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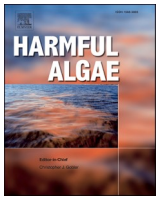

Spatiotemporal distribution of phycotoxins and their co-occurrence within nearshore waters

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ABSTRACT

Harmful algal blooms (HABs), varying in intensity and causative species, have historically occurred throughout the Chesapeake Bay, U.S.; however, phycotoxin data are sparse. The spatiotemporal distribution of phycotoxins was investigated using solid-phase adsorption toxin tracking (SPATT) across 12 shallow, nearshore sites within the lower Chesapeake Bay and Virginia's coastal bays over one year (2017-2018). Eight toxins, azaspiracid-1 (AZA1), azaspiracid-2 (AZA2), microcystin-LR (MC-LR), domoic acid (DA), okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxin-2 (PTX2), and goniodomin A (GDA) were detected in SPATT extracts. Temporally, phycotoxins were always present in the region, with at least one phycotoxin group (i.e., consisting of OA and DTX1) detected at every time point. Co-occurrence of phycotoxins was also common; two or more toxin groups were observed in 76% of the samples analyzed. Toxin maximums: 0.03 ng AZA2/g resin/day, 0.25 ng DA/g resin/day, 15 ng DTX1/g resin/day, 61 ng OA/g resin/day, 72 ng PTX2/g resin/day, and 102,050 ng GDA/g resin/day were seasonal, with peaks occurring in summer and fall. Spatially, the southern tributary and coastal bay regions harbored the highest amount of total phycotoxins on SPATT over the year, and the former contained the greatest diversity of phycotoxins. The novel detection of AZAs in the region, before a causative species has been identified, supports the use of SPATT as an explorative tool in respect to emerging threats. The lack of karlotoxin in SPATT extracts, but detection of *Karlodinium veneficum* by microscopy, however, emphasizes that this tool should be considered complementary to, but not a replacement for, more traditional HAB management and monitoring methods.

1. Introduction

Chesapeake Bay is the largest estuary in the United States, spanning 11,600 km^2 with a watershed that extends across 6 states from New York to Virginia. The Chesapeake Bay and the coastal bays along Virginia's Eastern Shore are highly productive, supporting many commercial and recreational fisheries, as well as an extensive aquaculture industry (Hudson 2018). Threats to these valuable estuarine and coastal waters are numerous and include issues like eutrophication (Kemp et al. 2005), seasonal hypoxia (Hagy et al. 2004), organic pollutants (Baker et al. 1994), the presence of harmful algae (Glibert et al. 2014), overfishing and habitat loss (Wilberg et al. 2011), and consequences of sea level rise (Eggleston and Pope 2013). At least 37 species of harmful algae have been documented and are known to co-occur across both space and time in the lower Chesapeake Bay (Marshall et al. 2009). Included in this list are both non-toxic species whose high biomass blooms can elicit negative effects, and toxic species that may have detrimental impacts on the ecosystem or human health through phycotoxin production.

Within this group of toxigenic HABs in the Chesapeake Bay are *Dinophysis* spp. and *Prorocentrum lima*, potential producers of okadaic

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acid and dinophysistoxins associated with the human health syndrome, diarrhetic shellfish poisoning (DSP) (Marshall et al. 2005, 2009). Pectenotoxins are also produced by *Dinophysis* spp. and are regulated globally in association with DSP (EFSA 2009); pectenotoxins, however, are not classified as DSP toxins in this work as they are unregulated in the U.S. Potential producers of yessotoxin, *Gonyaulax* spp., have been reported in Chesapeake Bay (Marshall et al. 2005, Rhodes et al. 2006). Toxigenic diatoms, *Pseudo-nitzschia* spp. have also been documented in the Bay (Marshall et al. 2009), along with the production of domoic acid associated with amnesic shellfish poisoning (ASP; Thessen and Stoecker 2008). Similarly, the Chesapeake Bay and its tributaries harbor *Karlodinium veneficum, Alexandrium monilatum*, and *Microcystis* spp., all of which produce phycotoxins, i.e., karlotoxins, goniodomins, or microcystins, respectively, with implications for animal health (Marshall et al. 2005, Deeds et al. 2006; Marshall and Egerton 2009, May et al. 2010; Amado and Monserrat 2010; Bukaveckas et al. 2018, Wolny et al. 2020b). To date, there have been no reported human health illnesses attributed to phycotoxin exposure in Chesapeake Bay or the coastal bays. The only precautionary closure in this area occurred in 2002 due to the presence of *Dinophysis*, but phycotoxin levels in water and shellfish meat samples were below the regulatory limit (Tango et al. 2002).

