.1 Oyster mortality

In the live cell bioassay, the larvae exposed to higher cell concentrations of *A. catenella* and *D. acuminata* exhibited moderate mortality by 96 h ($Acat_{500}$: 22.8 ± 8.7%; $Dacum_{1,000}$: 21.9 ± 7.0% standard error [SE], n = 10 wells per treatment; **Fig. 1**). Fed and Unfed controls and the $Acat_{10}$, $Acat_{100}$, $Acat_{1,000}$, $Dacum_{10}$, $Dacum_{100}$, $Dacum_{500}$ treatments, however, exhibited low mortality (Fed: 0.9 ± 0.9%; Unfed: 0%; $Acat_{10}$: 5.7 ± 4.6%; $Acat_{100}$: 9.2 ± 5.0%; $Acat_{1,000}$: 0%; $Dacum_{10}$: 3.9 ± 1.6%; $Dacum_{100}$: 2.0 ± 1.4%; $Dacum_{500}$: 2.8 ± 1.4% SE). Early in the live cell bioassay, at 24 h, there was no significant difference in mortality between any of the treatments (Tukey-Bonferroni: all $p > 0.0125$, **Supp. Table 1**). By 48 and 72 h, the highest *D. acuminata* treatment ($Dacum_{1,000}$) had significantly higher mortality than the Fed and Unfed controls (Tukey-Bonferroni: all $p < 0.0125$). At the end of the live cell bioassay, 96 h, $Dacum_{1,000}$ and $Acat_{500}$ treatments had significantly higher mortality than the Fed and Unfed controls (Tukey-Bonferroni: all $p < 0.0125$). Well nested in treatment was a significant predictor of mortality ($\lambda_{LR} = 96$, $p < 0.0001$), explaining 0.007% of the difference in mortality after accounting for the effects of time and treatment. The interaction between time and treatment was significant in the model (LMM: $F_{27, 270} = 4$, $p < 0.0001$). Time (LMM: $F_{3, 270} = 24$, $p < 0.0001$) and treatment (LMM: $F_{9, 90} = 5$, $p < 0.0001$) were significant predictors of mortality. Cell concentrations of *A. catenella* and *D. acuminata* stayed the same or increased over the 96 hours across all treatments (**Supp. Fig. 1**).

Similar to the live cell bioassay, the larvae in the lysate bioassay exposed to lysate from 1,000 cells/mL of *D. acuminata* exhibited low to moderate mortality by 96 h

(Dacum$_{1,000}$: 10.2 ± 4.0%; **Fig. 2**). Interestingly, larvae exposed to the co-exposure, i.e., lysate from 1,000 cells/mL of both *A. catenella and D. acuminata*, responded with less mortality (Acat$_{1,000}$ x Dacum$_{1,000}$: 6.2 ± 2.3% SE, n = 10 wells per treatment; **Fig. 2**). Larvae in the Fed and Unfed controls and the Acat$_{100}$ and Acat$_{1,000}$ lysate treatments exhibited no, to very low, mortality (Fed: 0%, Unfed: 2.0 ± 1.3%, Acat$_{100}$: 0%, Acat$_{1,000}$: 0.9 ± 0.9% SE, n = 10 wells per treatment). The At 24, 48, and 72 h, there were no significant differences in mortality between any of the treatments (Tukey-Bonferroni: all $p > 0.0125$, **Supp. Table 2**). At 96 h, the Dacum$_{1,000}$ and the Acat$_{1,000}$ x Dacum$_{1,000}$ lysate treatments had significantly higher mortality than the Fed control (Tukey-Bonferroni: all $p < 0.0125$). Well nested in treatment was a significant predictor of mortality ($\lambda_{LR} = 94$, $p < 0.0001$), explaining 0.004% of the difference in mortality after accounting for the effects of time and treatment. The interaction between time and treatment was significant in the model (LMM: $F_{15, 162} = 2$, $p < 0.0001$). Time (LMM: $F_{3, 162} = 8$, $p < 0.0001$) and treatment (LMM: $F_{5, 54} = 4$, $p < 0.0001$) were significant predictors of mortality.

In the pure toxin bioassay, all treatments that contained PTX2 led to rapid inactivity and mortality; the decision was made to terminate all treatments containing PTX2 at 48 h to collect samples for toxin analyses. All treatments that contained PTX2 exhibited moderate mortality by 48 hours (PTX2: 49.6 ± 5.8%, OA x PTX2: 50.0 ± 4.7%, PTX2 x STX: 36.5 ± 2.8%, OA x PTX2 x STX: 61.1 ± 4.9% SE, n = 10 wells per treatment; **Fig. 3**), and these treatments exhibited significantly higher mortality than the Carrier control at 24 h (Kruskal-Wallis: $\chi^2 = 47$, df = 7, $p < 0.0001$, Dunn Tests: all $p < 0.05$) and at 48 h (Kruskal-Wallis: $\chi^2 = 71$, df = 7, $p < 0.0001$, Dunn Tests: all $p < 0.05$). Larvae from the Carrier control, OA, STX, and OA x STX treatments exhibited very low

mortality by 96 h (Carrier: 1.0 ± 1.0%, OA: 1.0 ± 1.0%, STX: 1.0 ± 1.0%, OA x STX: 2.0 ± 1.3%). For these remaining treatments, well nested in treatment was a significant predictor of mortality ($\lambda_{LR} = 26$, $p < 0.0001$), explaining 0.002% of the difference in mortality after accounting for the effects of time and treatment. Time was a significant predictor of mortality (LMM: $F_{3, 117} = 4$, $p = 0.02$), while treatment was not (LMM: $F_{3, 36} = 0.2$, $p = 0.9$).

3.2 Oyster inactivity

In the live cell bioassay, depression of activity was observed early on in the highest treatments for both HAB species. At 24 h, the $Acat_{500}$, $Acat_{1,000}$, and $Dacum_{1,000}$ treatments had significantly higher inactivity than the Fed and Unfed controls (Tukey-Bonferroni: all $p < 0.0125$, n = 10 wells per treatment; **Fig. 4**, **Supp. Table 3**). By the end of the live cell bioassay, 96 h, all of the *A. catenella* treatments had significantly higher inactivity than the Fed and Unfed controls ($Acat_{10}$: 46.3 ± 11.7%, $Acat_{100}$: 82.1 ± 3.7%, $Acat_{500}$: 71.2 ± 7.0%, $Acat_{1,000}$: 89.8 ± 3.4%, Tukey-Bonferroni: all $p < 0.0125$; **Table 2**). With *D. acuminata*, however, recovery from toxicity was observed; larval inactivity generally peaked early on in the experiment (24 h: $Dacum_{10}$:19.2 ± 5.2%, $Dacum_{100}$:10.2 ± 5.6%, $Dacum_{500}$: 5.6 ± 4.0%, $Dacum_{1,000}$:48.2 ± 10.6% SE), but then activity resumed in some of these inactive larval oysters (**Fig. 4**). Well nested in treatment was a significant predictor of inactivity ($\lambda_{LR} = 53$, $p < 0.0001$), explaining 24% of the difference in inactivity after accounting for the effects of time and treatment. The interaction between time and treatment was significant in the model (LMM: $F_{27, 270} = 8$, $p < 0.0001$). Time (LMM: $F_{3, 270} = 20$, $p < 0.0001$) and treatment (LMM: $F_{9, 90} = 31$, $p < 0.0001$) were significant predictors of inactivity.

In contrast to the live cell bioassay, larval inactivity associated with *D. acuminata* generally increased with time during the lysate bioassay. From 48 – 96 h, the $Dacum_{1,000}$ lysate treatment led to significantly higher inactivity of oysters than Fed or Unfed controls (Tukey-Bonferroni: all $p < 0.0125$, n = 10 wells per treatment; **Fig. 5**, **Supp. Table 4**). Inactivity in the $Dacum_{1,000}$ lysate treatment peaked at 96 h at 37.7 ± 7.0% SE (**Table 3**). Additionally, lysate treatments made with *A. catenella* did not elicit significant oyster inactivity throughout the 96-h bioassay. Well nested in treatment was a significant predictor of inactivity ($\lambda_{LR} = 56$, $p < 0.0001$), explaining 42% of the difference in inactivity after accounting for the effects of time and treatment. The interaction between time and treatment was significant in the model (LMM: $F_{15, 162} = 2$, $p = 0.003$). Time (LMM: $F_{3, 162} = 3$, $p = 0.03$) and treatment (LMM: $F_{5, 54} = 16$, $p < 0.0001$) were significant predictors of inactivity.

All oysters were inactive in treatments that contained PTX2 in the pure toxin bioassay (100%), these treatments were significantly different from the Carrier control at both 24 h (Kruskal-Wallis: $\chi^2 = 73$, df = 7, $p < 0.0001$, Dunn Tests: all $p < 0.05$, n = 10 wells per treatment) and 48 h (Kruskal-Wallis: $\chi^2 = 73$, df = 7, $p < 0.0001$, Dunn Tests: all $p < 0.05$; **Table 4**). Inactivity in the other pure toxin treatments, OA, STX, OA x STX, over the full 96 h was low (< 6%; **Table 4**). For these other treatments, well nested in treatment was a significant predictor of inactivity ($\lambda_{LR} = 44$, $p < 0.0001$), explaining 19% of the difference in inactivity after accounting for the effects of time and treatment. Time and treatment were not significant predictors of inactivity in these other treatments (LMM: $F_{3, 117} = 0.5$, $p = 0.7$; $F_{3, 36} = 0.5$, $p = 0.7$, respectively).

3.3 Toxins in HAB cultures, water, and oysters

Initial hatchery water used in the bioassays did not contain detectable concentrations of PSTs or the DST, OA (< DL). Trace levels of PTX2 and another DST, dinophysistoxin-1 (DTX1), were detectable in various lots of initial hatchery water; these 2 toxins were below the limits of quantification (< LOQ), and therefore, were minimal compared to experimental concentrations used in this study.

The intracellular toxin profile for the *A. catenella* culture used in this study was dominated by C2, with 3.9 pg C2/cell (> 90%); trace levels of STX were detected, but were < LOQ. As expected, no PSTs were detectable in the dissolved, i.e., extracellular, fraction of the *A. catenella* culture, nor were PSTs quantifiable in the lysate due to the lack of a concentration step in the extraction methods; *A. catenella* culture, however, was pelleted and aspirated to concentrate toxins prior to analysis. This was similarly why well water was not collected for toxin quantification from treatments with *A. catenella* or pure PSTs.

The intracellular toxin profile for the *D. acuminata* culture used in this study was dominated by PTX2, with 8.0 pg PTX2/cell (90.0%), 0.4 pg OA esters/cell (4.4%), 0.1 pg DTX1/cell (1.1%), and 0.2 pg DTX1 esters/cell (2.2%; **Supp. Fig. 2**). Only PTX2 was quantifiable in the extracellular portion (0.2 pg PTX2/cell; 2.2%). OA was not detected in the intracellular or extracellular fractions of the culture. Lysate from *D. acuminata* had a similar profile to the culture, with 4.6 pg PTX2/cell equiv. (88.5%), 0.3 pg OA esters/cell equiv. (5.8%), 0.1 pg DTX1/cell equiv. (1.9%), 0.2 pg DTX1 esters/cell equiv. (3.8%), and OA was < LOQ.

Larval oysters in all of the bioassays bioaccumulated PTX2 (**Table 5**). In the live cell bioassay, oysters bioaccumulated increasing amounts of PTX2 with increasing cell concentrations of *D. acuminata*. Oysters exposed to the highest concentration of live *D. acuminata*, 1,000 cells/mL, accumulated similar PTX2 concentrations to oysters exposed to an equivalent *D. acuminata* lysate treatment (5.2 and 3.7 pg PTX2/oyster, respectively). Oysters exposed to concentrations of pure PTX2, equivalent to 10,000 cells/mL, during the pure toxin bioassay, contained an order of magnitude more toxin (40.2 – 50.0 pg PTX2/oyster) than oysters exposed to 1,000 *D. acuminata* cell equivalents/mL of live cells or lysate. PTX2 was not detected in control oysters. OA and DTX1 were not detected in any oyster sample from this study, with the exception of control oysters that contained trace DTX1, below the LOQ.

In the pure toxin bioassay, the PTX2 measured in triplicate well water samples declined over 48 hours, with, or without oysters. PTX2 concentrations in well water declined from 70.7 ± 14.5 SD ng PTX2/mL to 28.1 ± 3.7 SD ng PTX2/mL in control wells without oysters, and to 34.7 ± 5.9 SD ng PTX2/mL in wells with oysters. At 48 hours, one PTX2 control well without oysters contained OA, and one PTX2 well with oysters had DTX1, in both instances, these toxins were detected in trace amounts (<LOQ).

4. DISCUSSION

This is the first study to assess the effects of *A. catenella*, *D. acuminata*, OA, PTX2, and STX on larval eastern oysters (*C. virginica*), and the first study to assess the combined effects of *A. catenella* and *D. acuminata* lysates and toxins on any bivalve. Overall, STX and OA alone were not acutely toxic to the oysters, however, exposure to PTX2, live *A. catenella*, or live or lysed *D. acuminata* led to deleterious effects. Mortality was highest for oysters exposed to pure PTX2 (10,000 cells/mL equiv.), reaching about 50% by 48 h, followed by oysters exposed to live *A. catenella* or *D. acuminata* (500 or 1,000 cells/mL, respectively) that both exhibited significant mortality by 96 h (> 20%). Larvae were rendered inactive by 24 h when exposed to pure PTX2, while 24 h of exposure to 1,000 cells/mL of either live *A. catenella* or *D. acuminata* caused larval inactivity; this larval inactivity increased with *A. catenella* and decreased with *D. acuminata* over the 96-h bioassay. Co-exposures of oyster larvae to *A. catenella* and *D. acuminata* lysate exhibited effects more similar to *D. acuminata* lysate alone; additionally, effects of co-exposures to pure toxins were dominated by the effects of PTX2. The demonstrated toxicity of PTX2 to larval oysters along with the observed mortality during exposure to a high cell concentration of live *D. acuminata* (1,000 cells/mL), suggests that concentrated blooms of PTX2-producing *D. acuminata* pose a threat to survival for larval oysters. Furthermore, larval oysters exposed to live or lysed *D. acuminata* or pure PTX2 bioaccumulated PTX2 (**Table 5**). These findings provide new perspective on the implications for shellfish health of two HAB species traditionally associated with shellfish poisoning in humans.

4.1 Effects of *Alexandrium catenella* on larval oysters

In this study, larval oysters exposed to live *A. catenella* experienced significant inactivity and some mortality by 96 h, while *A. catenella* lysate and pure STX had no measured effect. Larval oyster inactivity was commonly observed by the end of the 96-h bioassay in response to exposure to live *A. catenella* across all cell concentrations tested (10 – 1,000 cells/mL, **Fig. 4**, **Table 2**). The highest cell concentration (1,000 cells/mL) resulted in significant inactivity throughout the 96-h bioassay. These findings are in agreement with previous studies that exposed larval oysters to PST-producing and non-PST-producing strains of *Alexandrium* spp., demonstrating that PSTs did not play a role in the observed inactivity (Basti et al. 2015, Castrec et al. 2020). Larval oysters swim to feed, respire, and locate substrate to set on, and therefore, inactivity may indicate reduced larval fitness and could lead to larval mortality.

Mortality was less prevalent than inactivity in this study; the only live cell *A. catenella* treatment to exhibit significant larval mortality during the bioassay was the 500 cells/mL treatment. This treatment exhibited significant larval mortality at 96 h, and at 72 h had the highest percentage of larval inactivity amongst the live cell treatments (**Table 2**). It is unlikely that the observed mortality when exposed to live *A. catenella* was due to physical interactions between the HAB and the oysters because there was no oyster mortality in the highest treatment (1,000 cells/mL). Oyster larvae did not ingest *A. catenella* (**Supp. Fig. 1**), as these cells are 3X the size of particles typically ingested by oysters at this life stage (Whedon and Kofoid 1936, Fritz et al. 1984). This suggested that interactions with toxins or bioactive compounds were with the extracellular fraction of

those compounds, limiting toxin exposure routes to superficial interactions with exposed tissues, i.e., gills, mantle, velum, and digestive epithelial tissues.

In contrast to treatments with live cells, neither larval inactivity nor mortality was observed when oysters were exposed to either *A. catenella* lysate or pure STX. In the pure toxin bioassay, larvae were exposed to a high concentration of purified STX (27 ng STX/mL) that was equivalent to 10,000 cells/mL of *A. catenella*, representing a concentration about an order of magnitude higher than the most extreme bloom conditions observed in the New England region, USA (Brosnahan et al. 2017). The lack of observed deleterious effects when larvae were exposed to this elevated level of toxin suggests that negative impacts from extracellular STX are not expected under typical field conditions. Similarly, Yan and colleagues (2001) found that exposure of scallop eggs to pure STX, even at extreme doses (5,900 ng STX/mL), did not replicate the toxicity of live *A. tamarense*, further supporting our finding that PSTs are of minimal threat to larval shellfish.

Despite its hydrophilic and polar nature (Wiese et al. 2010), extracellular STX is expected to be bioavailable to larval oysters. Previous studies have demonstrated the bioavailability and toxicity of dissolved STX to non-feeding fish larvae and embryos (Oberemm et a. 1999, Lefebvre et al. 2004, 2005). Additionally, various studies have implicated PSTs more generally with negative effects on adult shellfish (reviewed in Lassudrie et al. 2020). However, in the current study, dissolved STX was not toxic to larval oysters, and live cells of *A. catenella*, not lysate, were required to elicit a negative response. Together, these results indicate that other bioactive compounds were responsible for the observed deleterious effects, and that their production requires co-

incubation of HAB with a potential grazer. This theory was also proposed to explain toxicity in scallops (*P. maximus*) and another species of oyster (*C. gigas*) exposed to a non-PST-producing strain of *A. minutum* (Borcier et al. 2017, Castrec et al. 2018, 2020). Various bioactive compounds have been proposed as the cause of toxicity of *Alexandrium* spp. to aquatic life, including: lytic compounds, reactive oxygen species (ROS), and polyunsaturated fatty acids (PUFA; Yan et al. 2001, Marshall et al. 2003, Tillmann et al. 2008, Ma et al. 2009, Flores et al. 2012, Basti et al. 2015, Dorantes-Aranda et al. 2015, Mardones et al. 2015, Castrec et al. 2018, 2020, Supono et al. 2020). Further research is needed to identify the non-PST bioactive compounds produced by *Alexandrium* spp. and their mechanisms of toxicity.

4.2 Effects of *Dinophysis acuminata* on larval oysters

Larval oysters exposed to live or lysed *D. acuminata*, or pure PTX2, experienced significant inactivity and mortality, while exposure to pure OA had no measured effect. Larval oysters exhibited significant inactivity at 24 and 48 h in the 1,000 cells/mL live *D. acuminata* treatment, with significant larval mortality from 48 h on (21.9% by 96 h, **Table 2**). In the live cell bioassay, larval mortality increased with time, suggesting that live *D. acuminata* is acutely toxic to at least some larval oysters. Toxicity may be linked to extracellular compounds produced by this species, as *D. acuminata* is too large for oyster larvae to ingest and cells were not depleted during the bioassay (**Supp. Fig. 1**, Fritz et al. 1984, Park et al. 2019). Additionally, although larval inactivity was observed at 24 h during the live cell bioassay, larval inactivity decreased with time throughout the remainder of the bioassay and was not accounted for by the increase in larval mortalities, demonstrating recovery of some larvae. These effects were only significant in the highest

live cell concentration tested (1,000 cells/mL), a cell concentration occasionally seen in nature (Hattenrath-Lehmann et al. 2013, Brosnahan unpublished), suggesting that intense natural blooms of *D. acuminata* could stress wild larval oyster populations and reduce recruitment (i.e., survival to the juvenile stage).

Lysate from *D. acuminata* was toxic to larval oysters, with significant inactivity and mortality measured by 96 h; however, compared to an equivalent level treatment of live *D. acuminata*, the lysate was less toxic than live cells (approximately 10% lower mortality). Reduced toxicity was mirrored by the reduced PTX2 concentration of the lysate (**Supp. Fig. 2**). It should be noted that the concentrations of DTX1, DTX1 and OA esters, were similar between the *D. acuminata* intracellular culture and lysate samples (see **Section 3.3**), suggesting these toxins were more stable in seawater than PTX2. Pure PTX2 concentrations declined 60% over 48 h in control well water without oysters (pH 7.73, see **Section 3.3**). Interactions between extracellular PTX2 and the well surfaces were minimized through the use of glass inserts. Extracellular PTX2 may rapidly hydrolyze to less-toxic seco acids (Miles et al. 2006), while in the live treatments, cells may actively produce and release PTX2 throughout the bioassay.

PTX2 (179 ng/mL) led to rapid larval mortality (approx. 50% by 48 h) and complete (100%) larval inactivity when compared to the control or any OA treatments. This suggests that PTX2 played a significant role in the oyster inactivity and mortality in the *D. acuminata* live cell and lysate exposures in the current study. In a similar study using larval fish (*Cyprinodon variegatus*), exposure to pure PTX2 (0.0003 – 0.005 ng/mL) led to significant gill pathology and mortality by 96 h (Gaillard 2020). In the same study, live cells and lysate from a strain of *D. acuminata* that produced primarily

PTX2 and some OA and DTX1, did not cause any mortality, however, exposure to live cells caused some gill pathology. In the current study, a PTX2 concentration of 0.0003 ng/mL could be produced by 0.0375 cells/mL from the strain of *D. acuminata* used (DATC03, 8.0 pg PTX2/cell, **Supp. Fig. 2**), a low cell concentration that is observed in regions where *D. acuminata* occurs (Crespo et al. 2011, Brosnahan et al. 2017), further supporting the theory that PTX2 could be acutely toxic to larval shellfish in the environment.

Unlike PTX2, OA by itself was not acutely toxic to larval oysters. Oysters exposed to OA exhibited low inactivity (< 4%) throughout the bioassay and very low (1%) mortality by 96 h. These results corroborate those of a similar study that found no effect of pure OA (0.00006 ng/mL) on oyster gametes (*C. gigas*; Gaillard et al. 2020). Doses of OA higher than that tested in the current study (5.4 ng/mL), have been shown to produce toxic effects on aquatic organisms. OA at concentrations at or above 37.8 ng/mL reduced viability of larval mussels (*M. edulis*; De Rijcke et al. 2015), and concentrations 3,000 – 8,000 ng/mL were acutely toxic to larval zebrafish (*Danio rerio*; Figueroa et al. 2020). For the strain of *D. acuminata* used in the current study (DATC03), it would potentially take at least 94M cells/L (based on 0.4 pg OA/cell, **Supp. Fig. 2**) to reach an OA concentration of 37.8 ng/mL, indicating that OA is not involved in the acute toxicity of this strain of *D. acuminata* to larval shellfish.

Toxin profiles of *D. acuminata* strains from around the world vary (Fux et al. 2011, Tong et al. 2015); the strain used in this study primarily produced PTX2, but also produced OA esters, DTX1, and DTX1 esters (**Supp. Fig. 2**). The majority of PTX2 and all of the OA and DTX1 were intracellular in the *D. acuminata* culture. Tong and

colleagues (2015) suggested that strains of *D. acuminata* from the northwestern Atlantic, such as the one used in the current study, have low DST content compared to other strains of *D. acuminata* from around the world. Based on results from the current study, the reduced risk of DSP, however, may come with a tradeoff; i.e., strains with toxin profiles dominated by PTX2 may pose a higher risk to the health and survival of early shellfish life stages, and recruitment to wild populations.

4.3 Potential effects of co-exposure to *A. catenella* and *D. acuminata*

When larval oysters were co-exposed to either the lysate of *D. acuminata* and *A. catenella*, or a combination of their purified toxins, *D. acuminata* and PTX2 were responsible for the larval inactivity and mortality observed. The addition of *A. catenella* lysate reduced the negative effects of *D. acuminata* lysate on larval oyster activity, but significant larval oyster mortality associated with exposure to *D. acuminata* lysate was not changed by the addition of *A. catenella* lysate. In contrast, Rountos et al. (2019) found that *A. catenella* drove toxicity to larval fish, not *D. acuminata*, with a combined treatment of the live HAB species showing similar toxicity to *A. catenella* alone. This may indicate fundamental differences in the toxicity of these HAB species to fish (vertebrates) compared to bivalves (invertebrates), variations in exposure routes, the use of live cell versus lysate combined treatments, or it could belie differences in the bioactive compound production between HAB strains used in these different studies.

These two HAB species are known to co-occur in some locations and bloom concurrently (Hattenrath-Lehmann et al. 2013, Brosnahan et al. unpublished). The results of the current study suggest that larval shellfish present during bloom termination (i.e., cell lysis) of a combined bloom, will experience some associated mortality; however,

bloom termination of mono-specific *D. acuminata* blooms are expected to be more toxic by comparison. The current study focused on co-exposures of lysate and pure toxins to avoid confounding factors associated with cell-to-cell interactions between the HAB species, and interactions between individual HAB responses to the presence of a potential grazer. Co-exposures with live cells of *A. catenella* and *D. acuminata* should be pursued in future research once there is a better understanding of interactions between these HAB species and between potential grazers, as well as how these interactions impact production and release of toxins and other bioactive compounds.

Co-exposure of PTX2 with OA and/or STX did not alter the acute toxicity of PTX2 to larval oysters. PTX2 alone or in combination with OA and/or STX led to complete (100%) inactivity and 37 – 61% mortality of larval oysters by 48 h. Co-exposure to OA and STX had no measured effect on larval oysters, with no mortality and low (2%) inactivity by 48 h. Studies of combined effects (*in vivo* or *in vitro*) of HAB toxins are still rare (reviewed in Alarcan et al. 2018), and the authors are not aware of any studies that have combined pure STX with OA and/or PTX2. Given the complex and often concurrent nature of HAB species, combined effects studies should become commonplace as they are likely to provide realistic insight into the effects of HAB exposures in the environment.

4.4 Conclusion

In regions where *A. catenella* and *D. acuminata* co-occur with oyster spawning (Hattenrath-Lehmann et al. 2013, Loosanoff 1939), the sensitive, early life stages have the potential to be exposed to HAB cells and/or their toxins during or immediately following blooms. As demonstrated, exposure to live *A. catenella*, live or lysed *D.*

acuminata, or PTX2, could have negative consequences for larval oyster health and survival. The emergence of *D. acuminata* blooms (Brosnahan et al., unpublished) and nearly annual *A. catenella* blooms in the Nauset Marsh System, are not only detrimental to the shellfish industry (Crespo et al. 2011), but may also be impacting shellfish productivity in the area. Shellfish are not just vectors for human shellfish poisoning, in some instances larval oysters are negatively impacted by exposure to *A. catenella* and/or *D. acuminata*.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this chapter can be found in **APPENDIX II**.

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Table 1
Alexandrium catenella and *Dinophysis acuminata* bioassay treatments.

Bioassay	Treatments[a]	Cells/mL or Cells/mL equiv.	Species
Live Cell	Fed (Pav)[b]	25,000	*Pavlova pinguis*
	Unfed[b]	0	None
	Acat 10	10	*Alexandrium catenella*
	Acat 100	100	-
	Acat 500	500	-
	Acat 1,000	1,000	-
	Dacum 10	10	*Dinophysis acuminata*
	Dacum 100	100	-
	Dacum 500	500	-
	Dacum 1,000	1,000	-
Lysate	Fed (Pav)[b]	25,000	*Pavlova pinguis*
	Unfed[b]	0	None
	Acat 100	100	*Alexandrium catenella*
	Acat 1,000[b]	1,000	-
	Dacum 1,000[b]	1,000	*Dinophysis acuminata*
	Acat 1,000 x Dacum 1,000	[*]1,000	*Alexandrium catenella* & *Dinophysis acuminata*
Toxin	Carrier[b]	0	None
	OA	10,000	*Dinophysis acuminata*
	PTX2	10,000	*Dinophysis acuminata*
	STX	10,000	*Alexandrium catenella*
	OA x PTX2	10,000	*Dinophysis acuminata*
	OA x STX	[*]10,000	*Alexandrium catenella* & *Dinophysis acuminata*
	PTX2 x STX	[*]10,000	*Alexandrium catenella* & *Dinophysis acuminata*
	OA x PTX2 x STX	[*]10,000	*Alexandrium catenella* & *Dinophysis acuminata*

[a] Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, OA = okadaic acid, PTX2 = pectenotoxin-2, STX = saxitoxin
[b] Control treatments within each experiment
[*] Cells/mL equivalents of each algal species in the treatment, independently

Table 2
Inactivity and survival of larval oysters in the live cell bioassay at each timepoint.

Treatments[a]	24 h		48 h		72 h		96 h	
	Average *% surviving*	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**
Fed (Pav)	100.0 (0.0)	10.8 (3.2)	100.0 (0.0)	2.4 (1.8)	100.0 (0.0)	0.0 (0.0)	99.1 (0.9)	10.7 (4.0)
Unfed	100.0 (0.0)	10.1 (4.9)	100.0 (0.0)	6.2 (2.2)	100.0 (0.0)	4.3 (1.8)	100.0 (0.0)	5.7 (2.5)
Acat 10	100.0 (0.0)	26.6 (6.3)	100.0 (0.0)	11.7 (2.7)	100.0 (0.0)	9.4 (3.5)	94.3 (4.6)	46.3 (11.7) *
Acat 100	100.0 (0.0)	17.2 (5.2)	100.0 (0.0)	13.4 (5.4)	97.5 (2.5)	33.9 (9.9) †	90.8 (5.0)	82.1 (3.7) *
Acat 500	100.0 (0.0)	44.1 (5.8) *	98.8 (1.3)	24.6 (4.5)	98.8 (1.3)	85.6 (4.4) *	77.2 (8.7) *	71.2 (7.0) *
Acat 1,000	100.0 (0.0)	48.2 (4.0) *	100.0 (0.0)	53.3 (4.6) *	100.0 (0.0)	72.4 (5.0) *	100.0 (0.0)	89.8 (3.4) *
Dacum 10	100.0 (0.0)	19.2 (5.2)	99.2 (0.8)	8.2 (2.5)	97.1 (1.5)	7.1 (2.5)	96.1 (1.6)	14.5 (8.2)
Dacum 100	100.0 (0.0)	10.2 (5.6)	100.0 (0.0)	3.7 (2.8)	98.9 (1.1)	6.6 (3.1)	98.0 (1.4)	4.7 (2.1)
Dacum 500	100.0 (0.0)	5.6 (4.0)	100.0 (0.0)	5.9 (3.0)	100.0 (0.0)	5.8 (3.3)	97.2 (1.4)	4.0 (2.2)
Dacum 1,000	99.0 (1.0)	48.2 (10.6) *	88.6 (3.4) *	31.0 (8.3) †	83.9 (6.3) *	22.3 (5.9)	78.1 (7.0) *	14.5 (3.9)

Values indicate the treatment average with standard error given in parentheses.
Sample size was n = 10 wells per treatment.
[a] Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, numbers represent cell concentrations in cells/mL
[b] Percentage of surviving larval oysters that were inactive
*Value significantly different from the Fed and Unfed control values (Tukey-Bonferroni adjusted $\alpha = 0.0125$)
† Value significantly different from the Fed control value (Tukey-Bonferroni adjusted $\alpha = 0.0125$)

Table 3
Inactivity and survival of larval oysters in the lysate bioassay at each timepoint.

Treatments[a]	24 h		48 h		72 h		96 h	
	Average *% surviving*	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**
Fed (Pav)	100.0 (0.0)	0.9 (0.9)	100.0 (0.0)	2.0 (1.4)	100.0 (0.0)	2.2 (1.5)	100.0 (0.0)	2.0 (1.4)
Unfed	100.0 (0.0)	2.0 (1.3)	99.0 (1.0)	4.3 (3.4)	98.0 (1.3)	4.4 (1.8)	98.0 (1.3)	3.1 (1.6)
Acat 100	100.0 (0.0)	0.9 (0.9)	100.0 (0.0)	0.0 (0.0)	100.0 (0.0)	0.0 (0.0)	100.0 (0.0)	0.0 (0.0)
Acat 1,000	100.0 (0.0)	8.7 (4.4)	100.0 (0.0)	4.9 (1.7)	100.0 (0.0)	4.9 (2.2)	99.1 (0.9)	4.2 (1.7)
Dacum 1,000	100.0 (0.0)	14.2 (3.1) †	97.9 (1.4)	20.6 (6.6) *	93.8 (2.8)	27.4 (4.9) *	89.8 (4.0) *	37.7 (7.0) *
Acat 1,000 x Dacum 1,000	100.0 (0.0)	4.2 (2.3)	96.9 (1.6)	15.9 (4.4)	94.8 (2.3)	14.2 (4.4)	93.8 (2.3) *	13.1 (3.9)

Values indicate the treatment average with standard error given in parentheses.
Sample size was n = 10 wells per treatment.
[a] Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, numbers represent cell concentration equivalents (cells/mL equiv.)
[b] Percentage of surviving larval oysters that were inactive
*Value significantly different from the Fed and Unfed control values (Tukey-Bonferroni adjusted $\alpha = 0.0125$)
† Value significantly different from the Fed control value (Tukey-Bonferroni adjusted $\alpha = 0.0125$)

Table 4
Inactivity and survival of larval oysters in the pure toxin bioassay at each timepoint.

Treatments[a]	24 h		48 h		72 h		96 h	
	Average *% surviving*	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**	**Average *% surviving***	**Average *% inactive*[b]**
Carrier	100.0 (0.0)	4.3 (2.4)	100.0 (0.0)	2.0 (1.3)	99.0 (1.0)	1.1 (1.1)	99.0 (1.0)	1.1 (1.1)
OA	100.0 (0.0)	2.0 (1.3)	100.0 (0.0)	3.3 (2.2)	100.0 (0.0)	2.0 (2.0)	99.0 (1.0)	1.1 (1.1)
PTX2	79.0 (4.2) *	100.0 (0.0) *	50.4 (5.8) *	100.0 (0.0) *	-	-	-	-
STX	100.0 (0.0)	2.0 (1.3)	100.0 (0.0)	1.9 (1.3)	100.0 (0.0)	2.9 (1.5)	99.0 (1.0)	5.8 (2.1)
OA x PTX2	93.0 (2.1) *	100.0 (0.0) *	50.0 (4.7) *	100.0 (0.0) *	-	-	-	-
OA x STX	100.0 (0.0)	2.1 (1.4)	100.0 (0.0)	2.0 (1.3)	99.0 (1.0)	3.4 (1.7)	98.0 (1.3)	4.5 (1.8)
PTX2 x STX	90.3 (2.4) *	100.0 (0.0) *	63.5 (2.8) *	100.0 (0.0) *	-	-	-	-
OA x PTX2 x STX	82.0 (4.4) *	100.0 (0.0) *	38.9 (4.9) *	100.0 (0.0) *	-	-	-	-

Values indicate the treatment average with standard error given in parentheses.
Sample size was n = 10 wells per treatment.
[a] OA = okadaic acid, PTX2 = pectenotoxin-2, STX = saxitoxin
[b] Percentage of surviving larval oysters that were inactive
*Value significantly different from the Carrier control value (Benjamini-Hochberg adjusted $p < 0.05$)

Table 5
Oyster bioaccumulation of biotoxins associated with *Dinophysis acuminata*. N = 1 pooled oyster sample per treatment, results normalized to pg toxin/oyster.

Bioassay	Treatments[a]	Time collected (h)	# of oysters	Toxin (pg/oyster)		
				OA	DTX1	PTX2
N/A	Control	0	70*	<DL	<LOQ	<DL
Live Cell	Dacum 10	96	80	<DL	<DL	<DL
	Dacum 100	96	69	<DL	<DL	<LOQ
	Dacum 500	96	72	<DL	<DL	1.8
	Dacum 1,000	96	70	<DL	<DL	5.2
Lysate	Dacum 1,000	96	68	<DL	<DL	3.7
	Acat 1,000 x Dacum 1,000	96	65	<DL	<DL	2.5
Toxin	PTX2	48	74	<DL	<DL	40.2
	OA x PTX2	48	70	<DL	<DL	45.3
	PTX2 x STX	48	71	<DL	<DL	50.0
	OA x PTX2 x STX	48	70	<DL	<DL	47.9

Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*
DTX1 = dinophysistoxin-1, OA = okadaic acid, PTX2 = pectenotoxin-2, STX = saxitoxin
* Number of oysters estimated by volume from oyster density calculations

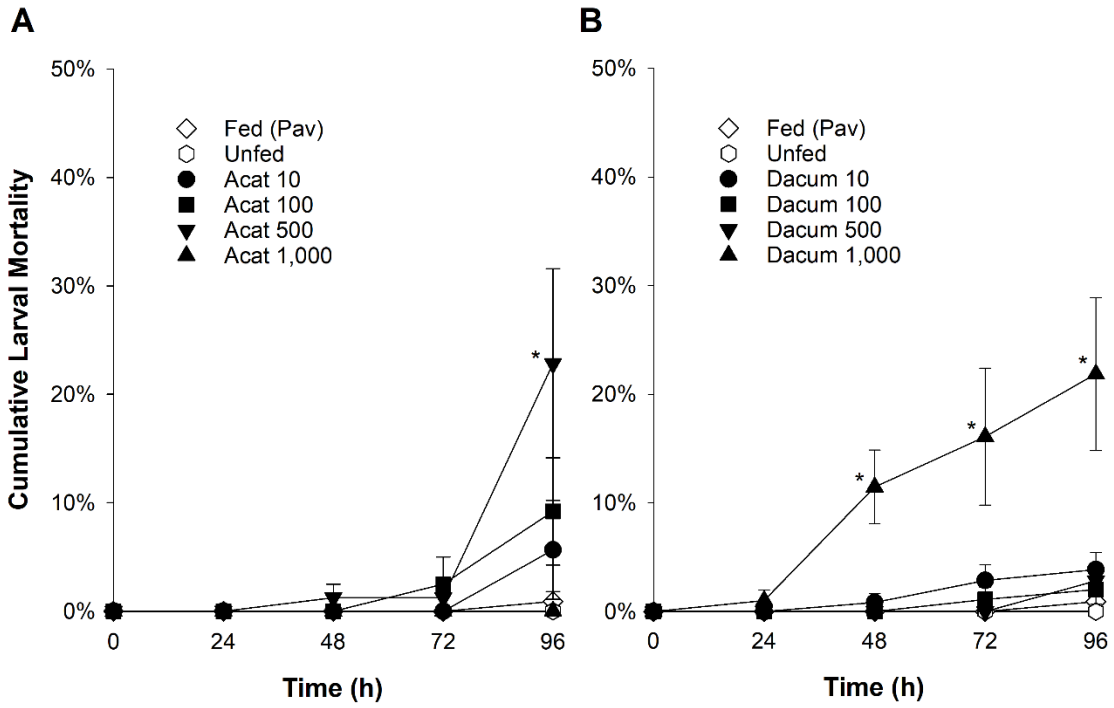

Figure 1
Cumulative larval mortality curves over time (hours) for the live cell bioassay, when larval oysters were exposed to (A) *Alexandrium catenella* (Acat), or (B) *Dinophysis acuminata* (Dacum), at four different initial cell concentrations (cells/mL). Error bars show standard error (n = 10 wells per treatment). Pav = *Pavlova pinguis*. Note the y-axis only goes up to 50% mortality. Datapoints that were significantly different from the Fed and Unfed controls are denoted by an asterisk.

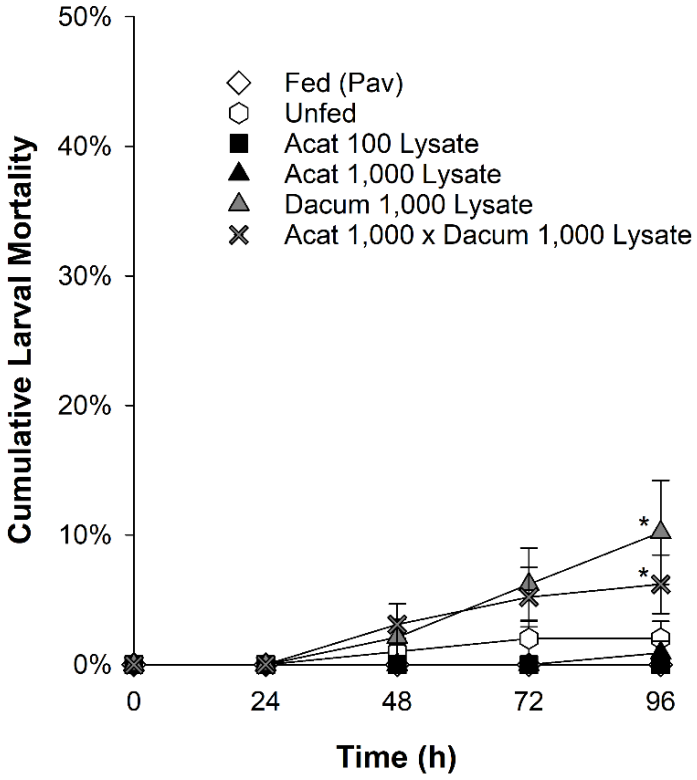

Figure 2
Cumulative larval mortality curve over time (hours) for the lysate bioassay, when larval oysters were exposed to *Alexandrium catenella* (Acat) and *Dinophysis acuminata* (Dacum) lysate treatments. Error bars show standard error (n = 10 wells per treatment). Pav = *Pavlova pinguis*, numbers represent cell concentration equivalents (cells/mL equiv.). Note the y-axis only goes up to 50% mortality. Datapoints that were significantly different from the Fed and Unfed controls are denoted by an asterisk.

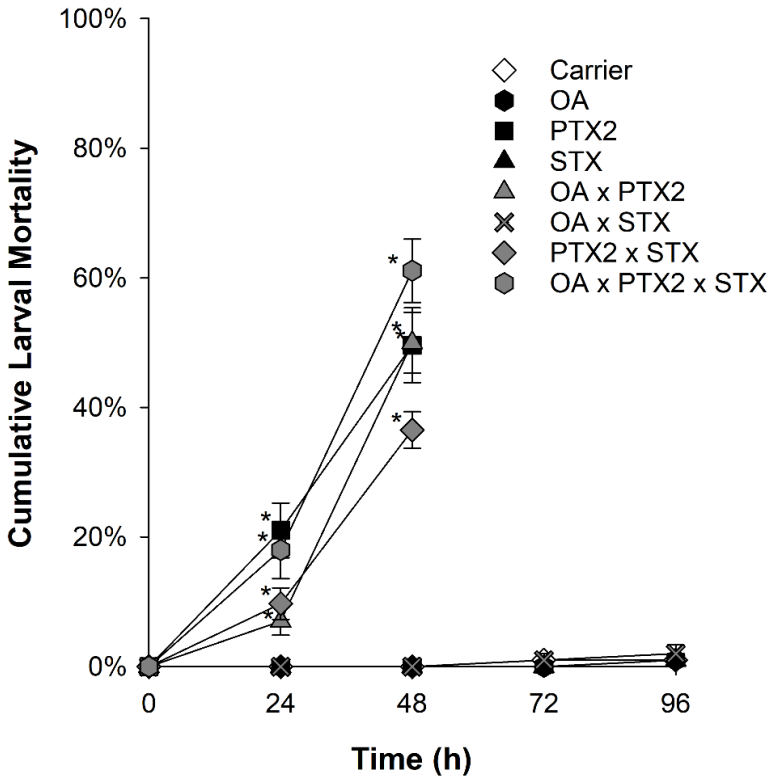

Figure 3
Cumulative larval mortality curve over time (hours) for the pure toxin bioassay, when larval oysters were exposed to okadaic acid (OA), pectenotoxin-2 (PTX2), and saxitoxin (STX), alone or in combination. Error bars show standard error (n = 10 wells per treatment). Note that PTX2, OA x PTX2, PTX2 x STX, and OA x PTX2 x STX treatments were terminated at 48 h. Datapoints that were significantly different from the Carrier control are denoted by an asterisk.

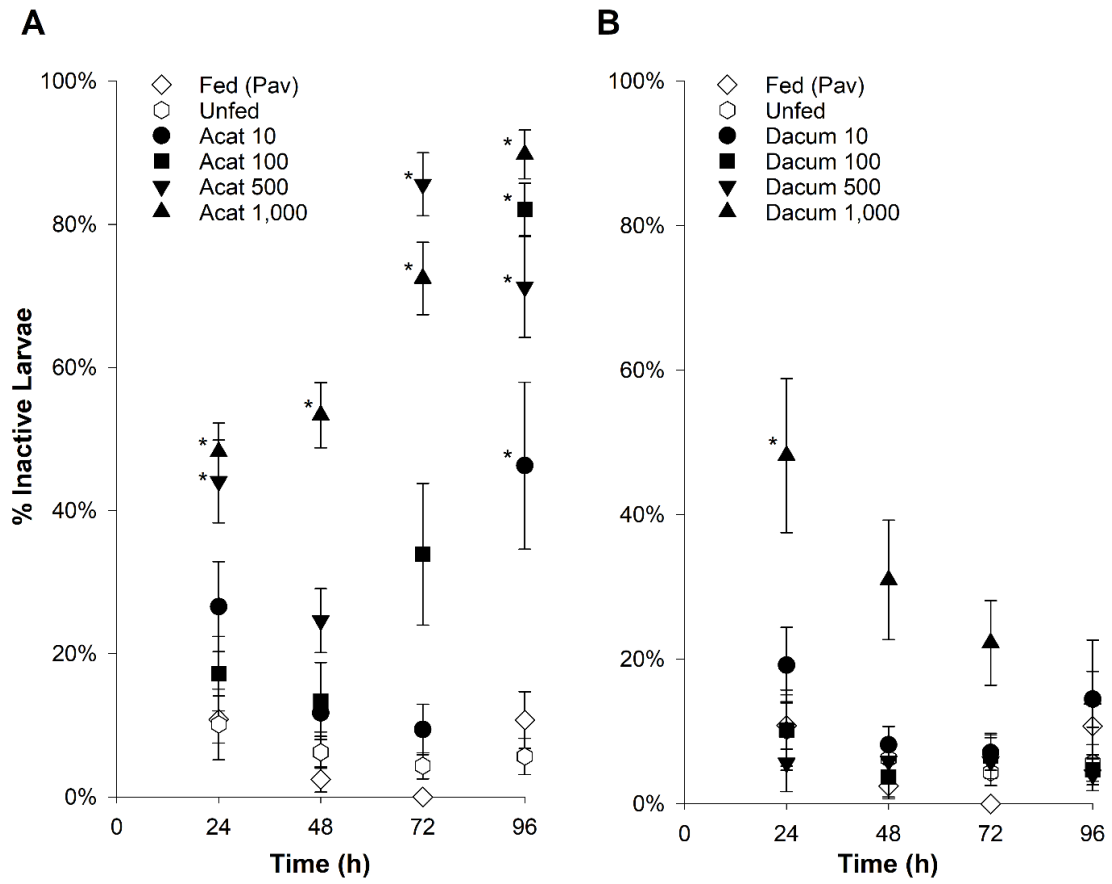

Figure 4
Percentage of inactive surviving larval oysters over time (hours) for the live cell bioassay, exposed to (A) *Alexandrium catenella* (Acat), or (B) *Dinophysis acuminata* (Dacum), at four different initial cell concentrations (cells/mL). Error bars show standard error (n = 10 wells per treatment). Pav = *Pavlova pinguis*. Datapoints that were significantly different from the Fed and Unfed controls are denoted by an asterisk.

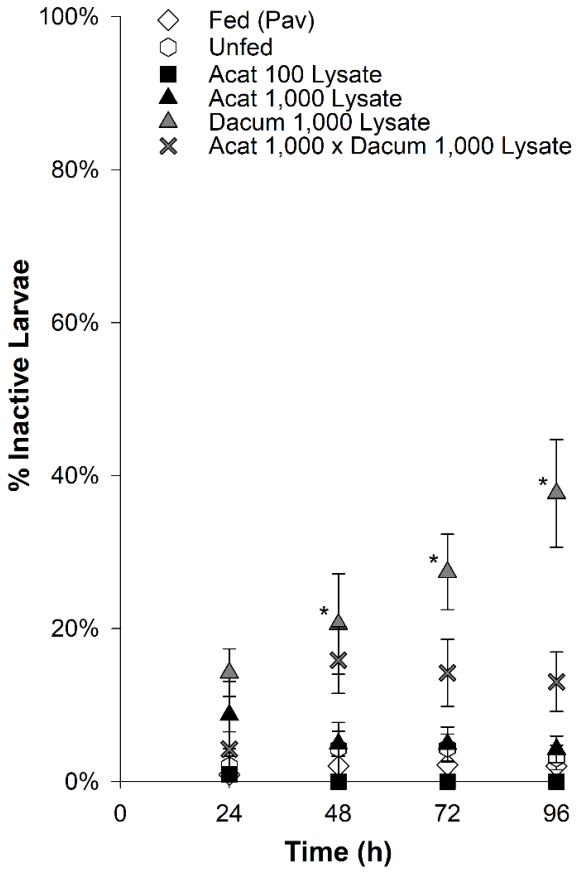

Figure 5
Percentage of inactive surviving larval oysters over time (hours) for the lysate bioassay, exposed to *Alexandrium catenella* (Acat) and *Dinophysis acuminata* (Dacum) lysate treatments. Error bars show standard error (n = 10 wells per treatment). Pav = *Pavlova pinguis*, numbers represent cell concentration equivalents (cells/mL equiv.). Datapoints that were significantly different from the Fed and Unfed controls are denoted by an asterisk.

CHAPTER 3

Comparison of marine and freshwater phycotoxins in oysters, solid phase adsorption toxin tracking devices (SPATTs), and particulate organic matter to inform HAB monitoring

ABSTRACT

To gain a baseline understanding of phycotoxin presence in the commercially-important eastern oyster (*Crassostrea virginica*), adult oysters from Chesapeake Bay, USA were screened for 13 phycotoxins over two years: azaspiracid-1 and 2 (AZA1, AZA2), domoic acid (DA), karlotoxin1-1 and 1-3 (KmTx1-1, KmTx1-3), microcystin-RR, LR, and YR (MC-RR, MC-LR, MC-YR), goniodomin-a (GDA), okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxin-2 (PTX2), and yessotoxin (YTX). Trace to low concentrations of AZA1, AZA2, DA, OA, and DTX1 were found in oysters, orders of magnitude below regulatory limits for associated human health syndromes. Phycotoxins more commonly associated with shellfish health, KmTx1-1, KmTx1-3, GDA, and PTX2, were also detected in oysters. Microcystins, MC-RR and MC-YR, were found in oyster tissue (maximum: 7.12 µg MC-RR/kg shellfish meat [SM]), indicating an urgent need for the development of regulatory limits for these freshwater phycotoxins in marine shellfish. Phycotoxins in oysters were compared to those detected in co-deployed solid phase adsorption toxin tracking (SPATT) devices and in particulate organic matter (POM, > 1 µm) to assess these metrics as monitoring tools. The dominant phycotoxin varied between oysters and SPATTs (DA and OA, respectively), and only four phycotoxins were detected in POM (AZA2, DA, MC-RR, PTX2) out of the 11 detected in oysters, indicating a mismatch in phycotoxin profiles between different compartments of the ecosystem. Despite this, DA in oysters was correlated to DA in POM collected two weeks prior (simple linear regression [LR]: $R^2 = 0.6$, $p < 0.0001$, $n = 39$), and PTX2 in oysters was well modeled by PTX2 in co-deployed SPATTs (LR: $R^2 = 0.4$, $p < 0.0001$, $n = 80$). Phycotoxin distribution varied with depth at nearshore, shallow sites (≤ 2 m); more

phycotoxins were detected in SPATTs deployed near-bottom than at surface (7 and 5, respectively), while the reverse was true for POM (3 near-bottom and 4 at surface). In the nearshore waters of the lower Chesapeake Bay, SPATTs or POM could be used for specific phycotoxin research and monitoring applications, but were not good indicators of overall phycotoxin bioaccumulation in oysters. Regional baseline phycotoxin data will help guide future phycotoxin monitoring and surveillance efforts.

KEYWORDS

solid phase adsorption toxin tracking (SPATT), domoic acid, pectenotoxin, goniodomin A, azaspiracid, okadaic acid, dinophysistoxin, karlotoxin, microcystin, monitoring, seafood safety, Chesapeake Bay, DSP, ASP, AZP, shellfish, oysters

1. INTRODUCTION

Along with fresh seafood consumption per capita, market demand for oysters in the USA has generally increased since 2000 (Botta et al. 2020, NMFS 2021). The USA commercial oyster fishery was valued over $250M in 2019 (NMFS 2021). In the same year, the ex-vessel dollar value (i.e., dollars received at time of first sale) of oyster (*Crassostrea virginica*) landings in the USA Middle Atlantic fishery region was $51M, with Virginia making up about $39M of that amount (NOAA Fisheries 2020). Virginia is home to a lucrative oyster fishery, ranking first in oyster production along the USA East Coast (Hudson 2019). Much of this oyster production occurs in the waters of the Chesapeake Bay.

The Chesapeake Bay is also home to an array of phycotoxins that have recently been detected via passive samplers, or solid phase adsorption toxin tracking devices (SPATTs, Onofrio et al. 2021). This same study found high levels of phycotoxin co-occurrence in the region, with 76% of samples detecting more than one phycotoxin. Furthermore, phycotoxins were detected year-round throughout the Virginia portion of the Bay (Onofrio et al. 2021), suggesting that oysters in the area are exposed to dissolved phycotoxins throughout grow-out and up to harvest.

Phycotoxins in the Bay are produced by a range of dinoflagellates, diatoms, raphidophytes, and cyanobacterial harmful algal bloom (HAB) species (Marshall 1996, Marshall and Egerton 2009). Some of these species are associated with seafood safety, while others are associated with finfish and shellfish health. Currently, there is no regional baseline data for the accumulation of these phycotoxins in shellfish. These data are necessary to assess risks to human health and identify those phycotoxins that may be

impacting resource sustainability through their accumulation in, and deleterious impacts on, shellfish.

Some phycotoxins that have been detected in the Bay can be associated with human syndromes from consuming shellfish that have bioaccumulated high concentrations of these phycotoxins; these phycotoxins and their associated syndromes include: azaspiracids (AZAs)—azaspiracid shellfish poisoning (AZP), domoic acid (DA)—amnesic shellfish poisoning (ASP), and diarrhetic shellfish toxins (DSTs)—diarrhetic shellfish poisoning (DSP). AZAs are produced by the *Amphidomataceae* family of dinoflagellates (Tillmann et al. 2017), but a causative organism has yet to be identified in the Chesapeake Bay (Onofrio et al. 2021). Diatoms from the DA-producing genus *Pseudo-nitzschia* have been documented in the Bay (Thessen and Stoecker 2008), as have DST-producing dinoflagellates *Dinophysis* spp. and *Prorocentrum lima* (Barbier et al. 1999, Marshall et al. 2005, Wolny et al. 2020a). In addition to these phycotoxins, the traditionally freshwater phycotoxins, microcystins (MCs), have been detected in the Bay along with MC-producing *Microcystis aeruginosa* (Tango and Butler 2008, Wood et al. 2014, Bukaveckas et al. 2017, 2018, Onofrio et al. 2021). MCs are hepatotoxins produced by *Anabaena*, *Aphanizomenon*, *Nostoc*, *Microcystis*, and *Planktothrix* cyanobacteria (Eriksson et al. 1990, Dawson 1998, Campos and Vasconcelos 2010). Concern has been raised about seafood safety in relation to MCs and shellfish, which can concentrate MCs (Miller et al. 2010, Mulvenna et al. 2012, Vareli et al. 2013, Preece et al. 2017, Camacho-Muñoz et al. 2021). Current HAB monitoring in Virginia is coordinated by the Virginia Department of Health (VDH) and consists of monthly sampling of water at over 60 stations throughout shellfish growing areas. The abundances

of phycotoxin-producing species are monitored, with subsequent testing of shellfish tissues for phycotoxins when elevated cell numbers are detected. To date, there have been no human illnesses documented to be caused by phycotoxin through consumption of Virginia shellfish, and only one precautionary shellfish harvest closure due to elevated *Dinophysis* in the Potomac River, however, only trace concentrations of DSTs were detected in exposed shellfish (Tango et al. 2004).

Other phycotoxins found in the Bay can negatively impact shellfish health. Pectenotoxin-2 (PTX2) is a phycotoxin produced by *Dinophysis* spp. that has been documented in the Bay (Onofrio et al. 2021). While regulated as a DST in the European Union and New Zealand (EFSA 2009, Boundy et al. 2020), PTX2 is not regulated elsewhere in the world because of dubious oral toxicity to mammals (Boundy et al. 2020 and references therein). This phycotoxin has however been documented to be harmful to shellfish health, reducing oyster fertilization success (Gaillard 2020, Gaillard et al. 2020). In the Bay, goniodomin A (GDA) is produced by the dinoflagellate *Alexandrium monilatum* (Hsia et al. 2006, Wolny et al. 2020b). Exposure to live or lysed *A. monilatum* has been shown to have negative impacts on shellfish health, inducing valve closure, reduced clearance rates, and mortality in oysters, clams, and mussels (Ray and Aldrich 1966, Sievers 1969, May et al. 2010, Pease 2016). While the precise mechanism of toxicity remains undetermined, GDA was detected in whelks that died during an *A. monilatum* bloom (Harding et al. 2009). *Karlodinium veneficum* is a dinoflagellate in the Bay that produces karlotoxins (KmTxs), including KmTx1-1 and KmTx1-3 (Brownlee et al. 2008, Stoecker et al. 2008, Bachvaroff et al. 2008, 2009, Adolf et al. 2009). After exposure to KmTx-producing *K. veneficum*, reduced clearance rates, reduced growth,

and/or mortality have been reported in oysters, clams, mussels, and scallops (Abbott and Ballantine 1957, Nielsen and Strømgren 1991, Galimany et al. 2008, Brownlee et al. 2008, Place et al. 2008).