Long-term phytoplankton abundance data have been collected since 1984 through the Chesapeake Bay Monitoring Program, with 14 long-term stations located throughout the lower Bay and its tidal tributaries. Additionally, the Virginia Department of Health (VDH), Virginia Institute of Marine Science (VIMS) and Old Dominion University monitor for the presence of potentially toxic and harmful algal bloom-forming species throughout the shellfish growing areas at over 60 stations on a monthly basis. Phycotoxin data, however, are sparse, leaving resource managers, health officials, and researchers without the necessary knowledge regarding the toxicity of local strains needed to prepare region-specific biotoxin contingency plans. Although there is no immediate cause for concern in the Bay, baseline knowledge of the current state of phycotoxin distribution in this region is important, especially considering the potential for phytoplankton assemblages to shift under changing environmental conditions (Wells et al. 2015). In addition, there has been a recent emergence of phycotoxins in the U.S. that can be associated with the human syndromes DSP (Campbell et al. 2010; Hattenrath-Lehmann et al. 2013; Trainer et al. 2013) and azaspiracid shellfish poisoning (AZP; Trainer et al. 2013; Kim et al. 2017). Along with the threats to human and animal health, an increased presence of HABs and phycotoxins would economically impact the expansive aquaculture industry in the Chesapeake Bay region. Understanding the current distribution of phycotoxins will provide necessary information for future research, monitoring, and mitigation.

To conduct a comprehensive screening of multiple phycotoxins, known or possibly emerging in the Chesapeake Bay and the Virginia coastal bays, the passive sampling technique solid-phase adsorption toxin tracking (SPATT) was employed. Since its introduction (MacKenzie et al. 2004), SPATT has been used in field studies to investigate a wide range of phycotoxin classes, or groups, ranging in polarity and size: DSP toxins, pectenotoxins (PTXs), azaspiracids (AZAs), cyclic imines, ciguatoxins, domoic acid (DA), paralytic shellfish poisoning toxins, microcystins, nodularins, anatoxin, or maitotoxins (Roué et al. 2018). As a semi-integrative, passive sampling technique, SPATT is useful for the detection of multiple toxins that may be present in low concentrations in the natural environment.

The goal of this study was to explore relative spatial and temporal trends of phycotoxins throughout lower Chesapeake Bay and Virginia coastal bays, at 12 near-shore sites, over the course of a 1-year field study using SPATT. Harmful algal cell presence was also monitored throughout the study using microscopy. The 14 marine and freshwater phycotoxins included in SPATT analyses were domoic acid (DA), pectenotoxin-2 (PTX2), okadaic acid (OA), dinophysistoxin 1 (DTX1), dinophysistoxin 2 (DTX2), yessotoxin (YTX), microcystin-RR (MC-RR), microcystin-YR (MC-YR), microcystin-LR (MC-LR), karlotoxin 1 (KmTx 1), karlotoxin 3 (KmTx 3), goniodomin A (GDA), azaspiracid-1 (AZA1), and azaspiracid-2 (AZA2). The latter two phycotoxins were investigated despite no record of causative organisms in the region as an example for how a phycotoxin-approach could identify emerging threats unnoticed by traditional light microscopy methods.

2. Material and methods

2.1. Field study

A field study was conducted for one year, between May 2017 and June 2018, within nearshore waters throughout the lower Chesapeake Bay and the Virginia coastal bays. Site selection and sampling were collaborative efforts by personnel from VIMS and the VDH Division of Shellfish Safety and Waterborne Hazards. Sampling was generally performed twice monthly, with more frequent sampling during periods of excessive biofouling in summer, and less frequent sampling due to inclement weather in winter. One SPATT was deployed 1 m from the bottom at each site and replaced with a new SPATT during each sampling event. Complementary surface water samples, 100-mL, were collected for microscopic analyses of phytoplankton cells.