The current study is a continuation of the work from a collaboration between the Virginia Institute of Marine Science (VIMS) and the VDH Division of Shellfish Safety and Waterborne Hazards to collect baseline data on phycotoxin spatiotemporal distribution in the Virginia portion of the Chesapeake Bay (Onofrio et al. 2021). The focus of this work was phycotoxins in oysters (*C. virginica*) and how phycotoxins in oysters related to other metrics. Efficient methods for monitoring a suite of phycotoxins in shellfish are needed to assess risks to seafood safety and shellfish health and productivity. The objectives of this study were to (1) establish baseline phycotoxin spatiotemporal distribution data in oysters for the Virginia portion of the Chesapeake Bay, (2) examine relationships between phycotoxin concentrations in oysters, SPATTs, and particulate organic matter (POM, > 1 µm) to assess whether oyster phycotoxin concentrations could be monitored or predicted from another metric, and to (3) examine the amount of variation in phycotoxin concentration with depth in SPATTs or in POM samples in shallow, near-shore waters, to determine best sampling practices.

2. MATERIALS & METHODS

2.1 Field study design

A field study was performed over six months in 2019 (January through June), and in 2020 (March through August), in nearshore waters of the Virginia-portion of the Chesapeake Bay. Four sites were sampled in each year, and were selected based on their proximity to shellfish growing areas and assignment to different sub-watersheds (**Fig. 1**, Onofrio et al. 2021). Due to personnel constraints in 2020, the Cherrystone site was replaced by the site at Wise Point. The five sites sampled during the current study were previously characterized by Onofrio and colleagues (2021); briefly, the sites were mesohaline (S = 5 – 18, Rappahannock) to polyhaline (S = 18 – 30, all other sites), with intermediate to quick flushing rates, low (0 - <0.5 µg/L, Wise Point) to medium (5 - 20 µg/L, all other sites) on the chl a eutrophic index, and shallow (≤ 2 m). Geomorphic settings of each site were classified: Rappahannock is a tidal creek, York is a tidal river, Lynnhaven and Cherrystone are tidal inlets, and Wise Point is a strait.

Adult oysters (30-134 mm, *C. virginica*) were deployed once per year at each site, approximately two weeks before the sampling period. Oyster cages were 0.3 m off the bottom and SPATTs were deployed above the oyster cage, i.e., 0.6 m from the bottom. Sampling occurred approximately every other week throughout the sampling period in each year for phycotoxin quantification in oyster meat (6 – 15 oysters pooled), SPATTs, and POM (> 1 µm), and the enumeration of cells in discrete surface water samples. To further compare phycotoxin concentrations and cell abundances between the surface and bottom, additional complementary water samples were collected approximately 0.5 m off

the bottom using a Niskin bottle, and an additional SPATT was deployed at each site just below surface using a float in 2019.

2.2 Sample preparation and phycotoxin extraction

After sampling, oysters were shucked, rinsed with ultrapure water to remove salts, and stored at -20 ºC until phycotoxin extraction. Extraction followed McNabb et al. (2005); briefly, 18 mL of 90% methanol (MeOH) was used to extract 2 g of pooled oyster homogenate, crude extract was centrifuged (3234 x g, 10 min, 4 ºC), the supernatant was aliquoted for alkaline hydrolysis (1 mL) and extra clean-up with hexane (2 mL).

SPATTs were constructed and prepared with Diaion® HP-20 resin (Fux et al. 2008). This resin readily adsorbs phycotoxins with a range of different polarities and sizes (Lane et al. 2010, Kudela 2011, McCarthy et al. 2014, Roué et al. 2018, Onofrio et al. 2021). SPATTs were stored, deployed, and extracted as described in Onofrio et al. (2021). Briefly, during extraction SPATTs were rinsed with ultrapure water to remove salts, resin was transferred to a PVDF 0.45-µm spin filter cup (Thermo Fisher Scientific, Waltham, MA, USA) within a 50-mL centrifuge tube, and the resin underwent sequential extractions with 10 mL of 35% MeOH and 2X 10 mL of 100% MeOH. The 35% MeOH extracts were collected separately; 100% MeOH extracts were pooled for each sample.

POM samples were prepared by filtering 200 mL of sample water through a Whatman 934-AH GFF filter (nominal pore size 1 µm). Filters were stored at -20 ºC until extraction. Filters were extracted in 2.0 mL of 100% MeOH, bath sonified for 30 min, centrifuged (3200 x g, 10 min, 4 ºC), and the supernatant collected.

An aliquot of every extracted sample, except 2019 SPATTs, was treated by alkaline hydrolysis to convert DST derivatives into the parent toxins, okadaic acid (OA)

and dinophysistoxin-1 (DTX1), following an adaptation of Villar-González et al. (2008). Briefly, 125 µL of NaOH 2.5N was added to 1 mL of sample extract, the mixture was heated to 76 ºC for 40 min and then neutralized with 125 µL of AcOH 2.5N.

2.3 Phycotoxin analysis

Sample extracts were analyzed for 13 phycotoxins (AZA1, AZA2, DA, MC-LR, MC-RR, MC-YR, KmTx1-1, KmTx1-3, GDA, YTX, PTX2, OA, DTX1) at VIMS using ultra-performance liquid chromatography-tandem mass spectrometry, with a trapping dimension and at-column dilution (UPLC-MS/MS with trap/ACD, Onofrio et al. 2020). Parent > daughter transitions, as presented in Onofrio et al. (2021), were used for quantitation, with the addition of a transition for DA: *m/z* 312.0 > 266.1, 30V, 15eV (Onofrio 2020). All extracts were 0.22-µm syringe filtered (PVDF, 13-mm, Durapore) and stored at -20 ºC for a maximum of two weeks before phycotoxin analysis. Injection volumes for each sample were 50 µL for oyster samples, and 100 µL for SPATTs and POM samples.

Standard curves were prepared in 100% MeOH using a series of 9 dilutions between 0.1 and 50 µg/L for all phycotoxins except AZA1 and AZA2, which were diluted between 0.004 and 2 µg/L. SPATTs from 2019 were run with the higher standard curve (0.1 – 50 µg/L) for AZAs and any samples with detectable AZA2 were rerun with an injection volume of 200 µL and a standard curve between 0.003 and 2 µg AZA2/L. KmTxs were not included in the standard curve due to limited purified material; a check standard of unknown concentration (~2 µg/L) containing KmTx1-1 and KmTx1-3 was run with each sample set to determine presence/absence. Instrument limits of detection (LOD) for the majority of phycotoxins were between 0.01 and 0.39 µg/L (Onofrio et al.

2020); LOD for DA and AZAs were 0.18 and 0.004 µg/L, respectively (Onofrio unpublished and this study). Blank injections of 100% MeOH were run after each set of 15 SPATTs or POM extracts, and after every 3 oyster extracts, to confirm that carryover was not occurring. In addition, 2 min of isocratic flow, 95% acetonitrile, was added to the end of each oyster run to provide better cleanup between injections. To confirm that retention times remained consistent, check standards (5 µg/L for each phycotoxin, or 0.12 µg/L of AZA1 and AZA2, with 3 µg/L of the other phycotoxins) or full standard curves were run after every 15-16 injections of extracts. SPATT and POM phycotoxin concentrations less than the limit of quantitation (LOQ) were represented as ½ LOD. Oyster phycotoxin concentrations less than the LOQ but with S/N ≥ 10 and a parent peak with S/N ≥ 3, were represented as ½ the concentration of the lowest quantifiable standard. Non-detects were represented as 0. Phycotoxin results in oysters were also presented as the percentage of samples that tested positive within the 81 extracts evaluated across all sites and time points.

The 2019, 35% MeOH extracts from SPATTs were analyzed at VDH for DA as described in Onofrio et al. (2021). Briefly, DA (ASP) ELISA kits (Abraxis Inc., Warminster, PA, USA) and an Abraxis plate reader were used to detect and quantify DA.

Phycotoxin standards were purchased from the National Research Council Canada: CRM-AZA1-b, CRM-AZA2-b, CRM-DA-g, CRM-YTX-c, CRM-PTX2-b, CRM-OA-d, CRM-DTX1-b. A mixed solution of MC-LR, MC-RR, and MC-YR was purchased from Sigma Aldrich (33578-1ML). KmTx1-1 and KmTx1-3 were purified from *K. veneficum* and provided by Dr. Allen Place (UMCES, Maryland). GDA was

purified from *A. monilatum* and provided by Drs. Thomas and Constance Harris (Harris et al. 2020).

2.4 Microscopic analysis

Microscopic analysis for HAB species was carried out in Lugol's preserved water samples as described in Onofrio et al. (2021), with the exception that in 2020, 5-mL aliquots were enumerated, improving the detection limit from 1 to 0.2 cells/mL. Briefly, HAB cells were identified and enumerated in Sedgewick Rafter counting chambers or well plates (Cellvis P12-1.5H-N, Mountainview, CA, USA) using light microscopy at 100 – 400X (Olympus CKX41, or Olympus 1X51 with Olympus DP73 digital camera and cellSens Standard software, Center Valley, PA, USA).

2.5 Statistical analysis

For phycotoxins that were detected in ≥ 10 oyster samples, linear regressions were used to determine whether phycotoxins in SPATTs (near-bottom), POM (surface), or HAB cell concentrations (surface), could be used as predictors of phycotoxin concentrations in oysters. Data were log10-transformed to meet assumptions of normality and centered. All linear regressions were re-run with a two-week (i.e., one sampling event) lag time, to see if predictor data from two weeks prior to oyster sampling improved model fit.

For phycotoxins that were detected in ≥ 10 SPATT samples, Wilcoxon signed-rank tests were used to test for any difference between phycotoxin concentrations at the surface and near-bottom. There were no phycotoxins that were detected in ≥ 10 POM samples, so no statistical comparisons of phycotoxin concentration between surface and near-bottom were made. Sampling events from 2019 were included in depth analysis,

with data from Rappahannock, York, Lynnhaven, and Cherrystone. The percentages of SPATTs and POM samples with each phycotoxin detected from samples collected at the surface and near-bottom were calculated for comparison.

Raw data for these analyses along with time series plots for AZA2, DA, and PTX2 for select sites, can be found in **APPENDIX III**. Statistical tests were performed in R Studio (2019) using R version 3.6.2.

3. RESULTS & DISCUSSION

This is the first comprehensive examination of phycotoxin concentrations in oysters in shallow, nearshore portions of the lower Chesapeake Bay, and the first to show that co-occurrence of phycotoxins in oysters was common (54% of oyster samples, n = 81). Of the 13 phycotoxins screened for in oysters during this study, 11 were detected: AZA1, AZA2, DA, MC-RR, MC-YR, KmTx1-1, KmTx1-3, GDA, PTX2, OA, and DTX1; MC-LR and YTX were not detected. The majority of oyster samples (84%, n = 81), contained at least one of these phycotoxins. DA had the highest cumulative concentration in oysters across all sampling time points and sites (**Table 1**), the greatest phycotoxin maximum in one sample of shellfish meat (SM, 579.42 µg DA/kg SM), and was one of the most prevalent phycotoxins in oyster samples (40%, n = 81, **Fig. 2**). Phycotoxins MC-RR, 7.12 µg/kg SM, and PTX2, 6.16 µg/kg SM, had the next highest phycotoxin maximums in oysters, followed by AZA1 and AZA2, 0.80 and 0.42 µg/kg SM, respectively. Phycotoxins KmTx1-1 and KmTx1-3 were present in oysters, but were not quantified in the current study, and trace amounts of MC-YR, GDA, OA, and DTX1 were detected in oysters, but amounts were below the respective limits of quantitation. Some of the phycotoxins detected in oysters are known to negatively impact shellfish health. Phycotoxins associated with seafood safety were also present, however, concentrations in oysters were well below regulatory limits (USFDA 2019). Further detail on Chesapeake Bay-specific results can be found below and in **APPENDIX IV**.

3.1 Shellfish health phycotoxins

The majority of oyster samples (64%, n = 81), contained at least one phycotoxin associated with shellfish health: GDA, KmTxs, and PTX2. These phycotoxins are not

expected to pose risks to seafood safety (Miles et al. 2004, Place et al. 2014, Boundy et al. 2020), but are detrimental to shellfish health (Place et al. 2008, Harding et al. 2009, Gaillard et al. 2020). Overall, the most commonly observed co-occurrence of phycotoxins in an oyster sample was the co-occurrence of KmTxs and PTX2. Phycotoxins of concern for shellfish health co-occurred in 12% of oyster samples (n = 81), in samples from all study sites and in both study years. This demonstrates the need for more research on combined effects of HAB phycotoxins on shellfish to better understand the ecological stressors and their consequences for shellfish populations.

Comparing shellfish health phycotoxins between metrics (oysters, SPATT, and POM), and between depths (surface and near-bottom), elucidated potential predictors of oyster phycotoxin load and improvements to monitoring in the Bay. There was no significant difference in concentrations of shellfish health phycotoxin between SPATTs at surface and near-bottom (**Fig. 3**), but slight variations with depth were observed with another matrix, POM. This indicates that dissolved phycotoxins (detected by SPATTs) were more evenly distributed with depth than particulate phycotoxins (detected by POM). More specifically, PTX2 was detected more often in surface, rather than near-bottom POM samples, while GDA was only detected near-bottom in one POM sample (**Table 2**). SPATTs were the best predictor of PTX2 concentrations in oysters; PTX2 in oysters was significantly correlated to PTX2 in SPATTs and POM collected at the same time as the oysters (LR SPATTs: $R^2 = 0.4$, $p < 0.0001$, $n = 80$; LR POM: $R^2 = 0.1$, $p = 0.01$, $n = 43$, **Table 3**). SPATTs collected two weeks before the oysters also exhibited a significant correlation with oyster PTX2 concentrations (LR: $R^2 = 0.3$, $p < 0.0001$, $n = 73$), indicating that SPATTs could potentially be used for early warning for this phycotoxin in

oysters. KmTxs were not detected in SPATTs or POM samples, demonstrating that these metrics could not be used to monitor exposure risk to KmTxs. The choice of metric for monitoring different shellfish health phycotoxins should consider the likely routes of exposure for shellfish, i.e., dissolved or particulate, as well as the depth the shellfish are filtering at, i.e., on a natural reef, in bottom cages, in floating cages. Currently, shellfish health phycotoxins are not monitored, however, should the need arise, SPATTs and POM should be further explored as a means to infer shellfish exposure to PTX2 and other shellfish health phycotoxins.

3.1.1 Goniodomin A (GDA)

GDA was occasionally detected in trace amounts in oyster samples in this study; however, GDA-producing *A. monilatum* were not detected in preserved samples. GDA has been implicated in mortality of whelks (*Rapana venosa*, Harding et al. 2009), where concentrations in afflicted whelk foot tissue were as high as 8.77 mg/kg SM. This study detected GDA in oyster samples from the Rappahannock site (**Fig. 4**), where *A. monilatum* cells have been identified (Wolny et al. 2020b) and GDA has been detected in SPATTs (**Table 1**, Onofrio et al. 2021), corroborating that *A. monilatum* is likely present at least this far north within the Chesapeake Bay. Despite the York site being a frequent hot-spot for *A. monilatum* blooms (Mackiernan 1968, Marshall and Egerton 2009, Wolny et al. 2020b) and GDA in SPATTs (**Table 1**, Onofrio et al. 2021), no GDA was detected in oysters at the York site during the current study.

GDA was detected in oysters in June and July in this study (**Fig. 4**). Blooms of GDA-producing *A. monilatum* generally occur in the Chesapeake Bay in the late summer (Mulholland et al. 2018, Wolny et al. 2020b, Onofrio et al. 2021), with GDA detectable

in SPATTs in the southern Chesapeake year-round, but peaking from August to October (Onofrio et al. 2021). GDA was also detected in surface and near-bottom SPATTs in the current study, and in one near-bottom POM sample (**Table 2**). As GDA is known to degrade rapidly in seawater (Onofrio et al. 2020), the presence of GDA in oysters, SPATTs, and POM before a bloom may implicate resuspended, or recently excysted, *A. monilatum* as the phycotoxin source; further study on this matter is needed. Cysts of *A. monilatum* are known to be widespread throughout the sediments of the lower Chesapeake Bay (Pease 2016, Van Hauwaert 2016), but cyst phycotoxin content has not been studied. Future studies of GDA in oysters should sample beyond August to reveal more information about oyster bioaccumulation of GDA during and after an *A. monilatum* bloom.

3.1.2 Karlotoxins (KmTxs)

This is the first known detection of KmTxs in oysters; however, KmTxs were not detected in SPATTs in this (**Table 1**), or a previous study (Onofrio et al. 2021). KmTxs are poorly soluble (Sheng et al. 2010) and degrade rapidly in the environment (Onofrio et al. 2021), leading to little sorption of KmTxs to SPATTs. Oysters, however, may feed directly on KmTx-producing *K. veneficum* cells (Brownlee et al. 2008) and accumulate KmTxs through diet. Furthermore, oysters exposed to 30,000 cells *K. veneficum*/mL, representing KmTx concentrations of 18.5 ng KmTxs/mL, exhibited reduced clearance rates (Place et al. 2008).

KmTxs were detected in oysters (January through May) when blooms of *K. veneficum* generally occur in the Chesapeake (Li et al. 2000, Glibert et al. 2007, Marshall and Egerton 2009). Cells of *K. veneficum* were detected at all sites. In 2019, cells of *K.*

veneficum were detected in preserved water samples in April and May, while in 2020, cells were detected March through July. All 2019 preserved water samples that had *K. veneficum* cells corresponded to oyster samples positive for KmTxs, while the majority of 2020 preserved water samples that had *K. veneficum* cells corresponded to oyster samples that were negative for KmTxs. KmTxs were almost exclusively detected in oyster samples from 2019, with the exception of two oyster samples that tested positive for KmTx1-1 from the York site in March and April 2020. In the current study, KmTx1-1 was more commonly detected in oyster samples than KmTx1-3 (28% and 5%, respectively, n = 81, **Fig. 2**). KmTx1-3 was only detected in oyster samples from April 2019, when KmTx1-1 was also present (**Fig. 5**), and often along with, or just preceding, *K. veneficum* cells in corresponding preserved water samples. Collectively, these data suggested that in 2020 there was reduced KmTx production, and/or the presence of a non-toxic strain of *K. veneficum*. Non-toxic and toxic strains of *K. veneficum* have been isolated from the Chesapeake Bay (Bachvaroff et al. 2009), and toxin production varies with nutrient availability, salinity, and temperature (Adolf et al. 2009, Fu et al. 2010, Place et al. 2012).

3.1.3 Pectenotoxin-2 (PTX2)

PTX2 was the most prevalent phycotoxin detected in oyster samples in this study (41%, n = 81, **Fig. 2**), and was the second-most abundant phycotoxin in oyster samples, reaching 6.16 µg PTX2/kg SM in one oyster sample (**Table 1**). There have been no studies on the effects of PTX2 on adult shellfish, however, two studies have looked into the effects of PTX2 on early life stages of oysters. Gametes of *C. gigas* exposed to PTX2 exhibited decreased fertilization success (Gaillard et al. 2020), and larval *C. virginica*

exposed to PTX2 exhibited inactivity followed by mortality (see **CHAPTER 2**). The distribution of oyster samples with PTX2 was widespread, but concentrated in the most southern sites, generally from April to June (**Fig. 6**) when cells of *Dinophysis* spp. were detected in water samples (March to June). Local strains of *Dinophysis* generally bloom in April and May, and exhibit phycotoxin profiles dominated by PTX2 (Fiorendino et al. 2020, Wolny et al. 2020a). PTX2 was detected on all SPATTs in the current study, suggesting persistence of this phycotoxin in the estuarine environment. PTX2 concentrations in SPATTs and in POM were significant predictors of PTX2 concentrations in oysters (LR SPATTs: $R^2 = 0.4$, $p < 0.0001$, $n = 80$; LR POM: $R^2 = 0.1$, $p = 0.01$, $n = 43$), while HAB cell concentration was not (LR: $R^2 = 0.01$, $p = 0.2$, $n = 73$, **Table 3**).

3.2 Seafood safety phycotoxins

The majority of oyster samples (67%, n = 81), contained at least one phycotoxin associated with seafood safety: AZAs, DA, DSTs (OA and DTX1), and MCs, however, phycotoxin concentrations in oysters were well below regulatory limits set to protect public health (USFDA 2019) and do not pose a current risk to the region. The majority of these phycotoxins are associated with well-known human health syndromes like AZP (AZAs), ASP (DA), and DSP (DSTs), while hepatotoxic MCs have been flagged as a potential seafood safety concern (Miller et al. 2010, Chorus and Welker, eds. 2021). In the current study, seafood safety phycotoxins co-occurred in 23% of oyster samples (n = 81), at least once at each site. The majority of co-occurrences were between AZAs and DA (68% of seafood safety phycotoxin co-occurrences, n = 44), demonstrating that oysters can co-accumulate at least low concentrations of multiple seafood safety

phycotoxins. These findings support the need for further study of chronic, sub-acute exposure to these phycotoxins, as well as a risk assessment of the combined effects of these phycotoxins for seafood safety.

3.2.1 Azaspiracids (AZAs)

This is the first known report of AZAs (AZA1 and AZA2) in shellfish on the eastern coast of the United States, specifically in the lower Chesapeake Bay. There are relatively few reports of AZAs or AZA-producing species in North America (Twiner et al. 2008, Trainer et al. 2013, Luo et al. 2016, Kim et al. 2017, Adams et al. 2020). Compared to other phycotoxins detected, AZA concentrations were low (**Table 1**), especially considering that AZA signals were enhanced due to the methodology (**APPENDIX V**). The highest concentration of AZAs detected (0.80 µg AZA1/kg SM) was three orders of magnitude below the regulatory limit for AZAs in shellfish (160 µg/kg SM, USFDA 2019), suggesting there is no current risk to seafood safety, but providing justification for regional monitoring of these phycotoxins, and for including them in regional biocontingency plans.

Of the AZA congeners included in the current analyses, AZA1 predominated; AZA1 was more widespread over time and space in oysters than AZA2 (**Fig. 7**). AZAs were detected at all sites and were detected from February through August, with no obvious seasonality (**Fig. 7**). AZA1 occurrence in oyster samples (23%) was almost double that of AZA2 (12%, **Fig. 2**). This is the opposite of AZA occurrence in SPATTs from this (**Table 1**) and a previous (Onofrio et al. 2021) study, that found AZA2 to be more prevalent, which may reflect differences in chemical stability, and/or uptake or detoxification rates between the analogs. Additionally, AZA1 and AZA2 did not co-occur

in oysters or SPATTs in the current study, however, AZA1 was only detected in one SPATT, in a trace amount. This is in contrast to a previous report of AZA1 and AZA2 co-occurrence in SPATTs (Onofrio et al. 2021). *Azadinium spinosum* has been shown to primarily produce AZA1 with small amounts of AZA2 (Tillmann et al. 2009), and *A. poporum* primarily produces AZA2 (Tillmann et al. 2016), however, AZA-producing species have not yet been identified to species-level within the Chesapeake Bay and so were not monitored as part of this study.

3.2.2 Domoic acid (DA)

DA was the most abundant phycotoxin detected in oysters in the lower Chesapeake Bay (**Table 1**), and one of the most prevalent phycotoxins in oyster samples (40%, n = 81, **Fig. 2**). DA was generally detected in concentrations at least an order of magnitude higher than other phycotoxins detected. This phycotoxin was detected in oysters from January to August, except during the month of June (**Fig. 8**). *Pseudo-nitzschia* spp. cells were only detected in one 2019 water sample from the York site in June, but in 2020, cells were detected at all four sites, generally from March through May, but occasionally in July and August. DA was more prevalent in oysters than in SPATTs from this study and a previous study (Onofrio et al. 2021), suggesting that SPATTs do not adequately reflect DA distribution in oysters in the lower Chesapeake. DA was not detected in a one-year SPATT study at the Cherrystone site (Onofrio et al. 2021), however, in the current study, DA was detected in oysters at that site from January to April in 2019, indicating that spatial distribution of DA may be more widespread in the lower Chesapeake than previously thought.

The current study found that oysters can accumulate low amounts of DA (0.58 mg DA/kg SM) at temperatures as cold as 5 ºC in the lower Chesapeake (**Fig. 8**). While this is novel information for the Bay, it is also in agreement with other studies that have concluded more generally that the same cold conditions that favor *Pseudo-nitzschia* blooms, make oysters unlikely to filter-feed enough biomass to accumulate concentrations of DA high enough to close harvest (regulatory limit: 20 mg/kg SM, USFDA 2019). This theory relies on evidence that (1) oysters feed inefficiently under temperatures of about 16 ºC (Loosanoff 1958), and (2) the optimum temperature for growth of *Pseudo-nitzschia* is around 9-10 ºC (Comeau et al. 2008, Anderson et al. 2010). In the current study, water temperatures were lowest in late January 2019, reaching < 5 ºC, coinciding with the highest DA concentration detected in an oyster sample (0.58 mg DA/kg SM at Lynnhaven). All oyster samples collected in January and February had low amounts of DA relative to the regulatory limit (20 mg/kg SM, USFDA 2019), with the exception of oyster samples from Rappahannock, where DA was never detected. This study provided evidence that oysters can accumulate DA at low temperatures, but found no evidence of DA accumulated to levels approaching concern for seafood safety. Furthermore, while chronic, sub-acute exposure may present a concern with some shellfish species (Lefebvre et al 2017, 2019), data from the current study suggests that eastern oysters are unlikely to present a chronic, sub-acute exposure risk. Oysters uptake DA less efficiently than other shellfish, such as mussels (Mafra et al. 2010). Additionally, DA concentrations in oysters in the current study were generally an order of magnitude lower than doses considered sub-acute (Lefebvre et al. 2017).

3.2.3 Diarrhetic Shellfish Toxins (DSTs)

DSTs, OA and DTX1, were rarely detected in oyster samples (2 and 1%, respectively, n = 81) and only in trace amounts, all in esterified forms (**Fig. 2**, **Table 1**). Even when oyster extracts were alkaline-hydrolyzed to convert derivatives to OA and DTX1 and the values were combined to assess risk, concentrations of DSTs were still trace (approximately 5.63 µg DST/kg SM), at least two orders of magnitude below regulatory limits for DSTs in shellfish (160 µg DST/kg SM, USFDA 2019). Additionally, DSTs were only found in oyster samples in May and June, and were detected at three of the five sites sampled (**Fig. 9**). This time frame puts DST occurrence in oysters right at the end of when *Dinophysis* cells were detected in water samples (March to June). Local *Dinophysis* strains generally bloom in April and May (Wolny et al. 2020a).

DSTs were detected on all SPATTs in the current study, suggesting the persistence of these phycotoxins in the estuarine environment beyond when *Dinophysis* cells were detected. In the Chesapeake Bay, despite the occurrence of *Dinophysis* cells (Marshall 1996, Marshall and Egerton 2009, Tango et al. 2004, Wolny et al. 2020a) and DSTs found in SPATTs in this and a previous study (Onofrio et al. 2021), shellfish appear to accumulate only trace amounts of DSTs (this study, Tango et al. 2004). This is in contrast to Long Island, NY, USA where DSTs in SPATTs and in shellfish were correlated, and shellfish accumulated high enough amounts of DSTs to warrant harvest closures (Hattenrath-Lehmann et al. 2018). Interestingly, these two estuarine systems on the East Coast, USA both harbor *D. acuminata* with a similar phycotoxin profile (Hattenrath-Lehmann et al. 2018, Fiorendino et al. 2020, Wolny et al. 2020a) but currently present very different risk levels for DSP. The apparent difference in risk is

partly explained by the types of shellfish that exceeded DST limits in Long Island, with mussels (*Mytilus edulis* and *Geukensia demissa*) accumulating much higher amounts of DSTs than oysters (*C. virginica*) or clams (*Mya arenaria*, Hattenrath-Lehmann et al. 2018). Mussels have been shown to accumulate much higher amounts of OA than oysters, clams, or scallops (Mafra et al. 2015 and references within). Furthermore, DSP risk may be linked to sustained, higher *Dinophysis* cell concentrations (>10 cells/mL, Hattenrath-Lehmann et al. 2018), which did not occur in the current study.

3.2.4 Microcystins (MCs)

MCs – traditionally freshwater phycotoxins – were detected in oysters in estuarine waters (**Fig. 10**). This study adds to a growing body of literature on MCs detected in estuarine and marine environments both in Virginia (Tango and Butler 2008, Wood et al. 2014, Bukaveckas et al. 2017, 2018, Onofrio et al. 2021) and globally (De Pace et al. 2014, Gibble and Kudela 2014, Preece et al. 2015, Gibble et al. 2016, Peacock et al. 2018), raising concern about human health impacts (Mulvenna et al. 2012, Vareli et al. 2013, Preece et al. 2017, Camacho-Muñoz et al. 2021) and the possibility of hepatotoxic shellfish poisoning (HSP, Miller et al. 2010).

The presence of MC-RR and MC-YR in oysters indicates an urgent need for the development of regulatory limits for freshwater phycotoxins like MCs, and increased monitoring for these phycotoxins in shellfish. MCs are currently not regulated in shellfish at the federal level in the USA. Existing guidance values for MCs in freshwater seafood have been summarized by the World Health Organization (WHO), and range from 5.6 to 51 µg/kg wet weight (Chorus and Welker, eds. 2021). Shellfish with concentrations of MCs well above these values have been found on the west coast of the USA (Gibble et al.

2016). The highest concentration of MC detected in the current study (7.12 µg MC-RR/kg SM) was on the low end of the range of existing guidance values, suggesting that a refined regulatory limit would assist in ensuring seafood safety. While signal enhancement of MC-RR in the current study was strong (174%, **APPENDIX V**), it is also well documented that recovery of MCs using methanolic extraction does not recover all MCs present in animal tissue that could still exhibit toxicity to the consumer (Williams et al. 1997, Smith et al. 2010, Chorus and Welker, eds. 2021). The detected concentrations of MCs in oyster samples in the current study strongly suggest that strategies for monitoring MCs in shellfish be developed and implemented immediately, and that research on seafood safety pertaining to MCs should be of high priority.

The sporadic occurrence of MCs in oysters from March through August (7%, n = 81, **Figs. 2**, **10**) suggests that these phycotoxins may enter the estuarine environment during or after episodic bloom events in freshwater that flow down estuary, or from spillover from freshwater ponds during rain events. MC-producing species, including *Microcystis* spp., *Oscillatoria* spp., *Dolichospermum* (formerly *Anabaena*) spp., and *Planktothrix* spp., were not observed in any water samples during this study, indicating either that cell detection methods may not have been sufficient, or that other producers may need to be explored in this system. While oysters can uptake particulate MCs (Gibble et al. 2016), MC-RR was detected in only one POM sample in the current study and oyster samples from the site did not exhibit quantifiable amounts of MCs. *Microcystis aeruginosa* lyses in seawater, releasing MCs (Miller et al. 2010), which persist in marine environments (Miller et al. 2010, Gibble and Kudela 2014). Shellfish are also capable of dissolved MC uptake (Gibble et al. 2016) and have been found to

biomagnify MCs to concentrations more than 100x greater than that of ambient water (Miller et al. 2010). In the current study, dissolved MCs were not detected in SPATTs; however, a previous study in the same system found sporadic MC-LR in SPATTs at the Lynnhaven site (Onofrio et al. 2021). It is unclear whether oysters in the current study accumulated MCs from the particulate and/or dissolved forms, or which organisms are the source of the phycotoxins.

3.3 Implications for phycotoxin monitoring and management

This study utilized a highly sensitive method for detecting a suite of phycotoxins in shellfish tissue that can be used for monitoring and early warning. The UPLC-MS/MS with trap/ACD method applied to oyster tissue successfully detected trace to low concentrations of a large range of phycotoxins pertinent to seafood safety (AZAs, DA, DSTs, MCs). Of the phycotoxins detected in this study that are currently regulated in shellfish in the USA (AZAs, DA, DSTs), all were detected at concentrations at least two orders of magnitude below current regulatory limits (USFDA 2019). The sensitivity of the method provides the opportunity to screen for phycotoxins well before concentrations approach a risk to seafood safety, creating opportunities for early warning and mitigation. Furthermore, this method may allow shellfish to be used as a tool to screen for the presence of phycotoxins produced by HAB species that are otherwise difficult to detect, either due to low cell concentrations (i.e., *Dinophysis*) or difficulty in microscopic identification (i.e., *Amphidomatace*).

Co-occurrence of phycotoxins was documented at all sites, and in both sampling years. Of the seafood safety phycotoxins, AZAs and DA co-occurred most commonly and at all sites except for Rappahannock, where DA was never detected (**Fig. 8**). Co-

occurrence of seafood safety phycotoxins in shellfish is common, and has been reported in mussels for DSTs and PSTs (Gago-Martinez et al. 1996, García et al. 2004, Hattenrath-Lehmann et al. 2018), DSTs and DA (Jester et al. 2009), as well as DSTs, PSTs, DA, and MCs (Peacock et al. 2018). A better understanding of how shellfish bioaccumulation of multiple phycotoxins may impact seafood safety is needed.

There was a lack of agreement between sample types; some phycotoxins found in oysters were not detected in complementary SPATTs or POM, phycotoxin profiles varied between sample types, and few samples contained detectable levels of HAB cells. Of the seafood safety phycotoxins detected in the current study, seven were detected in oysters (AZA1, AZA2, DA, MC-RR, MC-YR, OA, DTX1), while SPATTs and POM only picked up five or three of these phycotoxins, respectively (**Tables 1**, **4**). SPATTs failed to detect the MCs that were detected in oysters (MC-RR and MC-YR), and POM samples failed to detect AZA1, as well as any seafood safety phycotoxins that were detected in only trace concentrations in oysters (MC-YR, OA, DTX1). SPATTs in this and a previous study (Onofrio et al. 2021), primarily accumulated OA (**Table 1**), with DSTs representing 78% of the SPATT phycotoxin profile (n = 80, **Fig. 11**). In contrast, oysters and POM phycotoxin profiles were dominated by DA (oysters: 93%, n = 80, **Fig. 11**; POM: 99%, n = 43, **Table 4**). SPATTs and POM, therefore, did not accurately reflect the range of phycotoxins accumulated by oysters. Furthermore, HAB cell concentration data were sparse and showed little correspondence to the presence of phycotoxins in oysters (**Table 3**, **APPENDIX III**).

Given the inherent differences between sample type, it was not surprising that oysters, SPATTs, and POM accumulated phycotoxins differently. POM samples provided

discrete phycotoxin concentrations at the time of sampling. POM detected the particulate fraction of phycotoxins in a water sample, including intracellular phycotoxins as well as phycotoxins associated with particles > 1 µm. SPATTs and oysters, conversely, provided cumulative phycotoxin concentrations over the time deployed. SPATTs and oysters accumulated phycotoxins from the water column in fundamentally different ways. SPATTs were passive, adsorptive samplers that accumulated dissolved phycotoxins from the water. Whereas, oysters were active samplers, feeding, respiring, and interacting with large portions of water through time. Oysters were exposed to both dissolved and particulate phycotoxins, and are known to biotransform phycotoxins.

To address this general mis-match between oyster phycotoxin concentration and SPATTs, POM, or HAB cell concentration data, monitoring or predicting shellfish phycotoxin accumulation must be phycotoxin- and shellfish species-specific. In the Bay, SPATTs did not reflect the spatiotemporal accumulation of DSTs in oysters, and accumulated far higher amounts of DSTs than oysters (**Table 1**). A previous study found that oysters and clams did not accumulate DSTs in a manner that correlated to DST accumulation in SPATTs, while mussels did (Hattenrath-Lehmann 2018). SPATTs cannot be broadly compared with phycotoxin accumulation across shellfish species. When phycotoxin accumulation was comparable between oysters, SPATTs, and/or POM, correlations between sample types were only found for a few phycotoxins. Only three of the seafood safety phycotoxins found in oysters and SPATTs (AZA1, AZA2, DA), and two of those found in oysters and POM (AZA2, DA), had enough positive samples of both types to fit a linear regression ($n > 10$). While AZA1 in oysters was not correlated with AZA1 in SPATTs (LR: $R^2 = -0.01$, $p = 0.6$, $n = 80$, **Table 3**), SPATT phycotoxin

concentrations did have significant correlations with oyster phycotoxin concentrations for AZA2 (LR: $R^2 = 0.03$, $p = 0.05$, $n = 80$) and for DA (LR: $R^2 = 0.2$, $p < 0.0001$, $n = 80$, **Table 3**). For DA, SPATTs from two weeks prior to oyster sampling showed a significant correlation with oyster phycotoxin concentrations (LR: $R^2 = 0.2$, $p = 0.0003$, $n = 73$, **Table 3**), indicating that SPATTs could potentially be used for early warning for this phycotoxin in oysters. POM sampling for DA may provide an even better tool for surveillance or early warning of DA in oysters. DA in POM samples was strongly correlated to DA in oysters (LR: $R^2 = 0.5$, $p < 0.0001$, $n = 43$, **Table 3**). Furthermore, DA in POM samples from two weeks prior showed a stronger correlation (LR: $R^2 = 0.6$, $p < 0.0001$, $n = 39$, **Table 3**). Meanwhile, cell concentration of *Pseudo-nitzschia* spp. was not a significant predictor of DA concentrations in oysters (LR: $R^2 = -0.009$, $p = 0.6$, $n = 73$, **Table 3**). Challenges of associating *Pseudo-nitzschia* spp. cell counts with shellfish toxicity have been previously reported (Rowland-Pilgrim et al. 2019), because *Pseudo-nitzschia* spp. are known to vary in phycotoxin production by life stage, nutrient availability, and species strains (Bates 1998).

There were fine-scale differences in phycotoxin distribution with depth in nearshore, shallow water (≤ 2 m) sites in the lower Chesapeake Bay. Current standard sampling practices for HAB monitoring in Virginia involve the collection of water samples at the surface, and the deployment of SPATTs near-bottom. In 2019, SPATTs and POM samples were collected at the surface and near-bottom (0.5-0.6 m off bottom) for comparison of phycotoxin detection. More seafood safety phycotoxins were detected in POM samples collected at the surface rather than near-bottom. AZA2 and MC-RR were only detected in POM surface samples, in 7 and 2% of samples, respectively (n =

43), while DA was equally prevalent at both depths (**Table 2**). Conversely, deploying SPATTs near-bottom may be preferable to a surface deployment. AZA1 and DA were only detected in near-bottom SPATTs, and AZA2 was detected nearly twice as often in near-bottom SPATTs (**Table 2**), with higher AZA2 concentrations detected in near-bottom SPATTs (Wilcoxon signed-rank test, $V = 4$, $p = 0.02$, $n = 43$, **Fig. 3**). Between surface and near-bottom SPATTs, DTX1 and OA were equally prevalent and exhibited no difference in concentration with depth (Wilcoxon signed-rank test, $V = 48$, $p = 0.3$; $V = 572$, $p = 0.2$, respectively, $n = 43$, **Fig. 3**). At this time, the current standard of sampling phycotoxins in POM at the surface and in SPATTs near-bottom, represents best sampling practices for detecting seafood safety phycotoxins in POM and SPATTs in this region.

4. CONCLUSIONS

Based on the findings from this study, specific phycotoxin monitoring recommendations for the Virginia-portion of the Chesapeake Bay are included. Baseline data on the spatiotemporal distributions of phycotoxins in oysters in the Virginia-portion of the Chesapeake Bay were collected from late winter to summer, over two years, to assist future management decisions. Given that the unsampled time period (September – December) overlapped with the harvest season for oysters on public grounds in Virginia (Pertaining to Restrictions on Oyster Harvest, 2020), and that phycotoxins persist year-round in the Bay (Onofrio et al. 2021), future research into oyster phycotoxin bioaccumulation during this unsampled time period is advised. Lastly, monitoring approaches should continue to target sampling efforts to regions and time periods when shellfish may be legally harvested, with special attention to areas where phycotoxins have been prevalent. Lynnhaven is one such site, exhibiting the highest diversity of phycotoxins in oysters, and the highest amount of phycotoxins in oysters and POM samples (**Tables 1** and **4**). This site also accounted for almost half of the oyster samples with co-occurring seafood safety phycotoxins in this study. Lynnhaven is a southern, polyhaline, tidal inlet with an intermediate flushing rate (Herman et al. 2007, Onofrio et al. 2021) that hosts oyster harvest grounds (Trombly et al. 2016).

To continue to successfully monitor shellfish seafood safety in Virginia, it is recommended that SPATTs be used as an additional phycotoxin detection tool, and not as a replacement for the collection and testing of shellfish. SPATTs used in concert with deployed shellfish provided a more complete picture of the phycotoxins present. SPATTs should continue to be deployed near-bottom, and POM samples should continue to be

collected at the surface, to ensure the detection of a broad spectrum of phycotoxins present in the water column. SPATTs and POM phycotoxin samples may also prove useful for monitoring, or early detection, of DA in oysters. Shellfish phycotoxin testing should not be limited to oysters, as shellfish species are known to vary in phycotoxin uptake and elimination (Shumway 1990, Mafra et al. 2010, 2015, Reguera et al. 2014). Clams, another economically-important shellfish in the region (Hudson 2019), should be incorporated into phycotoxin testing when possible.

Currently, MCs present the most pressing matter for monitoring and management of phycotoxins in shellfish in the lower Chesapeake Bay. Human health effects of MCs have been well-documented (Mulvenna et al. 2012, Vareli et al. 2013, Preece et al. 2017, Camacho-Muñoz et al. 2021), and the possibility of transfer of MCs to humans through consuming shellfish has been discussed (Miller et al. 2010). As federal regulatory limits for MCs in shellfish do not currently exist, and WHO guidance values (Chorus and Welker, eds. 2021) suggest that oyster samples in the current study were potentially of concern for seafood safety, it is recommended that Virginia consider creating and implementing its own regulations on MCs in shellfish, at least until federal regulations are put into place. Additionally, increased monitoring for MCs in the lower Chesapeake Bay, and further research on the spatiotemporal distribution of MC events is recommended. As SPATTs failed to detect MCs, POM only detected one of the two MCs found in oysters, and cells were undetected by microscopy, it is recommended that oysters be deployed for state MC-monitoring purposes. More generally, given the discovery and prevalence of low concentrations of DA and AZAs in oysters, monitoring for these phycotoxins in shellfish is also recommended. Additionally, despite the sporadic

trace concentrations of DSTs detected in oysters in the current study, given the prevalence of *Dinophysis*, and DSTs in SPATTs in the Bay (Tango et al. 2004, Marshall and Egerton 2009, Wolny et al. 2020a, Onofrio et al. 2021), monitoring for DSTs should continue as usual.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this chapter can be found in **APPENDICES III - V**.

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Table 1

Spatial distribution of 11 phycotoxins across five sites in the Chesapeake Bay. Results are presented as cumulative phycotoxins at each site summed over all time points for oysters (µg/kg shellfish meat [SM]) and solid phase adsorption toxin tracking devices (SPATTs, µg/kg resin). Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020, all other sites were sampled in both years. Values below the limit of quantitation (<LOQ) were represented as ½ the concentration of the lowest quantifiable standard; values below the limit of detection (<LOD) were represented as zeroes.

Sample Type	Site (sample size)	Cumulative phycotoxins[a] (µg/kg SM or resin)									Sum of all toxins	Presence/Absence of phycotoxins[a] (+/-)	
		AZA1	AZA2	DA	MC-RR	MC-YR	GDA	PTX2	OA*	DTX1*		KmTx1-1	KmTx1-3
Oysters	Rappahannock (n =17)	0.83	0.86	0	0	11.25	9.00	0.45	0	5.63	28.02	+	+
	York (n = 21)	1.13	0	121.35	7.06	0	0	1.80	0	0	131.34	+	+
	Lynnhaven (n = 21)	3.40	0.75	1029.76	14.62	2.25	2.25	19.31	5.63	0	1077.97	+	+
	Wise Point (n = 10)	0.28	1.31	137.86	2.25	0	0	14.28	5.63	0	161.61	-	-
	Cherrystone (n = 11)	0.63	0	204.00	0	0	2.25	1.35	0	0	208.23	+	+
	Toxin Total (n = 80)	**6.27**	**2.92**	**1492.97**	**23.93**	**13.50**	**13.50**	**37.19**	**11.26**	**5.63**	**1607.17**		
SPATTs	Rappahannock (n =17)	0.31	0	0	0	0	40.16	121.67	370.50	204.21	736.85	-	-
	York (n = 21)	0	0.76	2.20	0	0	83.43	400.70	1207.92	656.64	2351.65	-	-
	Lynnhaven (n = 21)	0	1.47	9.40	0	0	142.94	831.15	1683.94	1044.83	3713.73	-	-
	Wise Point (n = 10)	0	1.22	3.67	0	0	2.90	851.49	1495.52	2417.50	4772.30	-	-
	Cherrystone (n = 11)	0	0	2.37	0	0	0	41.69	41.69	53.59	139.34	-	-
	Toxin Total (n = 80)	**0.31**	**3.45**	**17.64**	**0**	**0**	**269.43**	**2246.70**	**4799.57**	**4376.77**	**11713.87**		

[a]AZA1 = azaspiracid-1, AZA2 = azaspiracid-2, DA = domoic acid, MC-RR = microcystin-RR, MC-YR = microcystin-YR, GDA = goniodomin A, PTX2 = pectenotoxin-2, OA = okadaic acid, DTX1 = dinophysistoxin-1, KmTx1-1 = karlotoxin1-1, KmTx1-3 = karlotoxin1-3

* These phycotoxins are presented as the sum of the parent and derivative forms

Table 2
Percent of solid phase adsorption toxin tracking devices (SPATT) and particulate organic matter (POM) samples with each phycotoxin detected. Samples were collected in 2019 at surface and near-bottom (0.5 – 0.6 m from bottom).

Phycotoxin[a]	SPATTs[b]		POM[c]	
	Surface (n = 43)	Near-bottom (n = 44)	Surface (n = 43)	Near-bottom (n = 14)
AZA1	0	2	-	-
AZA2	14	23	7	0
DA	0	11	37	36
MC-RR	-	-	2	0
GDA	12	11	0	7
PTX2	100	100	14	7
OA	100	100	-	-
DTX1	35	36	-	-

[a]AZA1 = azaspiracid-1, AZA2 = azaspiracid-2, DA = domoic acid, MC-RR = microcystin-RR, GDA = goniodomin A, PTX2 = pectenotoxin-2, OA = okadaic acid, DTX1 = dinophysistoxin-1
[b] MC-RR was not detected in SPATTs
[c] AZA1, OA, and DTX1 were not detected in POM samples

Table 3

Linear regressions for four phycotoxins in oysters versus phycotoxin in solid phase adsorption toxin tracking devices (SPATT), in particulate organic matter (POM), or harmful algal bloom (HAB) cell concentration as determined by microscopy.
R^2 is reported with p-value and sample size. Significant regressions are bolded.

Lag Time	Predictor	AZA1[ab]	AZA2[b]	DA[c]	PTX2[d]
No lag	SPATT Phycotoxin	-0.01 (p = 0.6, n = 80)	**0.03 (p = 0.05, n = 80)**	**0.2 (p < 0.0001, n = 80)**	**0.4 (p < 0.0001, n = 80)**
	POM Phycotoxin	-	-0.02 (p = 0.7, n = 43)	**0.5 (p < 0.0001, n = 43)**	**0.1 (p = 0.01, n = 43)**
	Cell Concentration	-	-	-0.009 (p = 0.6, n = 73)	0.01 (p = 0.2, n = 73)
Two-week lag	SPATT Phycotoxin	-0.01 (p = 0.6, n = 73)	-0.009 (p = 0.6, n = 73)	**0.2 (p = 0.0003, n = 73)**	**0.3 (p < 0.0001, n = 73)**
	POM Phycotoxin	-	-0.02 (p = 0.7, n = 39)	**0.6 (p < 0.0001, n = 39)**	-0.02 (p = 0.6, n = 39)
	Cell Concentration	-	-	-0.02 (p = 1, n = 66)	-0.01 (p = 0.8, n = 66)

[a] Azaspiracid-1 (AZA1) was not detected in POM samples

[b] AZA concentrations were not compared to HAB cell concentrations because *Amphidomatace* spp. cells were too small to be identified by the microscopy methods applied

[c] *Pseudo-nitzschia* spp. was used for HAB cell concentration to compare to domoic acid (DA) concentration

[d] *Dinophysis* spp. was used for HAB cell concentration to compare to pectenotoxin-2 (PTX2) concentration

Table 4
Spatial distribution of four phycotoxins detected in particulate organic matter (POM) samples collected from surface waters across four sites in the Chesapeake Bay. All POM samples were collected in 2019. Results are presented as cumulative phycotoxins at each site summed over all time points in µg/L.

Site (sample size)	Cumulative phycotoxins[a] (µg/L)				Sum of all toxins
	AZA2	DA	MC-RR	PTX2	
Rappahannock (n =10)	0	1.65	0	0	1.65
York (n = 11)	0	203.37	0	5.56	208.93
Lynnhaven (n = 11)	0	1005.94	0	5.09	1011.03
Cherrystone (n = 11)	0.19	203.37	3.98	2.12	281.09
Toxin Total (n = 43)	**0.19**	**1485.75**	**3.98**	**12.78**	**1502.70**

[a]AZA2 = azaspiracid-2, DA = domoic acid, MC-RR = microcystin-RR, PTX2 = pectenotoxin-2

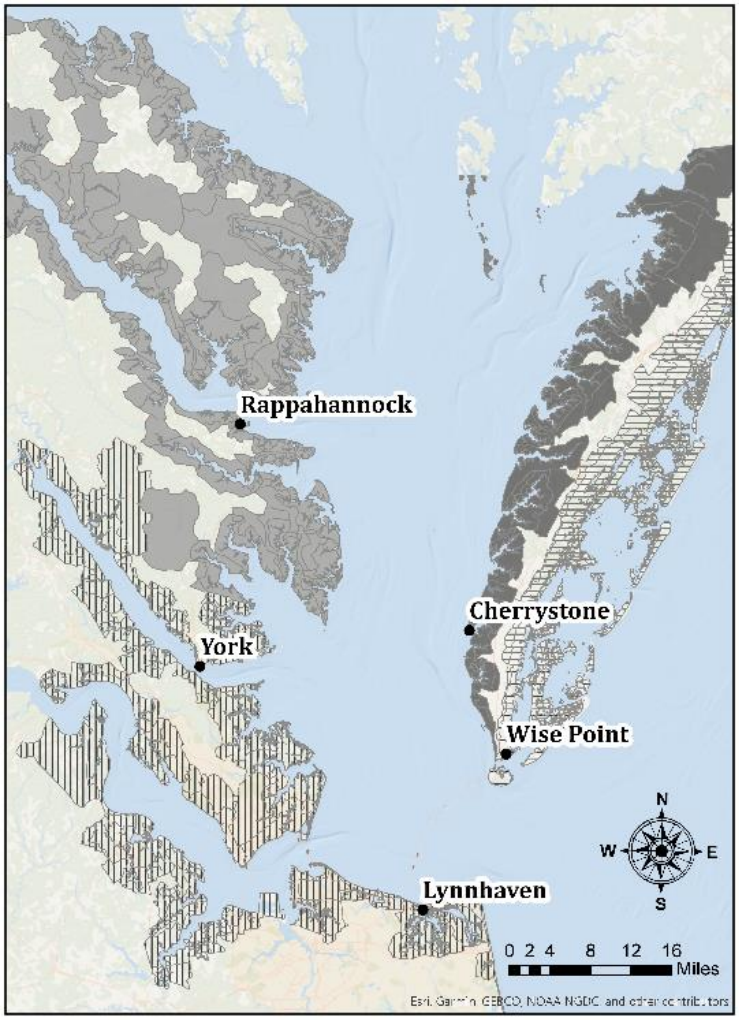

Figure 1
Locations of five sampling sites in the lower Chesapeake Bay. Shading represents regional watersheds, adapted from Onofrio et al. 2021 with credit to A. Roach, Virginia Dept. of Health.

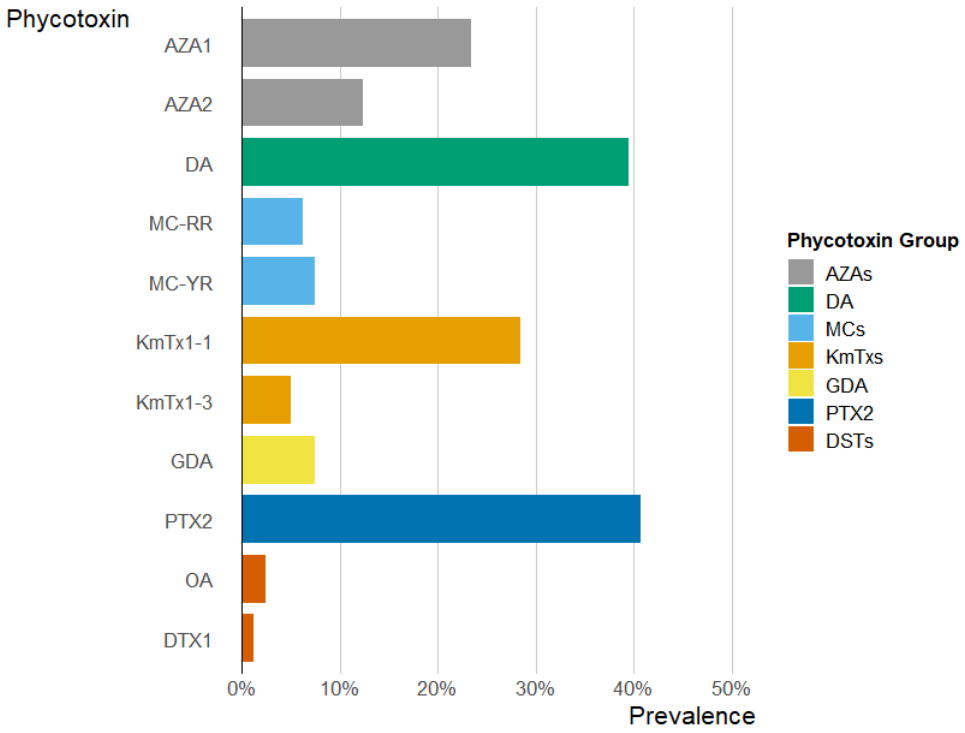

Figure 2
Prevalence of each phycotoxin in oyster samples (n = 81). Okadaic acid (OA) and dinophysistoxin-1 (DTX1), both diarrhetic shellfish toxins (DSTs), were detected in oysters in esterified forms. AZA1 = azaspiracid-1, AZA2 = azaspiracid-2, AZAs = azaspiracids, DA = domoic acid, MC-RR = microcystin-RR, MC-YR = microcystin-YR, MCs = microcystins, KmTx1-1 = karlotoxin1-1, KmTx1-3 = karlotoxin1-3, KmTxs = karlotoxins, GDA = goniodomin A, PTX2 = pectenotoxin-2.

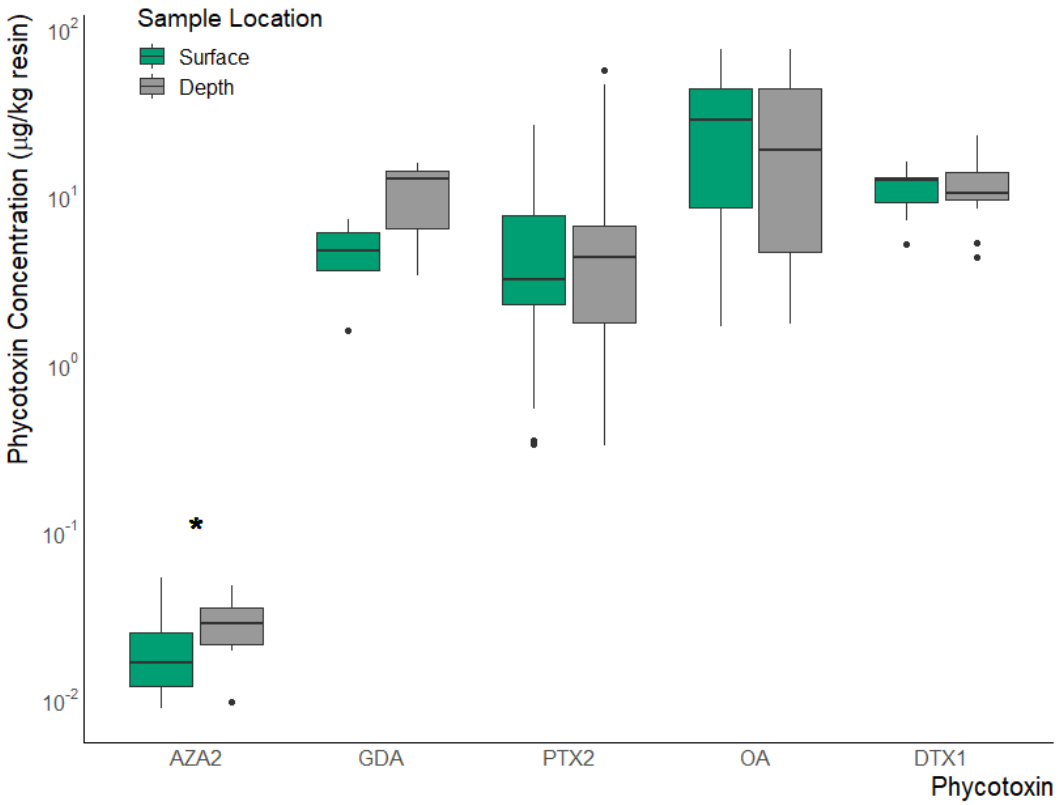

Figure 3
Box plot of 2019 phycotoxin concentrations (µg/kg resin) in solid phase adsorption toxin tracking devices (SPATT) deployed at the surface (green) or near-bottom (0.6 m off bottom, grey). Only phycotoxins detected in SPATTs at least once in surface and near-bottom are included. Note the $\log_{10}$ axis. Phycotoxin concentrations that were significantly different between surface and near-bottom SPATTs are denoted by an asterisk (Wilcoxon signed-rank test, $p < 0.05$). AZA2 = azaspiracid-2, GDA = goniodomin A, PTX2 = pectenotoxin-2, OA = okadaic acid, DTX1 = dinophysistoxin-1.