Twelve sites were selected for their geographical distribution and their relevance to shellfish growing areas (Fig. 1). The sites were divided into four regions based upon their watershed delineation (Fig. 1) and site characteristics (Table 1): the northern tributaries (sites 1, 2, and 3), the southern tributaries (sites 4, 5, and 6), the bayside Eastern Shore (sites 7, 8, and 9), and the coastal bays (sites 10, 11, and 12). Each site was located nearshore, in shallow waters ($\leq$ 2 m in depth), and was accessible by dock. Given that this study represented a broad spatial-scale survey over a 14-month period, it was important to put this study in the context of streamflow patterns that influence water quality and circulation within the Chesapeake Bay. Monthly Bay streamflow estimates, derived by using empirical relation curves that correlated streamflow at reference gauges (USGS 2020), over the study period (with antecedent months) and a longer-term thirty-year (1989-2018) climate interval are presented in Fig. 2. Bay-wide streamflow over the study period followed long-term seasonal discharge patterns of elevated discharge in late winter-early spring, followed by a recession from late spring through early fall driven by elevated evapotranspiration, and recovery beginning in late fall. In addition to seasonal patterns, monthly discharge rates fell within or were close to normal levels, as defined by the 1st and 3rd quartiles. Over the study period, primary mid to lower Bay tributaries exhibited similar patterns of streamflow with a notable exception of above normal rainfall in the upper watershed regions of the Rappahannock and James Rivers in May 2017 resulting in elevated streamflow for that month.

2.2. SPATT preparation and extraction

For this study, SPATTs were constructed with Diaion® HP-20, a commonly-used resin that has been applied to numerous phycotoxins ranging in polarity and size (Lane et al. 2010, Kudela 2011, McCarthy et al. 2014, Roué et al. 2018). SPATTs were prepared (Fux et al. 2008) and stored in containers of ultrapure water in the refrigerator for no longer than 4 weeks before use. After field deployment, SPATTs were stored frozen (-20°C) until phycotoxin extraction.

In preparation for bulk extraction of toxins, SPATTs were thawed and residual salts were removed by rinsing with ultrapure water. Resin was collected in a removable PVDF 0.45-μm spin filter cup (Thermo Fisher Scientific, Waltham, MA, USA), and placed within a capped, 50-mL centrifuge tube. Three sequential extractions were performed, using 1) 10 mL 35% methanol, 2) 10 mL 100% methanol, and 3) 10 mL 100% methanol with centrifugation at 1500 rcf for 15 minutes, 10°C (Onofrio 2020). The 35% methanol extract was stored separately, while the two 100% methanol extracts were pooled into one 20-mL extract. All extracts were stored at -20°C until toxin analysis.

Fig. 1. Map of 12 field sampling sites (black circles) in the lower Chesapeake Bay and Virginia coastal bays. Shading represents the watersheds associated with the four study regions: northern tributaries (light gray, sites 1, 2, and 3), southern tributaries (vertical lines, sites 4, 5, and 6), bayside Eastern Shore (dark gray, sites 7, 8 and 9), and coastal bays (horizontal lines, sites 10, 11, and 12).

2.3. Percent recovery from SPATT

Recovery efficiency was determined for the bulk extraction of phycotoxins from SPATT resin. Fresh SPATT discs were incubated for 24 hr in glass vials containing 12 phycotoxins, each at a final concentration 2.67 μg/L, in 0.2-µm filtered seawater, $S = 20$, from the York River, Chesapeake Bay, VA. Two phycotoxins were not included in this recovery experiment; DA recovery was described previously using a similar extraction sequence (Lane et al. 2010), and KmTx 1 was excluded due to a limited amount of available purified material. To quantify "toxin remaining in vial" after the 24 hr incubation, seawater was subjected to clean-up via solid-phase extraction (SPE) using 3-cc Oasis HLB 60 mg cartridges (Waters, Milford, MA, USA; Smith et al. 2018). Methanolic extracts from SPATT and seawater were analyzed by 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), to calculate percent recovery:

$$Recovery\ (\%) \frac{g\ toxin\ recovered\ off\ SPATT}{g\ toxin\ added\ to\ vial - g\ toxin\ remaining\ in\ vial} \times\ 100\%.$$

2.4. Toxin analysis

The 35% methanol SPATT extracts were analyzed for DA, in duplicate, at VDH using Domoic Acid (ASP) ELISA kits (Abraxis Inc., Warminster, PA, USA) and an Abraxis plate reader following the manufacturer's protocol, ON0021. Extracts were subject to a 1:2 dilution using the sample dilution buffer provided within the kit to achieve compatibility with the assay, i.e., reducing methanol to 17.5%. Samples that were positive for domoic acid upon first analysis, n=24, were concentrated using an Integrated SpeedVac® System (Thermo Fisher Scientific Inc., Waltham, MA, USA), reconstituted in ultrapure water, and analyzed again by ELISA for confirmation and quantitation. Domoic acid was then confirmed and quantified in 23 of these positive extracts.