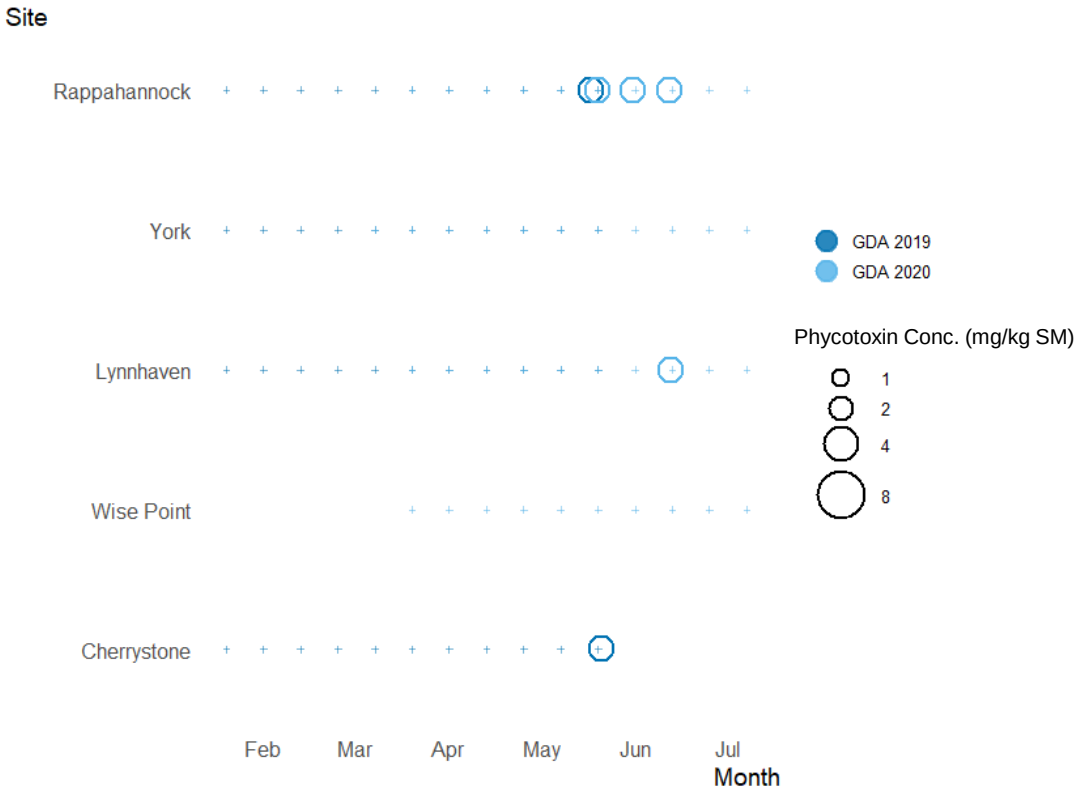

Figure 4
Oyster phycotoxin data (mg/kg shellfish meat [SM]) for goniodomin A (GDA) across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles are below the limit of quantitation (<LOQ) and are represented as ½ the concentration of the lowest quantifiable standard. Samples below the limit of detection (<LOD) are indicated by plus signs.

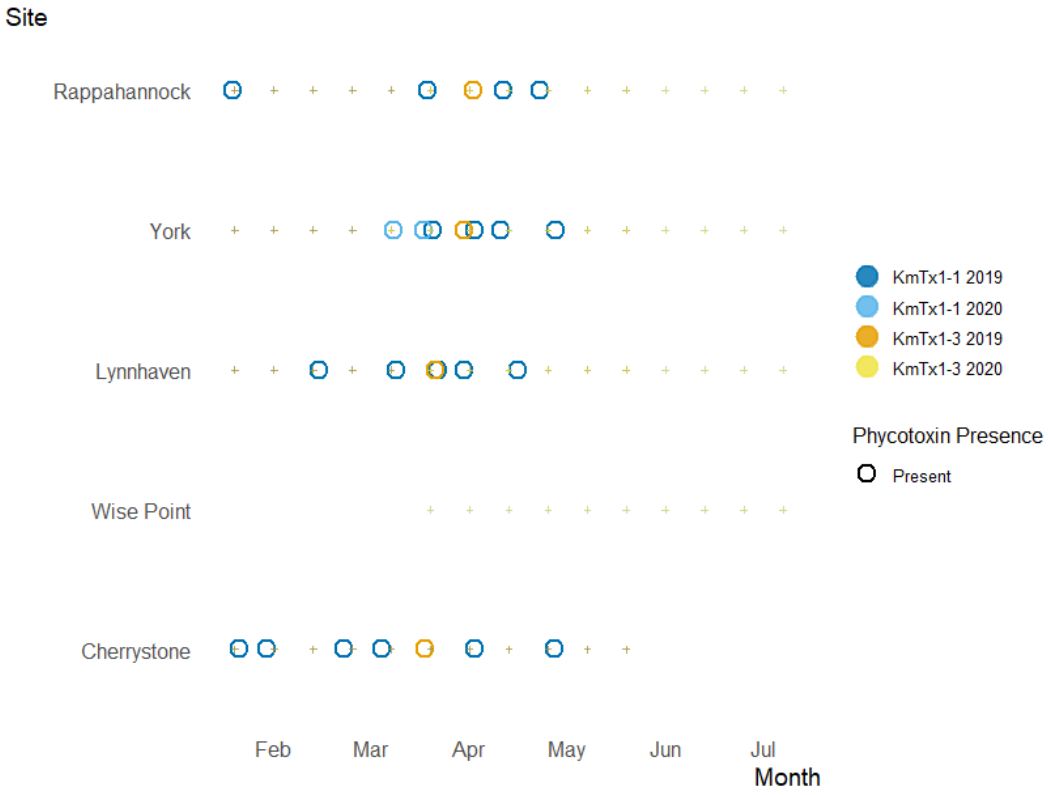

Figure 5
Oyster phycotoxin presence/absence data for karlotoxin1-1 and -3 (KmTx1-1 and KmTx1-3, respectively) across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles denote phycotoxin presence; phycotoxin absence is denoted with plus signs.

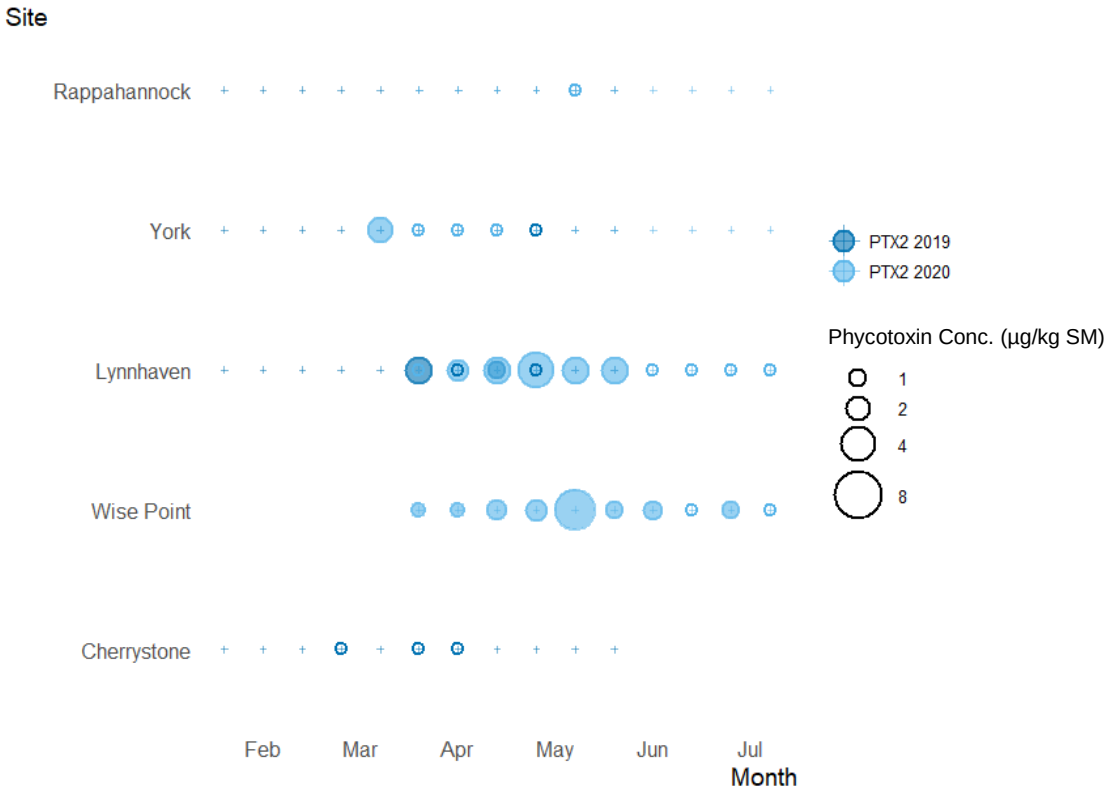

Figure 6
Oyster phycotoxin data (µg/kg shellfish meat [SM]) for pectenotoxin-2 (PTX2) across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles are below the limit of quantitation (<LOQ) and are represented as ½ the concentration of the lowest quantifiable standard. Samples below the limit of detection (<LOD) are indicated by plus signs.

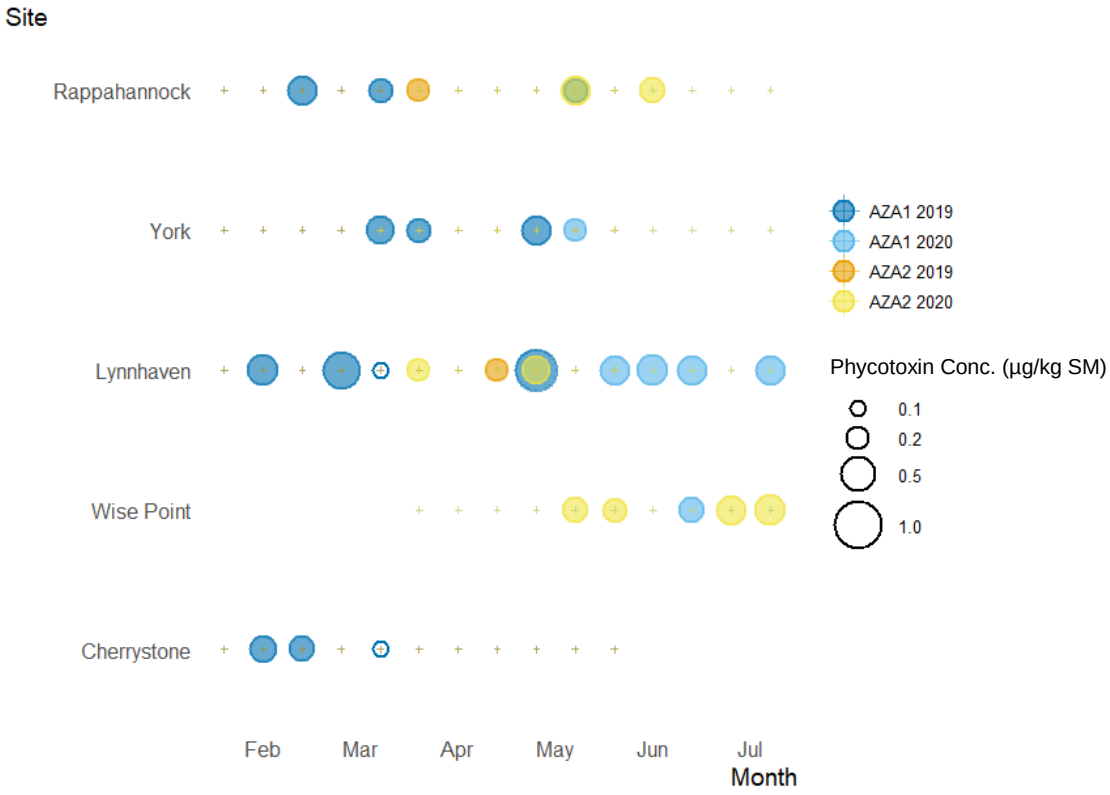

Figure 7
Oyster phycotoxin data (µg/kg shellfish meat [SM]) for azaspiracid-1 and -2 (AZA1 and AZA2, respectively) across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles are below the limit of quantitation (<LOQ) and are represented as ½ the concentration of the lowest quantifiable standard. Samples below the limit of detection (<LOD) are indicated by plus signs.

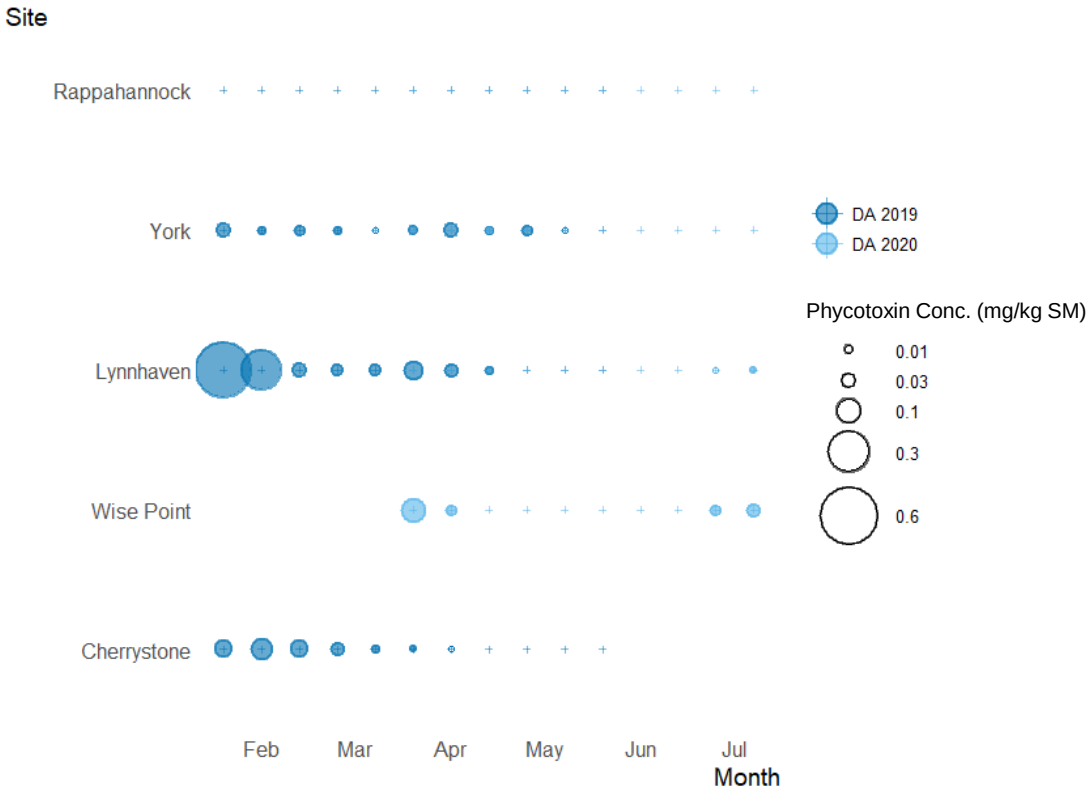

Figure 8
Oyster phycotoxin data (mg/kg shellfish meat [SM]) for domoic acid (DA) across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles are below the limit of quantitation (<LOQ) and are represented as ½ the concentration of the lowest quantifiable standard. Samples below the limit of detection (<LOD) are indicated by plus signs.

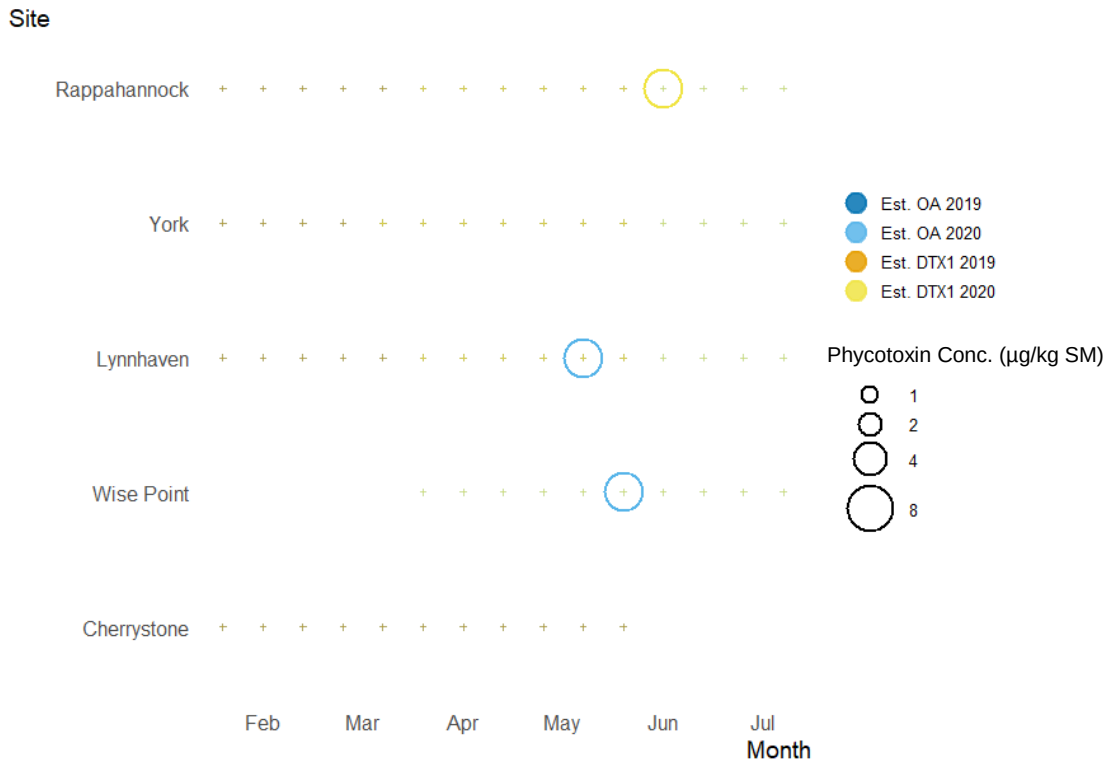

Figure 9
Oyster phycotoxin data (µg/kg shellfish meat [SM]) for diarrhetic shellfish toxins (DSTs) okadaic acid and dinophysistoxin-1 (OA and DTX1, respectively) in esterified forms, across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles are below the limit of quantitation (<LOQ) and are represented as ½ the concentration of the lowest quantifiable standard. Samples below the limit of detection (<LOD) are indicated by plus signs.

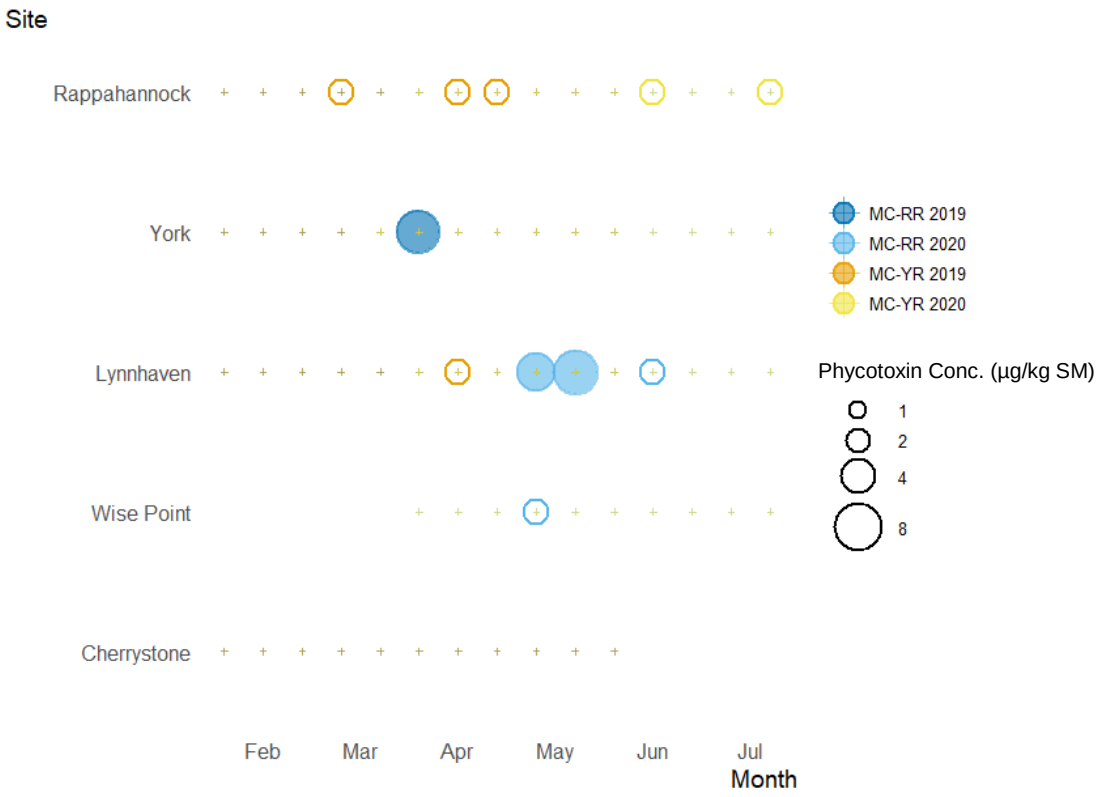

Figure 10
Oyster phycotoxin data (µg/kg shellfish meat [SM]) for microcystin-RR and -YR (MC-RR and MC-YR, respectively) across 5 sites within the lower Chesapeake Bay from January to June 2019 and from March to August 2020. Cherrystone was only sampled in 2019 and Wise Point was only sampled in 2020. Hollow circles are below the limit of quantitation (<LOQ) and are represented as ½ the concentration of the lowest quantifiable standard. Samples below the limit of detection (<LOD) are indicated by plus signs.

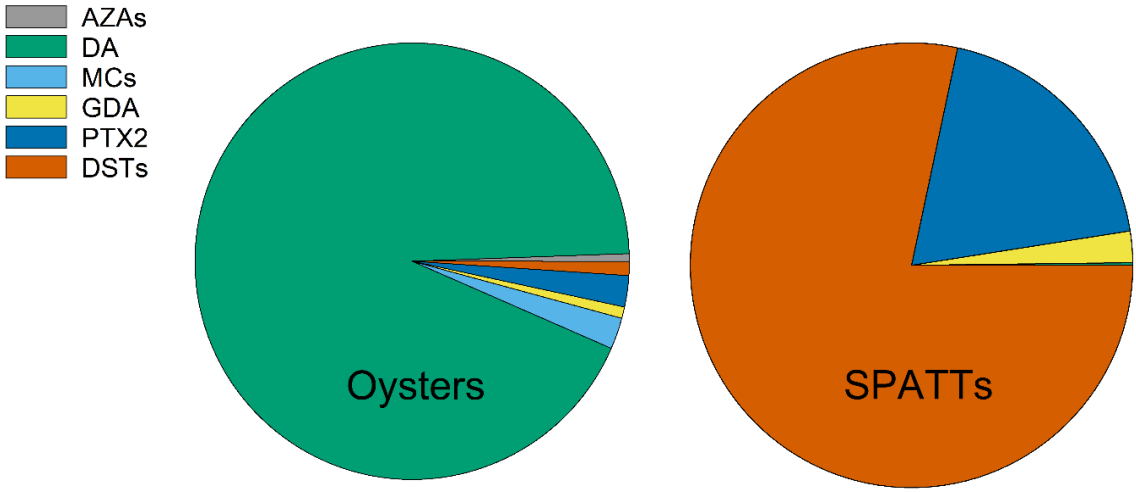

Figure 11
Comparison of oyster and solid phase adsorption toxin tracking devices (SPATT) cumulative phycotoxin profiles across all sites and time points by phycotoxin group (n = 80). Karlotoxins (KmTxs) were not included as they were not quantified in this study, however, KmTxs were present in oysters and absent in SPATTs. Diarrhetic shellfish toxins (DSTs) included okadaic acid and dinophysistoxin-1. AZAs = azaspiracids, DA = domoic acid, MCs = microcystins, GDA = goniodomin A, and PTX2 = pectenotoxin-2.

DISCUSSION

HAB species and toxins frequently co-occur in nature, interacting with oysters and other shellfish. Given the known detrimental effects of many of these HAB species and toxins on both shellfish health and seafood safety, the studies within furthered scientific understanding of HAB exposure to, and accumulation of phycotoxin from, some of these co-occurring HABs.

Effects of co-occurring HAB species on larval oysters

The effects of co-occurring HAB species and toxins on larval oysters were explored through two separate series of bioassays with *K. veneficum* and *P. cordatum*, or with *A. catenella* and *D. acuminata*. These are the first known HAB co-exposure studies with larval oysters. Further co-exposure studies should be encouraged, as these studies are more reflective of the complexities of reality. While exposure to *P. cordatum* led to some larval inactivity, *K. veneficum* demonstrated larval inactivity and mortality at all cell concentrations tested (1,000 – 50,000 cells/mL). Combined effects of exposure to *K. veneficum* and *P. cordatum* were no different from the effects of *K. veneficum* alone, suggesting that *K. veneficum* dominated interactions with larval oysters when *P. cordatum* was present. Exposure to live *A. catenella* caused larval inactivity at all cell concentrations tested (10 – 1,000 cells/mL), while *D. acuminata* live cell and lysate (1,000 cells/mL or equiv.) led to significant larval mortality by 96 h. Larval mortality associated with exposure to *D. acuminata* lysate (1,000 cells/mL equiv.) was not changed by the addition of *A. catenella* lysate (1,000 cells/mL equiv.) in co-exposure, suggesting that the observed toxicity of the combined treatment was driven by the *D. acuminata* lysate. Similarly, PTX2 drove the toxicity in combined pure toxin treatments. Even at extreme concentrations (10,000 cells/mL equiv.), exposure to pure OA and/or STX did

not cause inactivity or mortality in larval oysters. Contrastingly, PTX2 exposure with or without other toxins caused complete inactivity, and eventual mortality in larval oysters. PTX2 is thought to be responsible for the ill effects observed in larval oysters from exposure to *D. acuminata* live cell and lysate, although further study is needed to confirm this hypothesis. In these studies, co-exposures did not lead to synergistic effects on larval oysters. Effects are known to be species-specific between HABs and shellfish (Turner et al. 1998), therefore, future research should pursue co-exposure studies with different combinations of HAB and shellfish species.

During bioassays, the use of a combination of treatment types, i.e., live cell, lysate, pure toxin, etc., can help elucidate the potential sources of observed toxic effects. Other studies have compared toxigenic and non-toxigenic strains of HAB species to accomplish a similar feat (Basti et al. 2015, Castrec et al. 2020). The utilization of multiple bioassay treatment types is especially helpful to narrow down the possible sources of an observed toxic effect. Lysate and pure toxin treatments eliminate confounding factors associated with living cells, e.g., physical interactions, active production and release of compounds in response to a grazer, etc.; additionally, lysate treatments liberate intracellular components and modify interactions between those components and the bioassay organism.

Larval oysters were exposed to four different HAB species, each with unique properties, leading to different effects on larval oysters (**Fig. 1**). While all of these HAB species have been reported to have negative health impacts on early oyster life stages (Wikfors and Smolowitz 1995, Glibert et al. 2007, Stoecker et al. 2008, Mu and Li 2013, Basti et al. 2015, Gaillard et al. 2020), *A. catenella* and *D. acuminata* are best known for

their association with human syndromes PSP and DSP. Of the HAB species tested, *P. cordatum* was the only one that no toxins have yet been characterized for; some have suggested that live cell exposure is required to produce any toxic effects of *P. cordatum* (Wikfors and Smolowitz 1995). Another property of these HAB species that affected the breadth of interspecies interactions that could have occurred during the bioassays was cell size. With larval oysters, the size of the HAB cells in the exposure, and the size of the larvae, dictate whether or not larvae are exposed to intracellular components through digestion. Oyster larvae in these studies were too small to consume *A. catenella* or *D. acuminata*, meaning that any interaction with toxins or bioactive compounds had to be with the extracellular fraction. This limited toxin exposure routes to superficial interactions with tissues exposed to the media, primarily gills, mantle, and velum. By contrast, *K. veneficum* and *P. cordatum* were small enough to be consumed, making interactions with intracellular KmTxs possible. Overall, when designing HAB toxicity bioassays, consideration should go into the diversity of treatment types, as well as the specific properties of the HAB species in relation to the bioassay test organism.

Monitoring phycotoxins in oysters in the Chesapeake Bay

The Chesapeake Bay hosts a variety of phycotoxin-producing HAB species **(**Marshall 1996, Marshall and Egerton 2009). Recent efforts to establish baseline data for the region have shown that the Bay is host to a variety of phycotoxins (Onofrio et al. 2021). As a continuation of that work, phycotoxins were assessed in oysters in the Virginia-portion of the Chesapeake Bay, to establish which were present, whether co-accumulation in seafood occurred, and to compare sample types and approaches used in HAB monitoring. Eleven phycotoxins were detected in oysters: AZA1, AZA2, DA, MC-

RR, MC-YR, KmTx1-1, KmTx1-3, GDA, OA, DTX1, PTX2. Phycotoxins associated with shellfish poisoning syndromes were well below regulatory action limits. This study marked the first known detection of KmTxs in oysters, and the first detection of AZAs in shellfish on the eastern coast of the USA. Most importantly, this study documented concentrations of freshwater MCs in estuarine oysters at a level within the range of existing guidance values for MCs in freshwater seafood, summarized by the WHO (Chorus and Welker, eds. 2021), indicating a pressing need for the development of a federal regulatory limit for MCs in shellfish. Co-accumulation of multiple phycotoxins in oysters was common, but concentrations of these phycotoxins were very low. The co-accumulation of low concentrations of multiple phycotoxins supports the study of low-level chronic exposure to phycotoxins in humans, and suggests a need to better understand the combined effects of phycotoxins on mammalian systems. Furthermore, the exploration of the use of SPATTs and POM samples to detect phycotoxins, indicated that these metrics could be useful to supplement information provided by sentinel shellfish, but generally could not effectively replace oyster phycotoxin data.

Some shellfish species, like *C. virginica* have been shown to close or reduce feeding during HAB events (Shumway 1990, May et al. 2010), which may reduce exposure to, and accumulation of, phycotoxins. Because of this, oysters may not be the "best" sentinel species or bioindicators of phycotoxins in all regions, however, in the Chesapeake Bay, oysters are one of the primary shellfish species harvested (Hudson 2019). This work emphasizes the need for region-specific study to support seafood safety. The collection of baseline data on phycotoxins in oysters provided valuable data that can be used to assess future changes in phycotoxin spatiotemporal distribution in the Bay.

Conclusion

The findings from these studies explored the interactions between co-occurring HAB species and oysters; co-exposure to HAB species and toxins can impact larval oyster survival to recruitment, and can lead to co-accumulation of phycotoxins in adult oysters. Breakthrough of HAB species into oyster hatcheries is documented herein, and could potentially lead to mortality of young oysters. Many hatcheries already monitor for HABs, and should continue to do so. Frequent water changes may help mitigate any HAB-related issues by reducing static exposure to HABs. In the wild, larval oyster mortalities due to HABs likely already occur, efforts to compare records of HAB events to shellfish recruitment data are encouraged. Additionally, as adult oysters accumulate low concentrations of a wide variety of phycotoxins associated with issues for shellfish health as well as seafood safety, it is worthwhile to consider the management applications of these data. The detection of phycotoxins related to human illness at concentrations far below regulatory limits offers insight to managers on which HABs may become an issue in the future. Furthermore, for HAB species that are sparse, detection of associated phycotoxins in oysters may indicate their presence. This highly-sensitive method is a direct measure of risk to seafood safety; used in concert with other monitoring methods such as HAB cell concentrations, phycotoxin in SPATT, and remote sensing (Wolny et al. 2020), it offers promise as part of a regional early warning system. Finally, this research could not have been done without the participation of oyster aquaculturists and state seafood safety managers. Addressing the regional HAB monitoring and management needs of the future will require continued stakeholder involvement in

research, and the support of effective partnerships between regulatory and research agencies.

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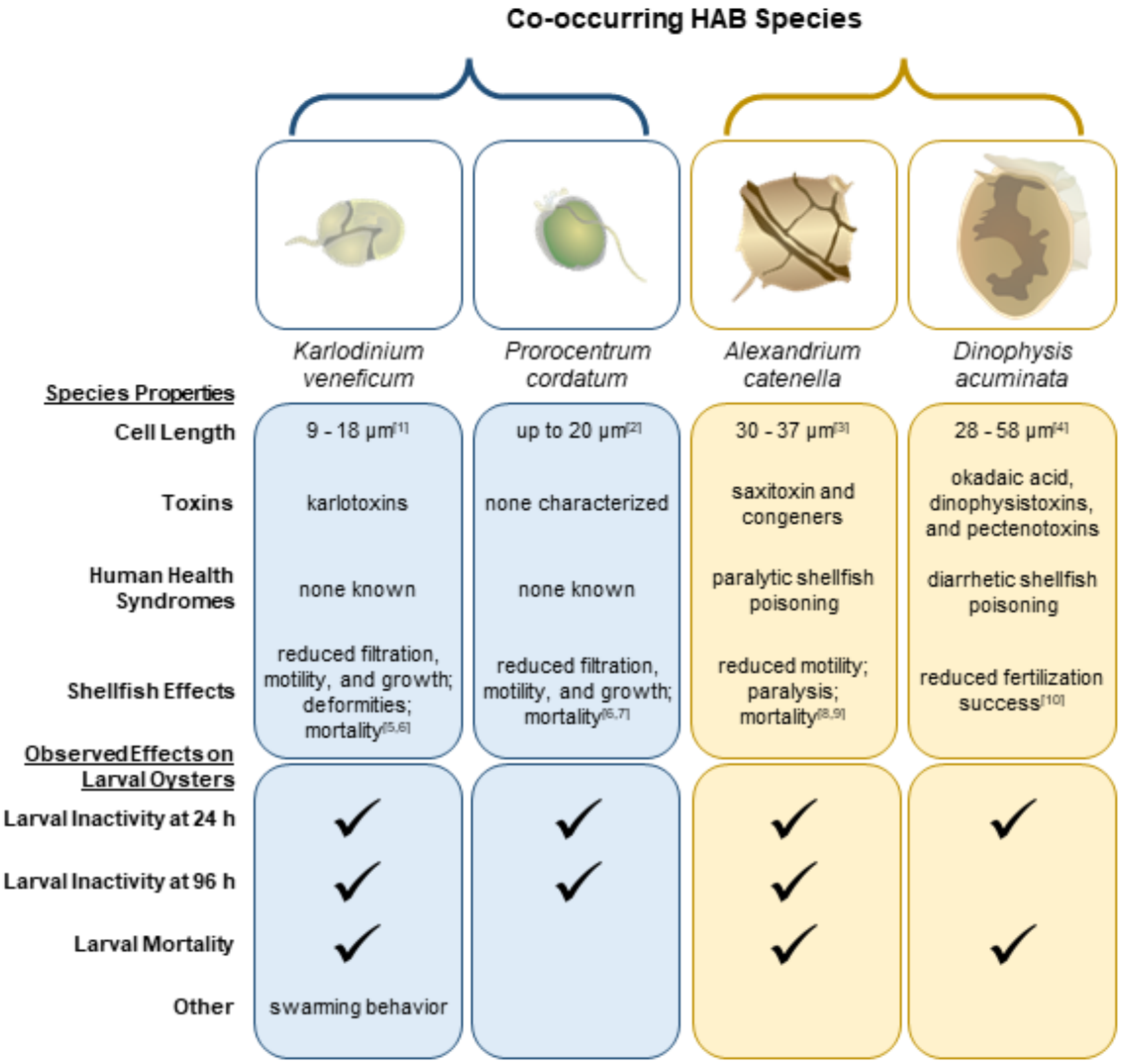


Figure 1
Summary of the co-occurring harmful algal bloom (HAB) species assessed, with species properties and the observed effects of live cell treatments of these HAB species on larval oysters during 96-h bioassays. Algae art attributed to Tracey Saxby and Jane Thomas, Integration and Application Network (ian.umces.edu/media-library). [1] Ballantine 1956. [2] Faust 1974. [3] Whedon and Kofoid 1936. [4] Park et al. 2019. [5] Reviewed in Place et al. 2012. [6] Glibert et al. 2007. [7] Reviewed in Heil et al. 2005. [8] Hégaret et al. 2007. [9] Basti et al. 2015. [10] Gaillard et al. 2020.

APPENDIX I

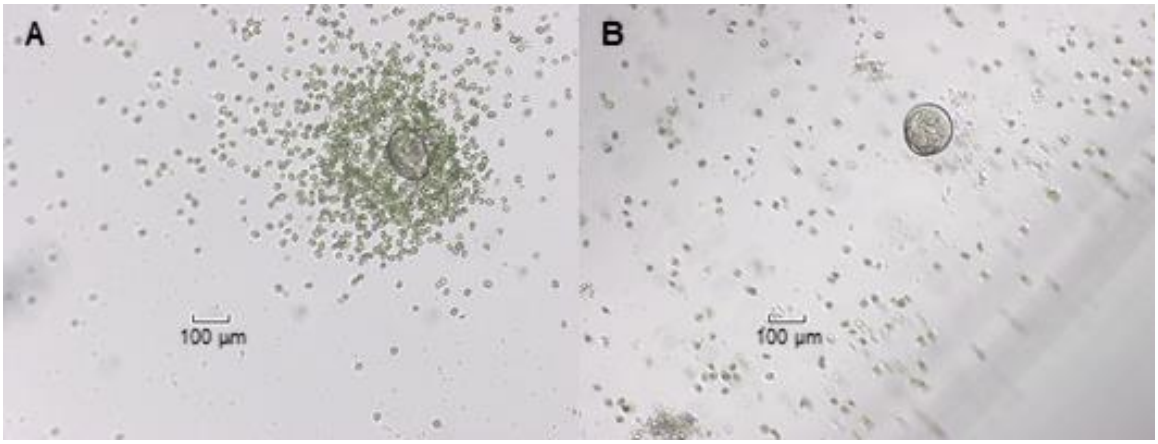
Supplementary Materials for Chapter 1

Larval oysters (*C. virginica*) were exposed to live HAB species *K. veneficum* or *P. cordatum* in 96-h bioassays (see **CHAPTER 1**). During these bioassays, live *K. veneficum* exposed to larval oysters (*C. virginica*) exhibited swarming behavior (**Supp. Fig. 1**), surrounding larval oysters, leading to larval mortality. Photos and videos from the experimental bioassays, as well as preliminary and follow-up studies, were used to capture this novel phenomenon using Infinity Analyze 6.5.4 (Lumenera Corp., Ottawa, Ontario, Canada) or CellSens Standard 1.12 (Olympus Corp., Shinjuku, Tokyo, Japan) software. Video captions are below. This phenomenon is fully described in **Ch. 1 Section 3.2.1** and further discussed in **Section 4.1.1**.

Videos can be found in the online version of this manuscript, at doi:10.1016/j.hal.2020.101965.

Video 1
Video from the dual harmful algal bloom (HAB) bioassay at 48 hours. In the foreground, a live, larval oyster with its cilia visible, tries to swim while being swarmed by *Karlodinium veneficum*. Another healthy, live, larval oyster swims in the background.

Video 2
Video from a follow-up study at 72 hours. The harmful dinoflagellate, *Karlodinium veneficum*, can be seen inside of the shell of a larval oyster, presumably feeding. Another healthy, live larval oyster swims in the background.



Supplemental Figure 1
(A) *Karlodinium veneficum* and (B) *Prorocentrum cordatum*, both at 35,000 cells/mL, with larval oysters at 24 hours in a preliminary bioassay.

APPENDIX II

Supplementary Materials for Chapter 2

Larval oysters (*C. virginica*) were exposed or co-exposed to live cell, lysate, or pure toxin from the HAB species *A. catenella* and/or *D. acuminata* in 96-h bioassays (see **CHAPTER 2**). Supplementary materials for these bioassays are included here for reference.

To detect differences in mortality and inactivity between treatments, least-squares means (LSM) were calculated for each treatment within the live cell and lysate bioassays as described in **Ch. 2 Section 2.7**. Briefly, arcsine-transformed larval mortality and inactivity data were used to compute LSMs for each treatment at each timepoint in R Studio (2019) using R version 3.6.1. Tukey's pairwise comparisons of treatments within each timepoint were used to compare LSMs with a significance level, $\alpha = 0.0125$. The results of the treatment comparison of LSMs of larval mortality for each treatment in the live cell bioassay (**Supp. Table 1**), and lysate bioassay (**Supp. Table 2**) are reported in **Ch. 2 Section 3.1**. The results of the treatment comparison of LSMs of larval inactivity for each treatment in the live cell bioassay (**Supp. Table 3**), and lysate bioassay (**Supp. Table 4**) are reported in **Ch. 2 Section 3.2**.

To indirectly confirm that larval oysters were not consuming *A. catenella* or *D. acuminata* cells, cell concentrations of *A. catenella* and *D. acuminata* were monitored during the live cell bioassay. Additional HAB treatment wells with larval oysters were sampled every 24 h throughout the 96-h bioassay, fixed, and counted by light microscopy, to put together a growth time series for each HAB species at all four initial cell concentrations tested (see **Ch. 2 Section 2.1.1**). The growth time series (**Supp. Fig. 1**) demonstrated that cell concentrations of both HAB species stayed the same or

increased over the 96-h bioassay (see **Ch. 2 Section 3.1**), suggesting larval oysters were not consuming these cells (see **Ch. 2 Section 4.1**).

The toxin profiles of the *D. acuminata* (DATC03, D. Anderson and M. Brosnahan, WHOI) culture and lysate used in **CHAPTER 2** were assessed and compared to assist in evaluating the results of the live cell and lysate bioassays. Culture and lysate samples were collected, extracted, and analyzed using ultra-performance liquid chromatography – tandem mass spectrometry with a trapping dimension and at-column dilution (UPLC-MS/MS with trap/ACD). Method details can be found in **Ch. 2 Section 2.3**. The resulting toxin profiles (**Supp. Fig. 2**) were reported in **Ch. 2 Section 3.3** and discussed in **Section 4.2**.

Supplemental Table 1
Least-squares means of arcsine-transformed larval mortality in the live cell bioassay at each timepoint.

Treatments*	24 h	48 h	72 h	96 h
Fed (Pav)	0.00 a	0.00 b	0.00 d	0.03 f
Unfed	0.00 a	0.00 b	0.00 d	0.00 f
Acat 10	0.00 a	0.00 b	0.00 d	0.11 f
Acat 100	0.00 a	0.00 b	0.05 d	0.17 f g
Acat 500	0.00 a	0.04 b c	0.04 d	0.39 g
Acat 1,000	0.00 a	0.00 b	0.00 d	0.00 f
Dacum 10	0.00 a	0.03 b	0.09 d e	0.13 f
Dacum 100	0.00 a	0.00 b	0.03 d	0.06 f
Dacum 500	0.00 a	0.00 b	0.00 d	0.09 f
Dacum 1,000	0.03 a	0.27 c	0.32 e	0.41 g

Standard error = 0.045 for all reported least-squares means values.
Letters denote groups of treatments that were or were not significantly different from one another based on Tukey's post hoc pairwise comparisons calculated within each level of time (Tukey-Bonferroni adjusted $\alpha = 0.0125$).
* Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, numbers represent cell concentrations (cells/mL)

Supplemental Table 2
Least-squares means of arcsine-transformed larval mortality in the lysate bioassay at each timepoint.

Treatments*	24 h	48 h	72 h	96 h
Fed (Pav)	0.00 a	0.00 b	0.00 c	0.00 d
Unfed	0.00 a	0.03 b	0.06 c	0.06 d
Acat 100	0.00 a	0.00 b	0.00 c	0.00 d
Acat 1,000	0.00 a	0.00 b	0.00 c	0.03 d e
Dacum 1,000	0.00 a	0.07 b	0.16 c	0.23 f
Acat 1,000 x Dacum 1,000	0.00 a	0.10 b	0.15 c	0.18 e f

Standard error = 0.034 for all reported least-squares means values.
Letters denote groups of treatments that were or were not significantly different from one another based on Tukey's post hoc pairwise comparisons calculated within each level of time (Tukey-Bonferroni adjusted $\alpha = 0.0125$).
* Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, numbers represent cell concentration equivalents (cells/mL equiv.)

Supplemental Table 3
Least-squares means of arcsine-transformed larval inactivity in the live cell bioassay at each timepoint.

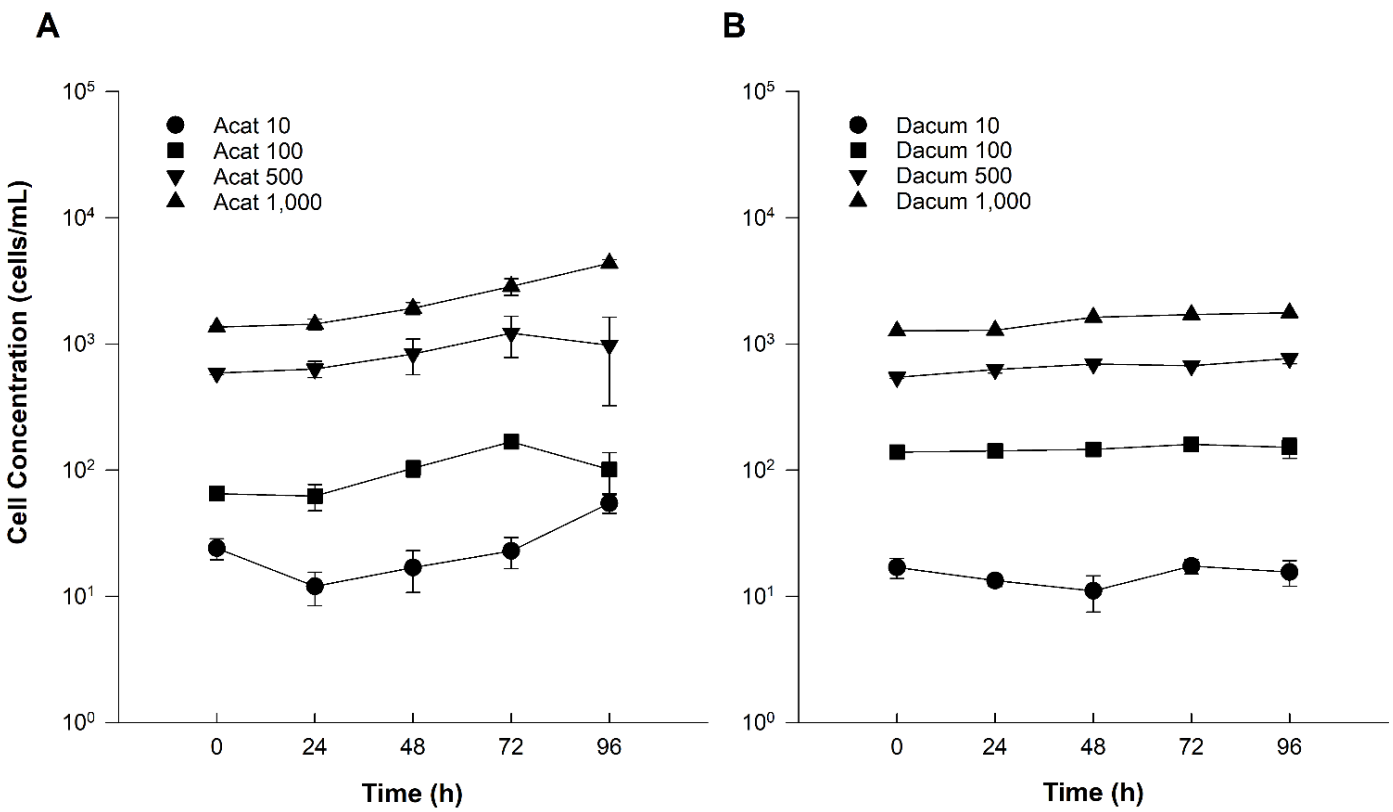
Treatments*	24 h	48 h	72 h	96 h
Fed (Pav)	0.26 a	0.07 c	0.00 g	0.25 j
Unfed	0.22 a	0.18 c d	0.13 g h	0.15 j
Acat 10	0.50 a b	0.31 c d	0.24 g h	0.77 k
Acat 100	0.34 a b	0.27 c d	0.56 h	1.18 k l
Acat 500	0.72 b	0.49 c e	1.25 i	1.05 k l
Acat 1,000	0.77 b	0.82 e	1.05 i	1.32 l
Dacum 10	0.40 a b	0.22 c f	0.21 g h	0.27 j
Dacum 100	0.20 a	0.09 c	0.16 g h	0.14 j
Dacum 500	0.11 a	0.15 c f	0.13 g h	0.11 j
Dacum 1,000	0.72 b	0.53 d e f	0.43 g h	0.32 j

Standard error = 0.082 for all reported least-squares means values.
Letters denote groups of treatments that were or were not significantly different from one another based on Tukey's post hoc pairwise comparisons calculated within each level of time (Tukey-Bonferroni adjusted $\alpha = 0.0125$).
* Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, numbers represent cell concentrations (cells/mL)

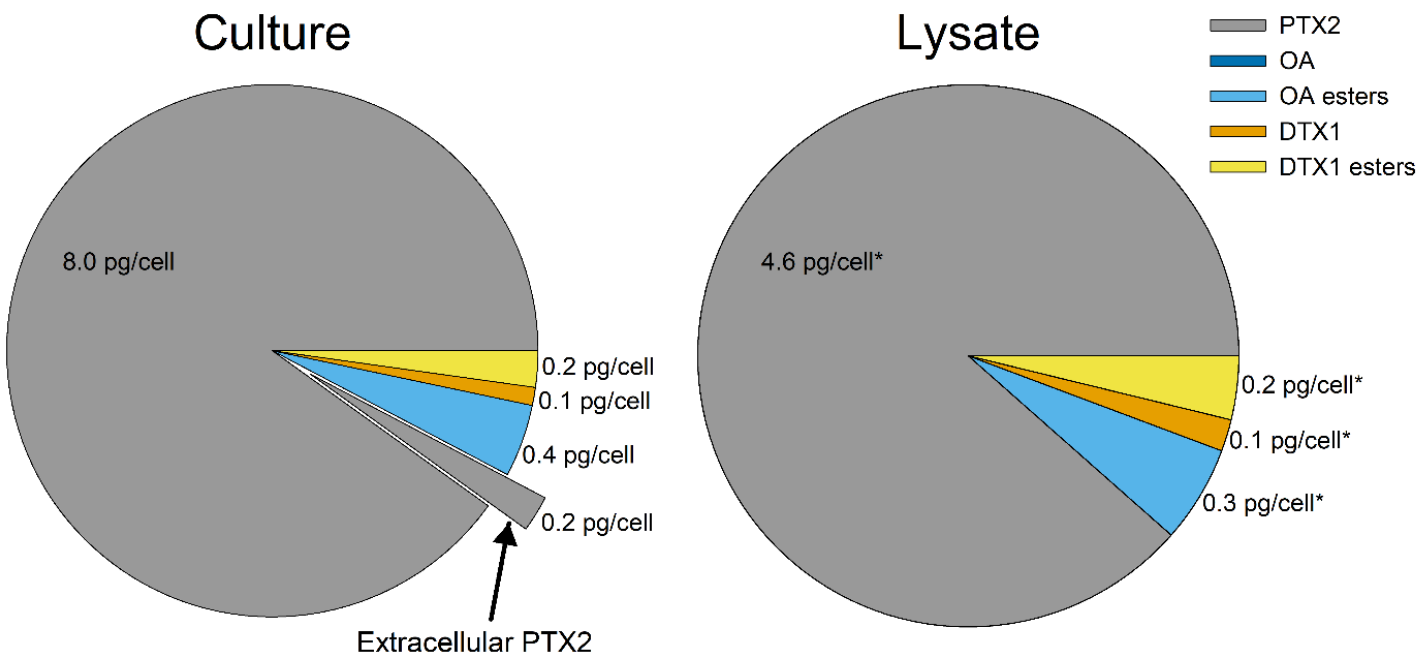
Supplemental Table 4
Least-squares means of arcsine-transformed larval inactivity in the lysate bioassay at each timepoint.

Treatments*	**24 h**	**48 h**	**72 h**	**96 h**
Fed (Pav)	0.03 a	0.06 c d	0.07 f g	0.06 i j
Unfed	0.06 a b	0.09 c d	0.13 f g	0.10 i j
Acat 100	0.03 a	0.00 d	0.00 g	0.00 j
Acat 1,000	0.21 a b	0.16 c d e	0.14 f g	0.13 i j
Dacum 1,000	0.34 b	0.39 e	0.54 h	0.65 k
Acat 1,000 x Dacum 1,000	0.11 a b	0.34 c e	0.30 f h	0.31 i

Standard error = 0.058 for all reported least-squares means values.
Letters denote groups of treatments that were or were not significantly different from one another based on Tukey's post hoc pairwise comparisons calculated within each level of time (Tukey-Bonferroni adjusted $\alpha = 0.0125$).
* Pav = *Pavlova pinguis*, Acat = *Alexandrium catenella*, Dacum = *Dinophysis acuminata*, numbers represent cell concentration equivalents (cells/mL equiv.)



Supplemental Figure 1
Growth time series of (A) *Alexandrium catenella* (Acat) and (B) *Dinophysis acuminata* (Dacum) at four different initial cell concentrations (cells/mL) when grown with larval oysters during the live cell bioassay. Error bars show standard error (n = 3 wells per treatment).



Supplemental Figure 2
Toxin profiles of the *Dinophysis acuminata* culture and lysate. Most of the toxins detected in the culture were intracellular, with the exception of 0.2 pg/cell of extracellular pectenotoxin-2 (PTX2). OA = okadaic acid, DTX1 = dinophysistoxin-1.
*Lysate units are pg/cell equivalents. Note that pg/cell is the same as ng/mL for a cell concentration of, or equivalent to, 1,000 cells/mL in this study.

APPENDIX III

Supplementary Materials for Chapter 3

Various field metrics were tested to see if they could serve as predictors of phycotoxin in adult oysters (*C. virginica*) in the Virginia-portion of the Chesapeake Bay. Metrics included solid phase adsorption toxin tracking devices (SPATTs), particulate organic matter (POM, > 1 µm), and HAB cell concentrations. Only phycotoxins that were detected in ≥ 10 oyster samples, i.e., AZA1, AZA2, DA, and PTX2, were used in the analyses, with data from both 2019 and 2020, and all sites (Ch. 3 Fig. 1). **Chapter 3 Section 2.5** describes in detail how the raw data from Table 1 was treated and used in linear regression models (Ch. 3 Table 3), using R Studio (2019) with R version 3.6.2. Significant correlations between the tested metrics and phycotoxin in oysters for PTX2 are highlighted in **Ch. 3 Section 3.1** and **Section 3.1.3**. Significant correlations between the tested metrics and phycotoxin in oysters for the AZAs and DA are highlighted in **Ch. 3 Section 3.3**. Further implications of these correlations for HAB monitoring and early warning are discussed in **Ch. 3 Section 4**.

For HAB monitoring, to assess fine-scale variation in phycotoxin distribution with depth, SPATTs and POM samples were deployed or collected, respectively, both at the water's surface and near-bottom (0.5 - 0.6 m, as described in **Ch. 3 Section 2.1**). All samples for these analyses were collected in 2019 at Rappahannock, York, Lynnhaven, and Cherrystone (Ch. 3 Fig. 1). For SPATTs, phycotoxins that were detected in ≥ 10 SPATT samples were included in the analysis, i.e., AZA1, AZA2, DA, GDA, PTX2, OA, DTX1 (Table 2, Ch. 3 Table 2). Wilcoxon signed-rank tests were performed using R Studio (2019) with R version 3.6.2 to test for differences between phycotoxin concentrations at the surface and near-bottom (see **Ch. 3 Section 2.5**, Ch. 3 Fig. 3). For POM samples, as mentioned in **Ch. 3 Section 2.5**, phycotoxins were detected in too few

samples for statistical comparisons, instead all of the POM sample raw data (**Table 3**) was summarized in **Ch. 3 Tables 2** and **4**. POM samples only detected AZA2, DA, MC-RR, GDA, and PTX2. Results of these fine-scale depth analyses can be found in **Ch. 3 Section 3.1** and its sub-sections, as well as in **Ch. 3 Sections 3.3** and **4**.