The 100% methanol SPATT pooled extracts were analyzed for the 13 remaining phycotoxins at VIMS using UPLC-MS/MS with trap/ACD (Onofrio et al. 2020). Parent > daughter transitions, as listed in Onofrio et al. (2020), were used for quantification, with the addition of transitions for KmTx 1: m/z 1361.7 > 1361.7, 70V, 2eV and 1361.7 > 937.7, 70V, 80eV (Bachvaroff et al. 2008); OA and DTX2: m/z 803.5 > 113.0, 80V, 60eV; and AZA2: m/z 856.4 > 820.3, 40V, 40eV. The injection volume for each sample was 100 μL, and standard curves were prepared in 100% methanol using a series of 9 dilutions between 0.1 and 50 μg/L. Limits of detection (LOD) were between 0.01 and 0.25 μg/L for all compounds, with the exception of KmTx 3 at 0.64 μg/L (Onofrio et al. 2020). All samples with detectable AZA2 were rerun with an injection volume of 200 μL, using a 9-point standard curve between 0.003 and 2 μg AZA2/L. During all analyses, blank injections of 100% methanol and injections of check standards, 5 μg/L for each toxin, were run after each set of 15 SPATT extracts to confirm that carryover was not occurring and that retention times remained consistent, respectively. All SPATT toxin data was normalized to ng toxin/g resin/day; concentrations less than

Table 1
Study site characterization. Salinity regime: mesohaline (S = 5-18), polyhaline (S = 18-30); study period mean value during time periods of SPATT deployment/retrieval; % seawater: % freshwater based on end member mixing of freshwater (S = 0) and adjacent oceanic waters off the bay mouth (S = 32, Austin 2002). Relative flushing rate as reported by Herman et al. (2007); open tidal river and strait associated stations were assigned quick rates. Chl a eutrophic index: low (0- <5 μg/L), medium (5-20 μg/L), and high (>20 μg/L); based on typical high concentration, in an annual cycle, determined as the 90th percentile (Bricker et al., 2007); average of 3-year interval (2016-2018; exception Station 6, Lynnhaven 1 year); data sets for extracted Chl a levels provided in footnotes.

Station ID & Waterbody	Geomorphic Setting	Salinity Regime Station Average (%Sea:%FW)	Relative Flushing Rate	Chl a Eutrophic Index (90% μg/L)
1 Great Wicomico River	Tidal River	Mesohaline 14.2 (44:56)	Slow	Medium (12.1[2])
2 Locklies Creek, Rappahannock	Tidal Creek	Mesohaline 14.5 (45:55)	Intermediate	Medium (15.5)
3 Gwynn's Island	Strait	Mesohaline 15.5 (48:52)	Quick	Medium (12.3[2])
4 York River	Tidal River	Polyhaline 20.1 (63:37)	Quick	Medium (15.5[3])
5 James River	Tidal River	Mesohaline 17.0 (53:47)	Quick	Medium (15.4[2])
6 Lynnhaven Inlet	Tidal Inlet	Polyhaline 21.6 (67:33)	Intermediate	Medium (15.0[1])
7 Onancock Creek	Tidal Creek	Mesohaline 17.1 (54:46	Intermediate	Medium (14.8[1])
8 Nassawadox Creek	Tidal Inlet	Polyhaline 18.3 (57:43)	Intermediate	Medium (17.0[1])
9 Cherrystone Inlet	Tidal Inlet	Polyhaline 21.9 (69:31)	Intermediate	Medium (12.6[1])
10 Wise Point	Strait	Polyhaline 28.3 (89:11)	Quick	Low (4.9)
11 Oyster Harbor	Embayment	Polyhaline 28.4 (89:11)	-	Medium (13.1[4])
12 Wachapreague Channel	Tidal Creek	Polyhaline 29.2 (91:9)	Intermediate	-

Data sources:
(1) CBP/VDEQ Shallow Water Monitoring Program (Stations: NSS001.78, OCN002.78, CRS001.80; Sampling interval: monthly).
(2) CBP Tidal Water Quality Monitoring (Stations: CB5.4W, LE 3.7, LE 5.3; Sampling interval: monthly).
(3) NOAA/NERRS Central Data Management Office (Station: York River Bridge; sampling interval: monthly).
(4) UVA Virginia Coast Reserve LTER (Station: Oyster Harbor; sampling interval: seasonal).