To visually compare phycotoxin and HAB cell concentration data over time, time series plots for phycotoxins in oysters, SPATTs, and POM, along with HAB cell concentrations, were plotted for select sites (**Ch. 3 Fig. 1**) and phycotoxins with enough data (AZA2, DA, and PTX2, data from **Table 1**). Phycotoxin in POM was only measured in 2019. Details for how data were collected can be found in **Ch. 3 Section 2**. For AZA2, oyster, SPATT, and POM phycotoxin data were plotted for Lynnhaven (**Fig. 1**) and Wise Point (**Fig. 2**). For DA, oyster, SPATT, and POM phycotoxin data were plotted along with *Pseudo-nitzschia* spp. cell concentrations for Lynnhaven (**Fig. 3**), Wise Point (**Fig. 4**), and York (**Fig. 5**). For PTX2, oyster, SPATT, and POM phycotoxin data were plotted along with *Dinophysis* spp. cell concentrations for Lynnhaven (**Fig. 6**), Wise Point (**Fig. 7**), and York (**Fig. 8**). AZA2 SPATT concentrations followed a similar pattern to oyster AZA2 concentrations over time (**Figs. 1** and **2**). DA concentrations were poorly related with *Pseudo-nitzschia* spp. cell concentrations (**Figs. 3**, **4**, **5**), but POM from two weeks prior to oyster samples gave some early warning of DA phycotoxin levels (**Fig. 5**) and SPATT phycotoxin concentrations showed some minor correlation with phycotoxin in oysters. For PTX2, it was visually difficult to see trends between phycotoxin samples or HAB cell concentrations, especially with SPATT PTX2 concentrations being much higher than oyster phycotoxin concentrations (**Figs. 6**, **7**, **8**). Observed trends, as well as

some less obvious trends, are reflected in the linear regression results in **Ch. 3 Table 3**, which are discussed in **Ch. 3 Sections 3** and **4**.

Table 1

Linear regression data: Oysters, SPATTs, and POM phycotoxin concentrations, and HAB cell concentrations. Phycotoxin concentrations below the limit of detection (LOD) are represented as zeroes. Oyster phycotoxin concentrations less than the limit of quantitation (LOQ) but with S/N ≥ 10 and a parent peak with S/N ≥ 3, were represented as ½ the concentration of the lowest quantifiable standard. SPATT and POM phycotoxin concentrations less than the LOQ were represented as ½ LOD. The LOD for quantifying cell concentrations in 2019 was 1 cell/mL, and in 2020 was 0.2 cells/mL. Blank cells signify that no data was collected.

Site	Date	AZA1		AZA2			DA				PTX2			
		Oyster (µg/kg SM)	SPATTs (µg/kg resin)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[a] (cells/mL)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[b] (cells/mL)
Rappahannock	1/22/2019	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	0.55	0.00	0
Rappahannock	2/6/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	0.59	0.00	0
Rappahannock	2/19/2019	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	0.92	0.00	0
Rappahannock	3/5/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	0.41	0.00	0
Rappahannock	3/22/2019	0.24	0.00	0.00	0.00	0.00	0.00	0.00	1.65	0	0.00	0.49	0.00	0
Rappahannock	4/2/2019	0.00	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0	0.00	0.32	0.00	0
Rappahannock	4/17/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	1.27	0.00	0
Rappahannock	5/1/2019	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	1.34		0
Rappahannock	5/17/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	1.71	0.00	0
Rappahannock	5/29/2019	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	1.01	0.00	0
Rappahannock	6/12/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	1.24	0.00	0
Rappahannock	5/26/2020	0.00	0.00	0.38	0.00		0.00	0.00		0	0.45	52.23		0
Rappahannock	6/12/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	29.63		0
Rappahannock	6/25/2020	0.00	0.00	0.28	0.00		0.00	0.00		0	0.00	23.21		0
Rappahannock	7/9/2020	0.00	0.00	0.00	0.00		0.00	0.00		0.4	0.00	3.30		0
Rappahannock	7/23/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	2.42		0
Rappahannock	8/6/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	1.01		0
York	1/26/2019	0.00	0.00	0.00	0.00	0.00	28.49	0.00	152.50		0.00	4.85	0.00	
York	2/7/2019	0.00	0.00	0.00	0.00	0.00	7.05	0.00	33.68	0	0.00	4.30	0.00	0
York	2/22/2019	0.00	0.00	0.00	0.00	0.00	14.99	2.20	7.61	0	0.00	3.59	0.00	0

Site	Date	AZA1		AZA2			DA				PTX2			
		Oyster (µg/kg SM)	SPATTs (µg/kg resin)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[a] (cells/mL)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[b] (cells/mL)
York	3/5/2019	0.00	0.00	0.00	0.00	0.00	7.17	0.00	0.00	0	0.00	3.00	0.00	0
York	3/19/2019	0.35	0.00	0.00	0.00	0.00	2.25	0.00	3.03	0	0.00	3.26	0.00	0
York	4/1/2019	0.23	0.00	0.00	0.00	0.00	9.88	0.00	4.92	0	0.00	6.37	0.00	0
York	4/15/2019	0.00	0.00	0.00	0.00	0.00	28.06	0.00	1.62	0	0.00	5.93	0.00	15
York	5/3/2019	0.00	0.00	0.00	0.00	0.00	6.34	0.00	0.00	0	0.00	11.00	1.95	0
York	5/14/2019	0.35	0.00	0.00	0.00	0.00	12.62	0.00	0.00	0	0.45	17.59	3.61	0
York	5/28/2019	0.00	0.00	0.00	0.00	0.00	2.25	0.00	0.00	0	0.00	14.22	0.00	0
York	6/10/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15	0.00	14.46	0.00	0
York	3/20/2020	0.00		0.00			0.00				2.24			
York	4/3/2020	0.00	0.00	0.00	0.00		0.00	0.00		1.2	0.45	72.53		0
York	4/17/2020	0.00	0.00	0.00	0.14		0.00	0.00		0.4	0.45	46.58		0.6
York	5/1/2020	0.00	0.00	0.00	0.14		2.25	0.00		0	0.45	51.34		0
York	5/15/2020	0.00	0.00	0.00	0.20		0.00	0.00		0	0.00	32.92		0
York	5/29/2020	0.21	0.00	0.00	0.18		0.00	0.00		0	0.00	36.23		0
York	6/12/2020	0.00	0.00	0.00	0.10		0.00	0.00		0	0.00	24.38		0
York	6/26/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	23.99		0
York	7/10/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	6.92		0
York	7/24/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	6.72		0
York	8/7/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	0.00	10.51		0
Lynnhaven	1/25/2019	0.00	0.00	0.00	0.04	0.00	579.42	6.04	943.54	0	0.00	5.29	0.00	0
Lynnhaven	2/5/2019	0.41	0.00	0.00	0.05	0.00	294.19	0.00	54.17	0	0.00	4.27	0.00	0
Lynnhaven	2/21/2019	0.00	0.00	0.00	0.04	0.00	29.08	0.00	2.61	0	0.00	5.62	0.00	0
Lynnhaven	3/5/2019	0.61	0.00	0.00	0.03	0.00	18.28	0.00	0.00		0.00	4.39	0.00	
Lynnhaven	3/18/2019	0.09	0.00	0.00	0.01	0.00	17.61	0.00	2.73	0	0.00	2.79	0.00	0
Lynnhaven	4/1/2019	0.00	0.00	0.00	0.02	0.00	50.72	0.00	1.34	0	2.38	12.74	1.14	0

Site	Date	AZA1		AZA2			DA				PTX2			
		Oyster (µg/kg SM)	SPATTs (µg/kg resin)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[a] (cells/mL)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[b] (cells/mL)
Lynnhaven	4/18/2019	0.00	0.00	0.00	0.02	0.00	26.37	0.00	1.55	0	0.45	25.23	1.29	0
Lynnhaven	4/29/2019	0.00	0.00	0.22	0.03	0.00	6.37	0.00	0.00		0.86	45.39	0.00	
Lynnhaven	5/15/2019	0.80	0.00	0.00	0.02	0.00	0.00	2.51	0.00	0	0.45	45.47	2.66	0
Lynnhaven	5/29/2019	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0	0.00	55.37	0.00	0
Lynnhaven	6/11/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.85	0.00	0	0.00	37.18	0.00	0
Lynnhaven	3/31/2020	0.00	0.00	0.20	0.23		0.00	0.00		8.4	0.45	72.52		0
Lynnhaven	4/16/2020	0.00	0.00	0.00	0.26		0.00	0.00		0	1.46	92.56		0
Lynnhaven	4/28/2020	0.00	0.00	0.00	0.20		0.00	0.00		0.4	2.34	109.98		0.4
Lynnhaven	5/12/2020	0.00	0.00	0.33	0.20		0.00	0.00		0.4	4.38	75.36		0
Lynnhaven	5/26/2020	0.00	0.00	0.00	0.18		0.00	0.00		1	2.46	49.96		0.2
Lynnhaven	6/10/2020	0.37	0.00	0.00	0.11		0.00	0.00		0	2.28	50.34		0.2
Lynnhaven	6/24/2020	0.40	0.00	0.00	0.00		0.00	0.00		0	0.45	53.47		0
Lynnhaven	7/7/2020	0.36	0.00	0.00	0.00		0.00	0.00		0	0.45	46.14		0
Lynnhaven	7/20/2020	0.00	0.00	0.00	0.00		2.25	0.00		0	0.45	21.82		0
Lynnhaven	8/3/2020	0.36	0.00	0.00	0.00		5.45	0.00		0	0.45	15.28		0
Wise Point	3/31/2020	0.00	0.00	0.00	0.23		85.79	0.83		15.8	0.68	114.23		0.2
Wise Point	4/16/2020	0.00	0.00	0.00	0.21		14.51	0.00		0	0.57	74.27		0.2
Wise Point	4/28/2020	0.00	0.00	0.00	0.16		0.00	0.00		0.4	1.32	63.08		0.2
Wise Point	5/12/2020	0.00	0.00	0.00	0.15		0.00	0.00		0	1.56	47.23		0
Wise Point	5/26/2020	0.00	0.00	0.28	0.23		0.00	0.00		0	6.16	118.42		1.8
Wise Point	6/10/2020	0.00	0.00	0.23	0.15		0.00	0.00		0	0.97	143.14		1.2
Wise Point	6/24/2020	0.00	0.00	0.00	0.00		0.00	0.00		0	1.12	116.04		0.4
Wise Point	7/7/2020	0.28	0.00	0.00	0.00		0.00	0.00		0	0.45	95.79		0
Wise Point	7/20/2020	0.00	0.00	0.38	0.00		14.76	0.00		0	0.99	31.43		0
Wise Point	8/3/2020	0.00	0.00	0.42	0.09		22.79	2.84		0.8	0.45	47.83		0

Site	Date	AZA1		AZA2			DA				PTX2			
		Oyster (µg/kg SM)	SPATTs (µg/kg resin)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[a] (cells/mL)	Oyster (µg/kg SM)	SPATTs (µg/kg resin)	POM (µg/L)	Cell Conc.[b] (cells/mL)
Cherrystone	1/25/2019	0.00	0.00	0.00	0.00	0.00	46.60	0.00	215.17	0	0.00	2.69	0.00	0
Cherrystone	2/5/2019	0.29	0.00	0.00	0.00	0.00	75.04	0.00	38.14		0.00	1.70	0.00	
Cherrystone	2/21/2019	0.25	0.00	0.00	0.00	0.00	44.00	2.37	21.48		0.00	1.85	0.00	
Cherrystone	3/5/2019	0.00	0.00	0.00	0.00	0.07	24.72	0.00	0.00	0	0.45	2.07	0.00	0
Cherrystone	3/18/2019	0.09	0.00	0.00	0.00	0.00	6.61	0.00	0.00	0	0.00	2.44	0.00	0
Cherrystone	4/1/2019	0.00	0.00	0.00	0.00	0.00	4.79	0.00	0.00	0	0.45	3.05	0.00	0
Cherrystone	4/18/2019	0.00	0.00	0.00	0.00	0.00	2.25	0.00	0.00	0	0.45	4.22	0.00	0
Cherrystone	4/29/2019	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00		0.00	4.47	0.00	
Cherrystone	5/15/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	6.29	2.12	0
Cherrystone	5/29/2019	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00		0.00	6.09	0.00	
Cherrystone	6/11/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	6.83	0.00	0

Abbreviations: AZA1 = azaspiracid-1, AZA2 = azaspiracid-2, DA = domoic acid, PTX2 = pectenotoxin-2, SPATTs = solid phase adsorption toxin tracking devices, POM = particulate organic matter (> 1 µm), SM = shellfish meat.

[a] Cell concentrations for DA are *Pseudo-nitzschia* spp.

[b] Cell concentrations for PTX2 are *Dinophysis* spp.

Table 2

Raw data for SPATT phycotoxin depth comparison. Phycotoxin concentrations below the limit of detection (LOD) are represented as zeroes. SPATT phycotoxin concentrations less than the limit of quantitation (LOQ) were represented as ½ LOD. Blank cells signify that no data was collected.

Site	Date	AZA1 (µg/kg resin)		AZA2 (µg/kg resin)		DA (µg/kg resin)		GDA (µg/kg resin)		PTX2 (µg/kg resin)		OA (µg/kg resin)		DTX1 (µg/kg resin)	
		SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom
Rappahannock	1/22/2019	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.53	0.55	4.93	3.55	0.00	0.00
Rappahannock	2/6/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.81	0.59	8.40	4.63	0.00	0.00
Rappahannock	2/19/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.97	0.92	9.78	8.08	0.00	0.00
Rappahannock	3/5/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.93	0.41	11.31	6.61	0.00	0.00
Rappahannock	3/22/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.32	0.49	8.24	5.62	0.00	0.00
Rappahannock	4/2/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.32	10.31	6.63	0.00	0.00
Rappahannock	4/17/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34	1.27	13.21	11.20	0.00	0.00
Rappahannock	5/1/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.67	1.34	17.64	8.14	0.00	0.00
Rappahannock	5/17/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.50	1.71	17.47	13.10	0.00	0.00
Rappahannock	5/29/2019	0.00	0.00	0.00	0.00	0.00	0.00	1.56	3.29	1.91	1.01	12.91	1.92	0.00	0.00
Rappahannock	6/12/2019	0.00	0.00	0.00	0.00	0.00	0.00	6.00	13.86	2.54	1.24	14.55	4.28	0.00	0.00
York	1/26/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.65	4.85	30.04	30.90	0.00	0.00
York	2/7/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.55	4.30	27.66	31.08	0.00	0.00
York	2/22/2019	0.00	0.00	0.00	0.00	0.00	2.20	0.00	0.00	3.56	3.59	37.69	39.65	0.00	0.00
York	3/5/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.04	3.00	34.82	36.20	0.00	0.00
York	3/19/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.09	3.26	36.69	37.71	0.00	0.00
York	4/1/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.25	6.37	42.19	42.82	0.00	0.00
York	4/15/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.34	5.93	38.86	39.75	0.00	0.00
York	5/3/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.80	11.00	44.37	42.48	12.31	10.17
York	5/14/2019	0.00	0.00	0.00	0.00	0.00	0.00	3.52	6.31	25.91	17.59	45.65	26.07	15.76	8.90
York	5/28/2019	0.00	0.00	0.00	0.00	0.00	0.00	4.60	15.52	11.81	14.22	42.40	34.63	12.81	9.85

Site	Date	AZA1 (µg/kg resin)		AZA2 (µg/kg resin)		DA (µg/kg resin)		GDA (µg/kg resin)		PTX2 (µg/kg resin)		OA (µg/kg resin)		DTX1 (µg/kg resin)	
		SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom	SPATT Surface	SPATT Near-bottom
York	6/10/2019	0.00	0.00	0.00	0.00	0.00	0.00	7.10	12.35	10.85	14.46	35.54	33.81	13.87	10.08
Lynnhaven	1/25/2019	0.00	0.00	0.05	0.04	0.00	6.04	0.00	0.00	3.77	5.29	43.81	41.27	0.00	0.00
Lynnhaven	2/5/2019	0.00	0.00	0.03	0.05	0.00	0.00	0.00	0.00	2.52	4.27	47.51	46.82	0.00	0.00
Lynnhaven	2/21/2019	0.00	0.00	0.01	0.04	0.00	0.00	0.00	0.00	1.52	5.62	44.50	56.74	0.00	0.00
Lynnhaven	3/5/2019	0.00	0.00	0.02	0.03	0.00	0.00	0.00	0.00	2.76	4.39	53.27	54.42	0.00	0.00
Lynnhaven	3/18/2019	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	4.64	2.79	57.20	55.59	0.00	0.00
Lynnhaven	4/1/2019	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	9.85	12.74	74.05	69.90	5.06	5.19
Lynnhaven	4/18/2019	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	4.60	25.23	57.03	72.88	7.68	12.53
Lynnhaven	4/29/2019	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	11.35	45.39	57.90	69.43	12.67	17.35
Lynnhaven	5/15/2019	0.00	0.00	0.00	0.02	0.00	2.51	0.00	0.00	14.77	45.47	41.51	59.55	11.74	22.28
Lynnhaven	5/29/2019	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	14.61	55.37	46.92	56.62	12.43	22.24
Lynnhaven	6/11/2019	0.00	0.00	0.00	0.00	0.00	0.85	0.00	0.00	11.74	37.18	31.57	52.92	9.11	20.04
Cherrystone	1/25/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.03	2.69	3.03	2.69	0.00	0.00
Cherrystone	2/5/2019		0.00		0.00		0.00		0.00		1.70		1.70		0.00
Cherrystone	2/21/2019	0.00	0.00	0.00	0.00	0.00	2.37	0.00	0.00	2.38	1.85	2.38	1.85	0.00	0.00
Cherrystone	3/5/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.54	2.07	2.54	2.07	0.00	0.00
Cherrystone	3/18/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.64	2.44	1.64	2.44	0.00	0.00
Cherrystone	4/1/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.04	3.05	3.04	3.05	0.00	4.20
Cherrystone	4/18/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.20	4.22	3.20	4.22	6.99	8.27
Cherrystone	4/29/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.99	4.47	3.99	4.47	8.83	9.58
Cherrystone	5/15/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.31	6.29	8.31	6.29	12.77	10.18
Cherrystone	5/29/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.13	6.09	7.13	6.09	10.68	11.23
Cherrystone	6/11/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.57	6.83	11.57	6.83	12.29	10.14

Abbreviations: AZA1 = azaspiracid-1, AZA2 = azaspiracid-2, DA = domoic acid, GDA = goniodomin A, PTX2 = pectenotoxin-2, OA = okadaic acid, DTX1 = dinophysistoxin-1, SPATT = solid phase adsorption toxin tracking device

Table 3

Raw data for POM phycotoxin depth comparison. Phycotoxin concentrations below the limit of detection (LOD) are represented as zeroes. POM phycotoxin concentrations less than the limit of quantitation (LOQ) were represented as ½ LOD. Blank cells signify that no data was collected.

Site	Date	AZA2 (µg/L)		DA (µg/L)		MC-RR (µg/L)		GDA (µg/L)		PTX2 (µg/L)	
		POM Surface	POM Near-bottom	POM Surface	POM Near-bottom	POM Surface	POM Near-bottom	POM Surface	POM Near-bottom	POM Surface	POM Near-bottom
Rappahannock	1/22/2019	0.00		0.00		0.00		0.00		0.00	
Rappahannock	2/6/2019	0.00		0.00		0.00		0.00		0.00	
Rappahannock	2/19/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rappahannock	3/5/2019	0.00		0.00		0.00		0.00		0.00	
Rappahannock	3/22/2019	0.00	0.00	1.65	1.05	0.00	0.00	0.00	0.00	0.00	0.00
Rappahannock	4/2/2019	0.00		0.00		0.00		0.00		0.00	
Rappahannock	4/17/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rappahannock	5/1/2019										
Rappahannock	5/17/2019	0.00		0.00		0.00		0.00		0.00	
Rappahannock	5/29/2019	0.00		0.00		0.00		0.00		0.00	
Rappahannock	6/12/2019	0.00		0.00		0.00		0.00		0.00	
York	1/26/2019	0.00		152.50		0.00		0.00		0.00	
York	2/7/2019	0.00		33.68		0.00		0.00		0.00	
York	2/22/2019	0.00	0.00	7.61	6.77	0.00	0.00	0.00	0.00	0.00	0.00
York	3/5/2019	0.00		0.00		0.00		0.00		0.00	
York	3/19/2019	0.00	0.00	3.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
York	4/1/2019	0.00		4.92		0.00		0.00		0.00	
York	4/15/2019	0.00	0.00	1.62	6.33	0.00	0.00	0.00	0.00	0.00	0.00
York	5/3/2019	0.00		0.00		0.00		0.00		1.95	
York	5/14/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.61	0.00
York	5/28/2019	0.00		0.00		0.00		0.00		0.00	

Site	Date	AZA2 (µg/L)		DA (µg/L)		MC-RR (µg/L)		GDA (µg/L)		PTX2 (µg/L)	
		POM Surface	POM Near-bottom	POM Surface	POM Near-bottom	POM Surface	POM Near-bottom	POM Surface	POM Near-bottom	POM Surface	POM Near-bottom
York	6/10/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.63	0.00	0.00
Lynnhaven	1/25/2019	0.00		943.54		0.00		0.00		0.00	
Lynnhaven	2/5/2019	0.00		54.17		0.00		0.00		0.00	
Lynnhaven	2/21/2019	0.00	0.00	2.61	2.68	0.00	0.00	0.00	0.00	0.00	0.00
Lynnhaven	3/5/2019	0.00		0.00		0.00		0.00		0.00	
Lynnhaven	3/18/2019	0.00		2.73		0.00		0.00		0.00	
Lynnhaven	4/1/2019	0.00		1.34		0.00		0.00		1.14	
Lynnhaven	4/18/2019	0.00		1.55		0.00		0.00		1.29	
Lynnhaven	4/29/2019	0.00		0.00		0.00		0.00		0.00	
Lynnhaven	5/15/2019	0.00		0.00		0.00		0.00		2.66	
Lynnhaven	5/29/2019	0.00		0.00		0.00		0.00		0.00	
Lynnhaven	6/11/2019	0.00		0.00		0.00		0.00		0.00	
Cherrystone	1/25/2019	0.00		215.17		0.00		0.00		0.00	
Cherrystone	2/5/2019	0.00		38.14		0.00		0.00		0.00	
Cherrystone	2/21/2019	0.00	0.00	21.48	25.04	0.00	0.00	0.00	0.00	0.00	0.00
Cherrystone	3/5/2019	0.07		0.00		0.00		0.00		0.00	
Cherrystone	3/18/2019	0.00	0.00	0.00	0.00	3.98	0.00	0.00	0.00	0.00	0.00
Cherrystone	4/1/2019	0.00		0.00		0.00		0.00		0.00	
Cherrystone	4/18/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cherrystone	4/29/2019	0.05		0.00		0.00		0.00		0.00	
Cherrystone	5/15/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.12	4.52
Cherrystone	5/29/2019	0.07		0.00		0.00		0.00		0.00	
Cherrystone	6/11/2019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Abbreviations: AZA2 = azaspiracid-2, DA = domoic acid, MC-RR = microcystin-RR, GDA = goniodomin A, PTX2 = pectenotoxin-2, POM = particulate organic matter (> 1 µm)

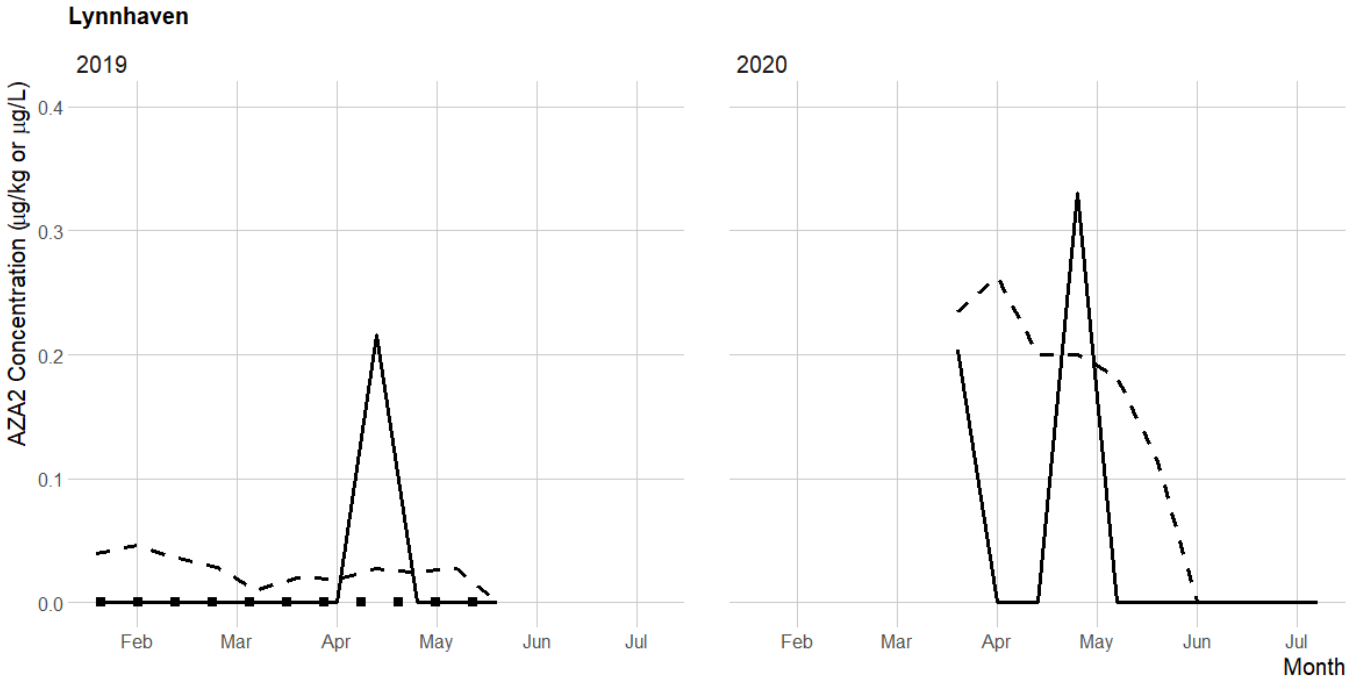


Figure 1
Lynnhaven AZA2
Time series of azaspiracid-2 (AZA2) at Lynnhaven in 2019 and 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]), dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin), and dotted line = phycotoxin in particulate organic matter (POM, > 1 µm; µg/L). Phycotoxin in POM was only measured in 2019.

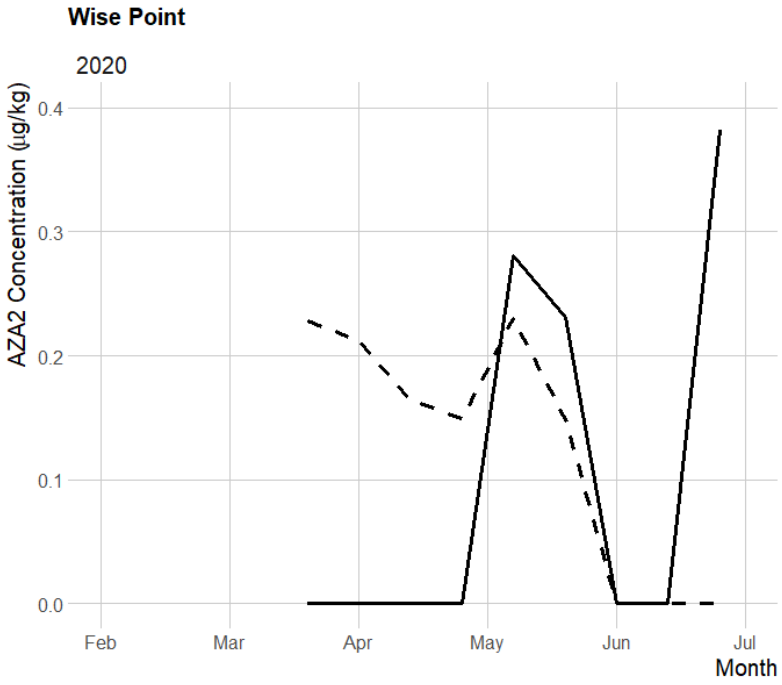


Figure 2
Wise Point AZA2
Time series of azaspiracid-2 (AZA2) at Wise Point in 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]) and dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin).