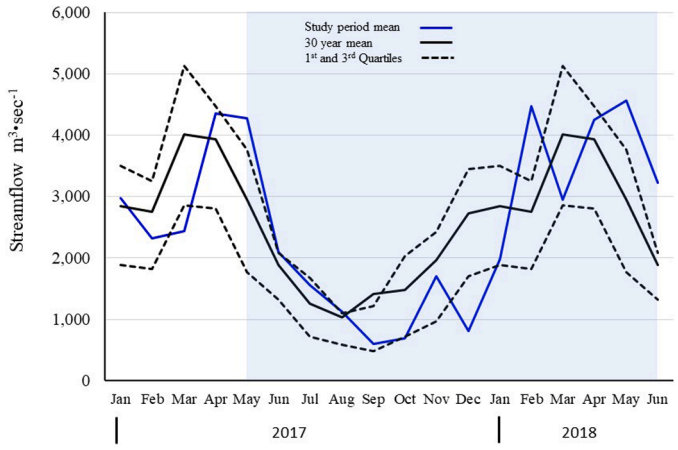

Fig. 2. Study period (blue solid line) and long-term climate interval (1989-2018; black solid line) monthly Bay streamflow estimates and normal flow range defined by 1st and 3rd quartile ranges (dashed lines). Study period is shaded. Data source: USGS (2020).

the limit of quantification are represented as ½ LOD, and non-detects are represented as 0. Toxin results are also presented as the percentage of samples that tested positive within the 321 extracts evaluated over all sites and time points.

Standards for the percent recovery experiment were purchased from the National Research Council Canada: CRM-AZA1-b, CRM-AZA2-b, CRM-DTX1-b, CRM-DTX2-b, CRM-OA-d, CRM-PTX2-b, CRM-YTX-c. A microcystin-RR, -YR, -LR mixed solution was purchased from Sigma Aldrich (33578-1ML). Karlotoxin 1 (KmTx 1) and karlotoxin 3 (KmTx 3) were purified from *Karlodinium veneficum* and provided by Dr. Allen Place (UMCES, Maryland). Goniodomin A was purified from *Alexandrium monilatum* by Drs. Thomas and Constance Harris (Harris et al. 2020).

Alkaline hydrolysis was used to convert DSP toxin derivatives into the parent toxins OA and DTX1 following the methods of Villar-Gonzalez et al. (2008). Due to the considerable number of samples, 321, alkaline hydrolysis was performed on select SPATT extracts. Extracts were selected from a site within the Chesapeake Bay (site 4) and the coastal bays (site 12) across all four seasons: July 2017, October 2017, January 2018, and April 2018. The samples were analyzed by UPLC-MS/MS with trap/ACD as written above, with a 100-μL injection volume.

2.5. Microscopic analyses

Surface water samples were analyzed for harmful algal species. Algal cells were enumerated using a 1-mL Sedgewick Rafter counting chamber and light microscopy at 100x (Olympus 1 × 51 with Olympus DP73 digital camera and cellSens Standard software, Center Valley, PA, USA). Larger volumes of water, 4 – 25 mL, were qualitatively evaluated for less abundant genera (i.e. *Dinophysis* and *Pseudo-nitzschia* spp.) and data were represented as presence or absence. Live samples were used for initial observation and identification, e.g., based on swimming pattern, while samples preserved with Lugol's solution (Carolina Biological Supply Company, Burlington, NC, USA) were enumerated. The lower detection limit for quantitative analysis was 1 cell/mL.

3. Results

3.1. Percent recovery from SPATT

The bulk SPATT extraction method was successful for the recovery of multiple phycotoxins from Diaion® HP-20 resin, resulting in percent

recoveries >87% in 100% methanol for all but PTX2 (Table 2). The low percent recovery for PTX2 indicates that the reported amounts of PTX2 in SPATT extracts are likely artificially low using this extraction method. Recoveries higher than 100% suggest signal enhancement due to matrix effects, potentially leading to an overestimation of the amounts of these toxins. This extraction method was, however, deemed sufficient given the screening application for which it was to be used. Future studies focused more heavily on PTX2, MC-RR, or GDA should consider optimizing the extraction method to improve extraction efficiency and/or further reduce matrix effects.