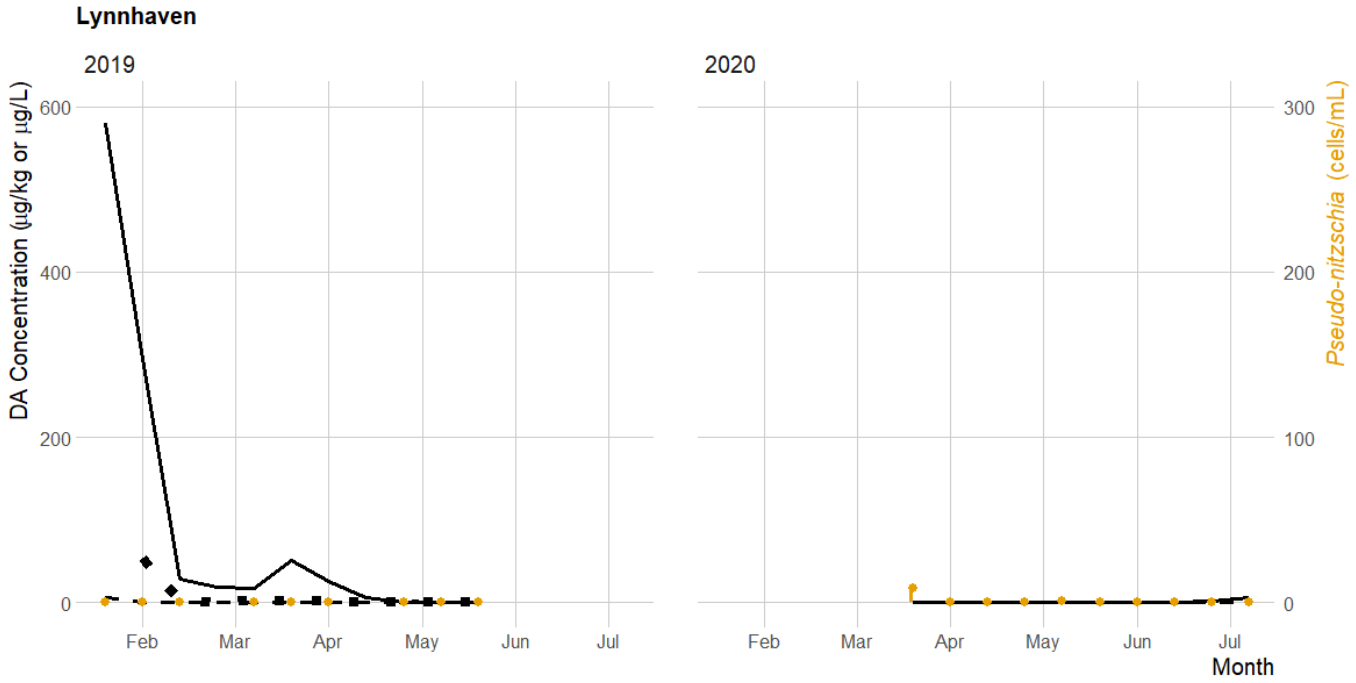


Figure 3
Lynnhaven DA
Time series of domoic acid (DA) and *Pseudo-nitzschia* at Lynnhaven in 2019 and 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]), dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin), and dotted line = phycotoxin in particulate organic matter (POM, > 1 µm; µg/L). Lollipops signify *Pseudo-nitzschia* cell concentration (cells/mL) as determined by light microscopy (yellow). Note the dual y-axes and scales. Phycotoxin in POM was only measured in 2019.

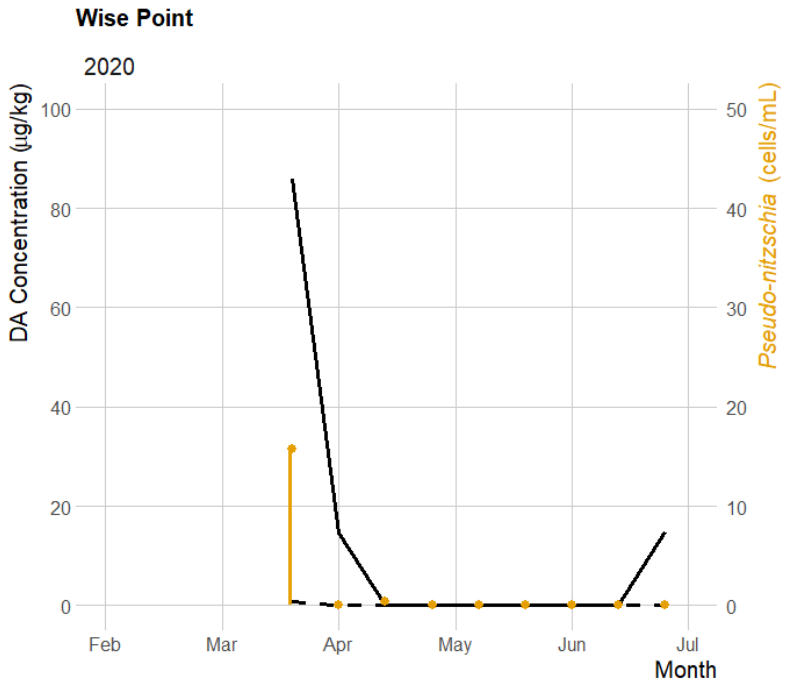


Figure 4
Wise Point DA
Time series of domoic acid (DA) and *Pseudo-nitzschia* at Wise Point in 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]) and dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin). Lollipops signify *Pseudo-nitzschia* cell concentration (cells/mL) as determined by light microscopy (yellow). Note the dual y-axes and scales.

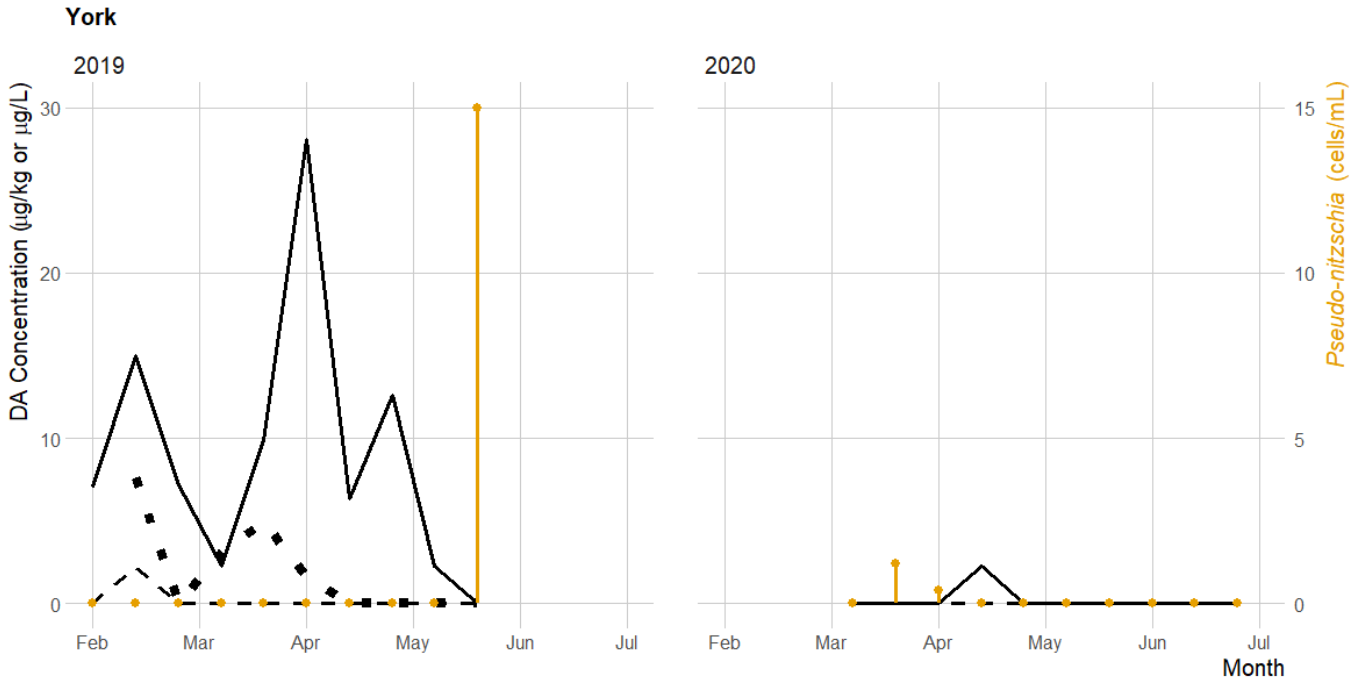


Figure 5
York DA
Time series of domoic acid (DA) and *Pseudo-nitzschia* at York in 2019 and 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]), dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin), and dotted line = phycotoxin in particulate organic matter (POM, > 1 µm; µg/L). Lollipops signify *Pseudo-nitzschia* cell concentration (cells/mL) as determined by light microscopy (yellow). Note the dual y-axes and scales. Phycotoxin in POM was only measured in 2019.

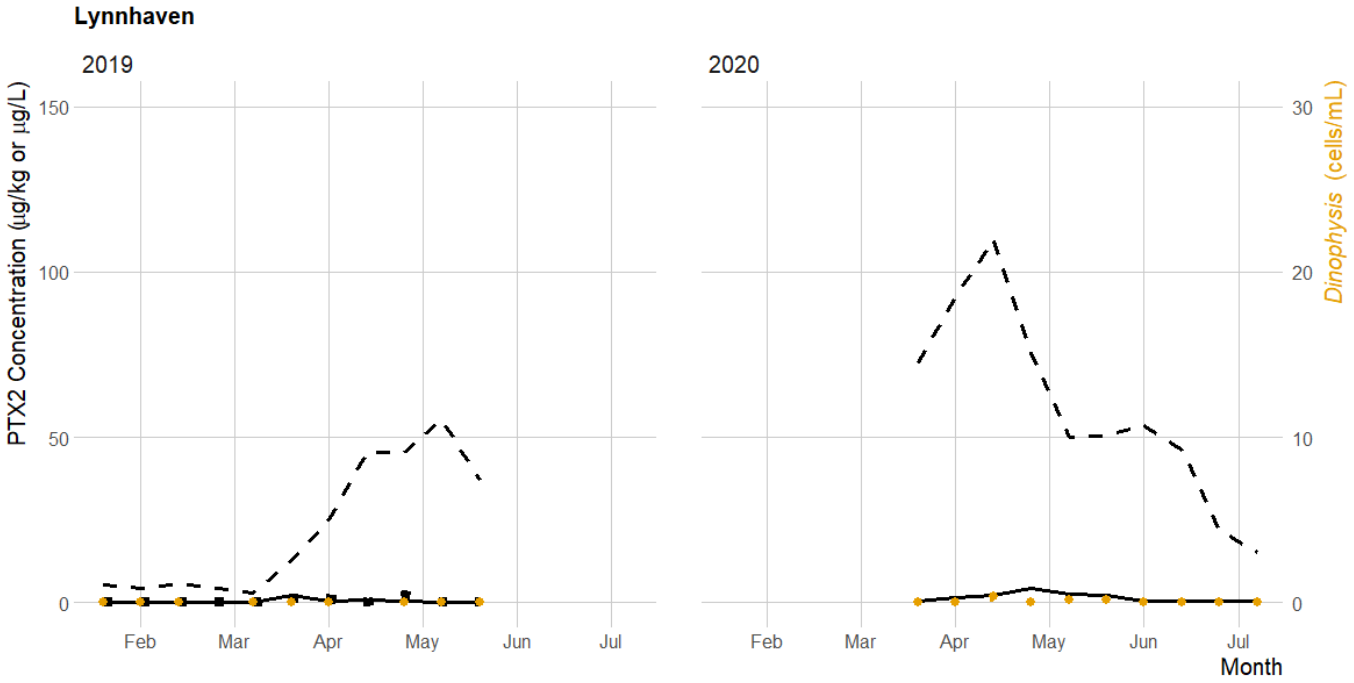


Figure 6
Lynnhaven PTX2
Time series of pectenotoxin-2 (PTX2) and *Dinophysis* at Lynnhaven in 2019 and 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]), dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin), and dotted line = phycotoxin in particulate organic matter (POM, > 1 µm; µg/L). Lollipops signify *Dinophysis* cell concentration (cells/mL) as determined by light microscopy (yellow). Note the dual y-axes and scales. Phycotoxin in POM was only measured in 2019.

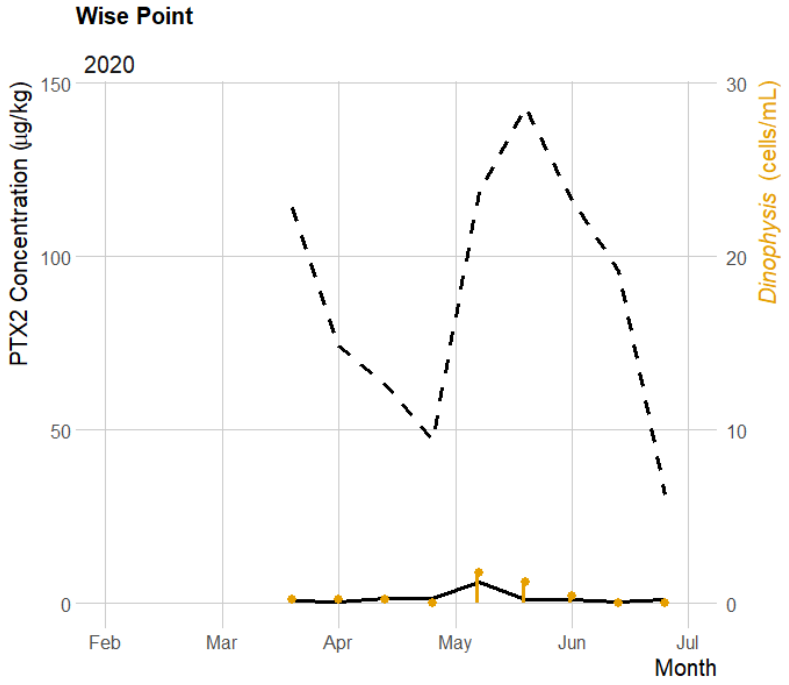


Figure 7
Wise Point PTX2
Time series of pectenotoxin-2 (PTX2) and *Dinophysis* at Wise Point in 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]) and dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin). Lollipops signify *Dinophysis* cell concentration (cells/mL) as determined by light microscopy (yellow). Note the dual y-axes and scales.

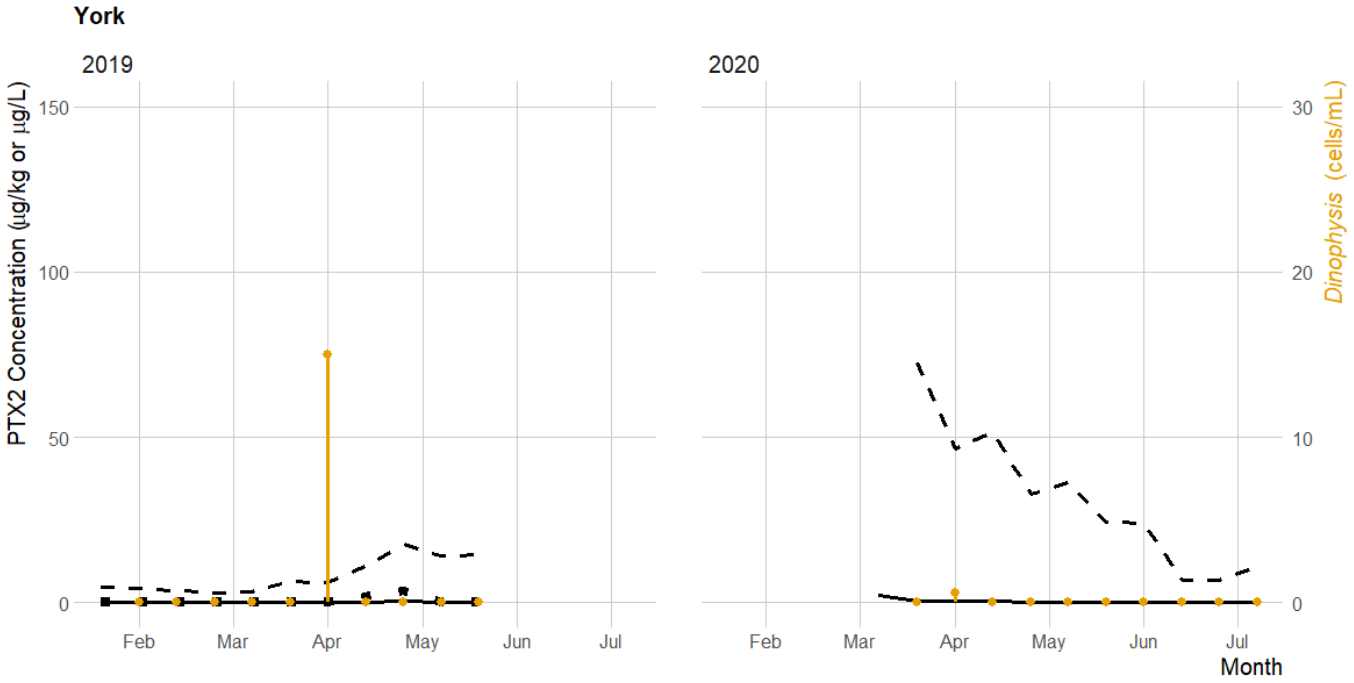


Figure 8
York PTX2
Time series of pectenotoxin-2 (PTX2) and *Dinophysis* at York in 2019 and 2020. Solid line = phycotoxin in oysters (µg/kg shellfish meat [SM]), dashed line = phycotoxin in solid phase adsorption toxin tracking devices (SPATT, µg/kg resin), and dotted line = phycotoxin in particulate organic matter (POM, > 1 µm; µg/L). Lollipops signify *Dinophysis* cell concentration (cells/mL) as determined by light microscopy (yellow). Note the dual y-axes and scales. Phycotoxin in POM was only measured in 2019.

APPENDIX IV

Chesapeake Bay Phycotoxin Distribution

Chesapeake Bay-specific results from **CHAPTER 3** are presented in more detail here. These results contain oyster, solid phase adsorption toxin tracking (SPATT), and particulate organic matter (POM, > 1 µm) phycotoxin data from all sites (**Ch. 3 Fig. 1**) and both sampling years, 2019 and 2020. Methods for sample collection and analysis can be found in **Ch. 3 Section 2**. Phycotoxin prevalence, concentration, co-occurrence, seasonal trends, spatiotemporal distribution, and fine-scale variation with depth are reported. More general trends are reported and discussed in **Ch. 3 Sections 3** and **4**.

Shellfish health phycotoxins

The majority of oyster samples (64%), contained at least one phycotoxin included in a phycotoxin group associated with shellfish health: GDA, KmTxs, and PTX2. At least one of these phycotoxin groups was detected each sampling year (2019 & 2020) and at each sampling site. These phycotoxin groups co-occurred in 12% of oyster samples, at York, Lynnhaven, and Cherrystone. The majority of co-occurrences were between KmTxs and PTX2; the only other co-occurrence was between GDA and PTX2 at Lynnhaven on July 7, 2020. Some seasonal trends of shellfish health phycotoxins in oysters were detected, with KmTxs more prevalent in the winter and spring, and PTX2 more prevalent in the spring to early summer. Trace amounts of GDA were present in the early summer.

Goniodomin A (GDA)

GDA occurred in trace amounts in 7% of oyster samples (**Ch. 3 Fig. 2**, **Ch. 3 Table 1**). Three sites had oyster samples with GDA, Rappahannock, with three positive samples, and Lynnhaven and Cherrystone each with one (**Ch. 3 Fig. 4**). GDA was only found in oyster samples from June and July. GDA was detected in 12% of SPATTs at

surface and in 11% of SPATTs near-bottom (n = 43 and 44, respectively). GDA was only detected in one, near-bottom POM sample (7%, n = 14), from the York site on June 10, 2019 (2.6 µg GDA/L).

Karlotoxins (KmTxs)

KmTx1-1 was present in 28% of oyster samples, while KmTx1-3 was present in only 5% of oyster samples (**Ch. 3 Fig. 2**). KmTxs were detected at Rappahannock, York, Lynnhaven, and Cherrystone (**Ch. 3 Fig. 5**, **Ch. 3 Table 1**). KmTx1-1 was found January through May, while KmTx1-3 was found once at each of the above sites in April. KmTx1-3 always co-occurred with KmTx1-1.

Pectenotoxin-2 (PTX2)

PTX2 was the most prevalent phycotoxin in oysters, occurring in 41% of samples (**Ch. 3 Fig. 2**), however it occurred in lower amounts than DA (**Ch. 3 Table 1**). PTX2 was detected from March to August and was found at all five sites, but was only found sporadically and in trace amounts at Rappahannock and Cherrystone (**Ch. 3 Fig. 6**). PTX2 was most prevalent at Lynnhaven and Wise Point, peaking at Wise Point in May 2020 at 6.16 µg/kg SM. OA detected in oysters always co-occurred with PTX2, while DTX1 did not.

Seafood safety phycotoxins

The majority of oyster samples (67%), contained at least one phycotoxin included in a phycotoxin group associated with seafood safety: AZAs, DA, DSTs and MCs. At least one of these phycotoxin groups was detected each sampling year (2019 & 2020) and at each sampling site. These phycotoxin groups co-occurred in 23% of oyster samples, and at least once at each site, but primarily at Lynnhaven (9 of 19 co-occurrences). The

majority of co-occurrences were between AZAs and DA, at York, Lynnhaven, Wise Point, and Cherrystone. Two oyster samples had co-occurrence of three of these phycotoxin groups, Rappahannock on June 25, 2020 had AZAs, DSTs, and MCs, and York on April 1, 2019 had AZAs, DA, and MCs.

Azaspiracids (AZAs)

AZA1 & 2, both associated with Azaspiracid Shellfish Poisoning (AZP), were detected in oysters in the lower Chesapeake Bay. AZA1 & 2 did not co-occur in oyster samples. Both AZAs were generally detected in concentrations at least an order of magnitude lower than other phycotoxins detected in this study (**Ch. 3 Fig. 8**, **Ch. 3 Table 1**). AZA1 was found at all five sites but was found most frequently, and in the highest amounts, at Lynnhaven, where it peaked in May 2019 at 0.80 µg/kg SM. AZA1 was detected from February to August, while AZA2 was detected from March to August and at Rappahannock, Lynnhaven, and Wise Point. AZA2 peaked at Wise Point in August 2020 at 0.42 µg/kg SM. AZA1 occurrence in oyster samples (23%) was almost double that of AZA2 (12%, **Ch. 3 Fig. 2**). The current study found AZAs in oysters and trace amounts of AZA1 in one SPATT at Rappahannock, a site where AZAs had not previously been detected (Onofrio et al. 2021). Lynnhaven exhibited prevalent AZA1 in oysters, this site was one of two sites where AZA1 was detected in SPATTs in a previous study (Onofrio et al. 2021); unlike this previous study, AZA1 was found at all sites sampled, and was not limited to southern tributaries. Additionally, Lynnhaven and Wise Point had been noted as sites with year-round AZA2 in SPATTs (Onofrio et al. 2021), and in the current study these sites supported AZA2 in oysters.

Domoic acid (DA)

DA, associated with Amnesic Shellfish Poisoning (ASP), was the most abundant phycotoxin detected in oysters in the lower Chesapeake Bay (**Ch. 3 Table 1**). DA was generally detected in concentrations at least an order of magnitude higher than other phycotoxins detected. DA was found at all sites except the northernmost, Rappahannock, and was more prevalent in the winter and spring (**Ch. 3 Fig. 9**). The highest amounts of DA were detected at Lynnhaven, peaking at 580 µg/kg SM or 0.58 mg/kg SM. This phycotoxin was detected from January to August, however, it was not detected at any site in June. DA occurred in 40% of oyster samples (**Ch. 3 Fig. 2**).

Diarrhetic Shellfish Toxins (DSTs)

OA and DTX1, both associated with Diarrhetic Shellfish Poisoning (DSP), were found in trace amounts in oysters in esterified forms (**Ch. 3 Table 1**). OA and DTX1 rarely occurred in oyster samples (2 and 1%, respectively, **Ch. 3 Fig. 2**) and did not co-occur. DSTs were detected in the late spring, once each at Rappahannock, Lynnhaven, and Wise Point. OA was detected twice, once at Lynnhaven in May 2020, and once at Wise Point in June 2020, while DTX1 was detected only once, at Rappahannock in June 2020 (**Ch. 3 Fig. 7**).

Microcystins (MCs)

MC-RR & -YR, both hepatotoxins, were detected in oysters in the lower Chesapeake Bay, while MC-LR was not. MC-RR and MC-YR did not co-occur in oyster samples. MCs were detected sporadically at Lynnhaven and Rappahannock, and once each at York and Wise Point. MC-RR was found sporadically in April through June at three sites, York, Lynnhaven, and Wise Point (**Ch. 3 Fig. 10**). There were three oyster

samples with quantifiable MC-RR, two at Lynnhaven on May 12 and 26, 2020 (5.25 and 7.12 µg/kg SM, respectively) and one at York on April 1, 2019 (7.06 µg/kg SM). MC-YR was found sporadically in March through August in trace amounts, primarily at Rappahannock, but also in one sample from Lynnhaven. MC-RR & -YR occurrence in oyster samples was low and comparable between congeners (6 and 7%, respectively, **Ch. 3 Fig. 2**). MC-RR was detected in only one POM sample from Cherrystone on March 18, 2019 (4.0 µg MC-RR/L), however, oyster samples from this site never exhibited quantifiable amounts of MCs.

Reference

Onofrio, M.D., Egerton, T.A., Reece, K.S., Pease, S.K.D., Sanderson, M.P., Jones III, W., Yeargan, E., Roach, A., DeMent, C., Wood, A., Reay, W.G., Place, A.R., Smith, J.L., 2021. Spatiotemporal distribution of phycotoxins within nearshore waters of the Chesapeake Bay and Virginia coastal bays. Harmful Algae 103, 101993. doi:10.1016/j.hal.2021.101993.

APPENDIX V

Phycotoxin Percent Response from Oyster Matrix

Goal

Shellfish extractions to detect and/or quantify phycotoxins are inherently "dirty", i.e., the extraction contains other compounds that may interfere with the detection of phycotoxins (MacKenzie et al. 2004). The purpose of this analysis was to determine the enhancement or suppression of phycotoxins of interest in oyster (*C. virginica*) matrix, to better understand whether phycotoxin concentrations were over- or under-estimated in the study. Phycotoxin concentrations with signal enhancement due to matrix interference will result in artificially inflated phycotoxin concentration values compared to methanolic (MeOH) controls, while signal suppression due to matrix interference will result in artificially low phycotoxin concentration values compared to MeOH controls.

Methods

Percent response, i.e., enhancement or suppression of analytes during chemical analysis via LC-MS/MS with trap/ACD, was determined for 11 phycotoxins in extracted, pooled oyster matrix (AZA1, AZA2, DA, DTX1, GDA, MC-LR, MC-RR, MC-YR, OA, PTX2, YTX). KmTxs were excluded due to a limited amount of available purified material. Triplicate oyster matrices and triplicate 90% MeOH controls were spiked to a final concentration of 5 µg/L with each phycotoxin, or 0.2 µg/L for AZAs. Spiked samples were analyzed as described in **Ch. 3 Section 2.3** by UPLC-MS/MS with trap/ACD to calculate percent response:

$$\%\ Response\ = \frac{peak\ area\ of\ phycotoxin\ in\ oyster\ matrix}{peak\ area\ of\ phycotoxin\ in\ 90\%\ MeOH} \times 100$$

Results

Ion enhancement and suppression of the 11 analytes was examined in oyster extracts by comparing spiked matrix to 90% methanolic controls. Overall, percent

responses were above 100% in oyster matrix relative to methanolic controls for all tested phycotoxins, indicating ion enhancement (102 – 179%, **Table 1**). The exception was OA which demonstrated slight suppression (92% response). Signal enhancement potentially led to overestimation of the amounts of these phycotoxins in oysters. The phycotoxins with the greatest signal enhancement, MC-LR (227%) and YTX (189%) were not detected in oysters in this study. Further discussion of signal enhancement for these phycotoxins can be found in **Ch. 3 Section 3**.

Reference

MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P., Selwood, A., 2004. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that stimulates the biotoxin contamination of filter feeding bivalves. Toxicon 44(8), 901-918. doi:10.1016/j.toxicon.2004.08.020.

Table 1
Percent response of 11 phycotoxins in oyster matrix. The average percent response of triplicate samples is reported with standard deviation (SD).

Phycotoxin	Oyster Matrix % Response (SD)
AZA1	144 (3)
AZA2	135 (11)
DA	147 (4)
DTX1	102 (7)
GDA	125 (31)
MC-LR	227 (41)
MC-RR	174 (20)
MC-YR	179 (34)
OA	92 (5)
PTX2	119 (23)
YTX	189 (13)

AZA1 = azaspiracid-1, AZA2 = azaspiracid-2, DA = domoic acid, DTX1 = dinophysistoxin-1, GDA = goniodomin A, MC-LR = microcystin-LR, MC-RR = microcystin-RR, MC-YR = microcystin-YR, OA = okadaic acid, PTX2 = pectenotoxin-2, YTX = yessotoxin