3.2. DSP toxins and pectenotoxins

The DSP toxins OA and DTX1 were detected in all samples (100%), i.e., at every time point from all 12 sites, and PTX2 was detected in all but one of these samples. Dinophysistoxin-2, another phycotoxin associated with global DSP, was not detected in any of the field samples. Instrumental blanks included during toxin analyses were consistently negative for DSP toxins and PTX2, indicating that the observed persistent presence was not due to carryover between samples. OA was always found in greater relative quantities than DTX1 in SPATT extracts. The coastal bays region showed higher relative amounts of DSP toxins and PTX2 compared to the sites within Chesapeake Bay (Figs. 3, 4, 5). The highest recorded amount of DSP toxins and PTX2 on SPATTs occurred on July 31, 2017 at the coastal bay site #10 (61 ng OA/g resin/day, 15 ng DTX1/g resin/day, and 72 ng PTX2/g resin/day). The maximum composite DSP toxin load on SPATTs, i.e., OA + DTX1, was 76 ng DST/g/resin/day.

Fine-scale temporal variations in all three toxins were observed at the site level; however, a general trend was apparent: maximum phycotoxin loads on SPATT were detected at all sites between summer and fall (Figs. 3, 4, 5). If evaluating OA, DTX1, and PTX2 together at representative sites, the coastal bays region peaked in these phycotoxins more than two months earlier than the Chesapeake Bay (Fig. 6). In addition to timing, toxin profiles varied between the Bay and coastal bays; OA dominated the toxin profile during the phycotoxin peak within the Bay (Fig. 6A), while in the coastal bays, PTX2 was found in relatively equal amounts to OA (Fig. 6B).

Overall, free OA and DTX1, i.e., parent structures, were more abundant in SPATT extracts than esterified forms (Fig. 7). Esterified OA was more abundant in the Chesapeake Bay than coastal bays region in every season except for summer (Fig. 7A). A similar trend was apparent for DTX1, where in both the Chesapeake Bay and the coastal bays, esterified DTX1 was more abundant in winter and spring than in summer and fall (Fig. 7B).

3.3. Goniodomin A

Goniodomin A was detected in SPATT extracts from all four regions,

Table 2
Percent recovery of 12 phycotoxins extracted in 100% methanol from Diaion® HP-20 SPATT resin using the bulk extraction method. The average percent recovery +/- standard deviation of triplicate samples is reported.

Toxin	Percent Recovery +/- standard deviation
MC-RR	156 +/- 15
MC-LR	99 +/- 9
MC-YR	99 +/- 5
AZA1	90 +/- 2
AZA2	118 +/- 9
KmTx 3	90 +/- 11
GDA	152 +/- 29
PTX2	15 +/- 8
YTX	90 +/- 9
OA	100 +/- 2
DTX2	87 +/- 3
DTX1	88 +/- 5

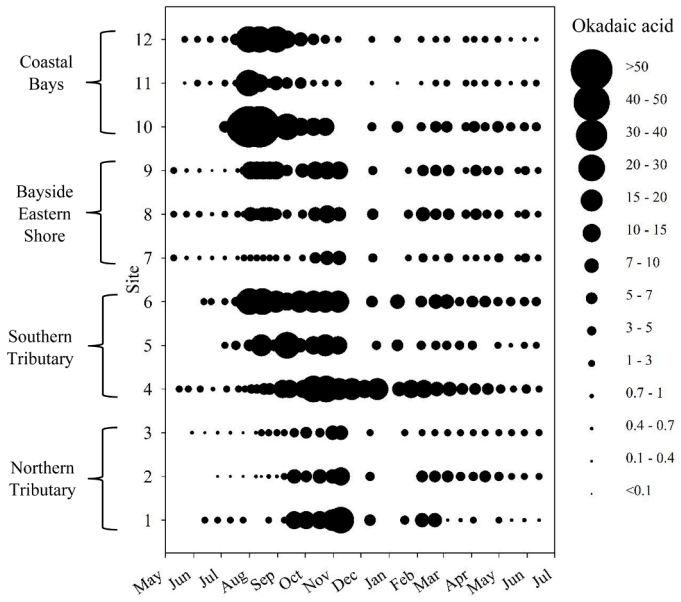

Fig. 3. SPATT toxin data (ng OA/g resin/day) for okadaic acid (OA) across 12 sites within the lower Chesapeake Bay and the coastal bays from May 2017 – June 2018.

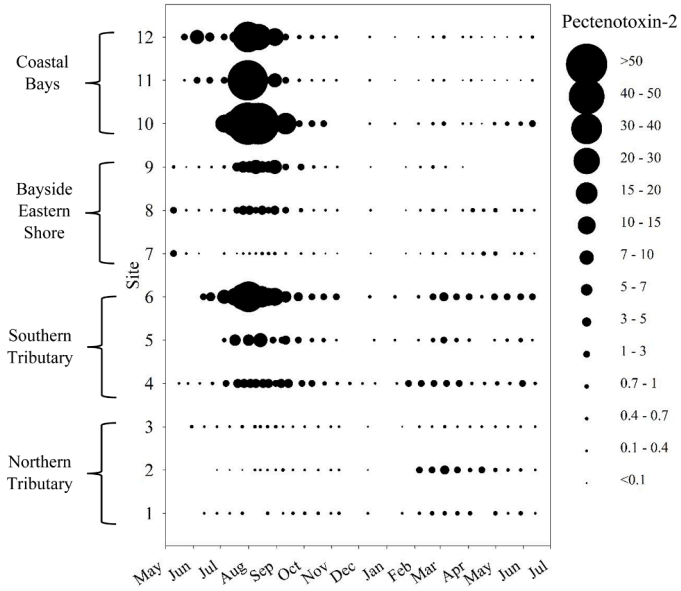

Fig. 4. SPATT toxin data (ng PTX2/g resin/day) for pectenotoxin-2 (PTX2) across 12 sites within the lower Chesapeake Bay and the coastal bays from May 2017 – June 2018.

and at all sites sampled except two of the coastal bay sites: 11 and 12 (Fig. 8). Overall, GDA was detected in 50% of the samples collected. The phycotoxin was most prevalent within the southern tributary region; 94% of SPATT extracts from this region were positive for GDA. Seasonally, GDA amounts in SPATT extracts peaked during the warmer months of late summer and early fall, in all regions. The highest recorded amount at 102,050 ng GDA/g resin/day was from a SPATT collected from the southern tributary region during late summer (site 4; Fig. 8). Goniodomin A was also prevalent in the other Chesapeake Bay regions, with 65% of the SPATT extracts from the northern tributary region and 38% from the bayside Eastern Shore region testing positive. A seasonal period of interruption was observed during the colder months in these two regions, i.e., an absence of GDA in winter and early spring. In

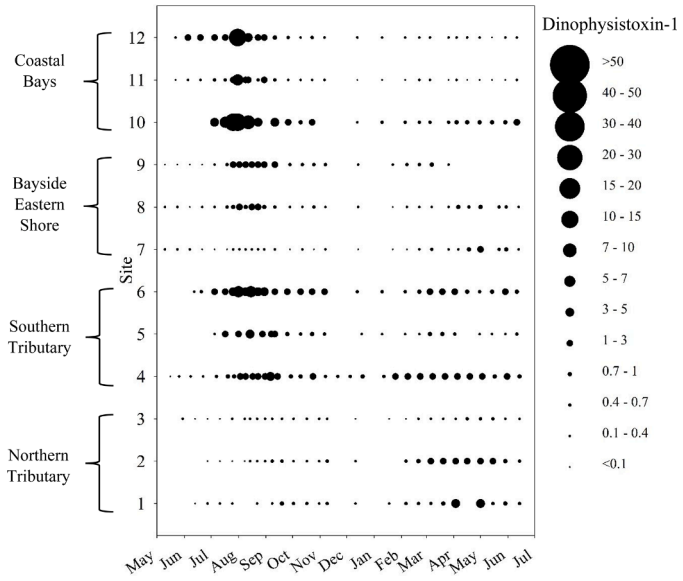

Fig. 5. SPATT toxin data (ng DTX1/g resin/day) for dinophysistoxin-1 (DTX1) across 12 sites within the lower Chesapeake Bay and the coastal bays from May 2017 – June 2018.

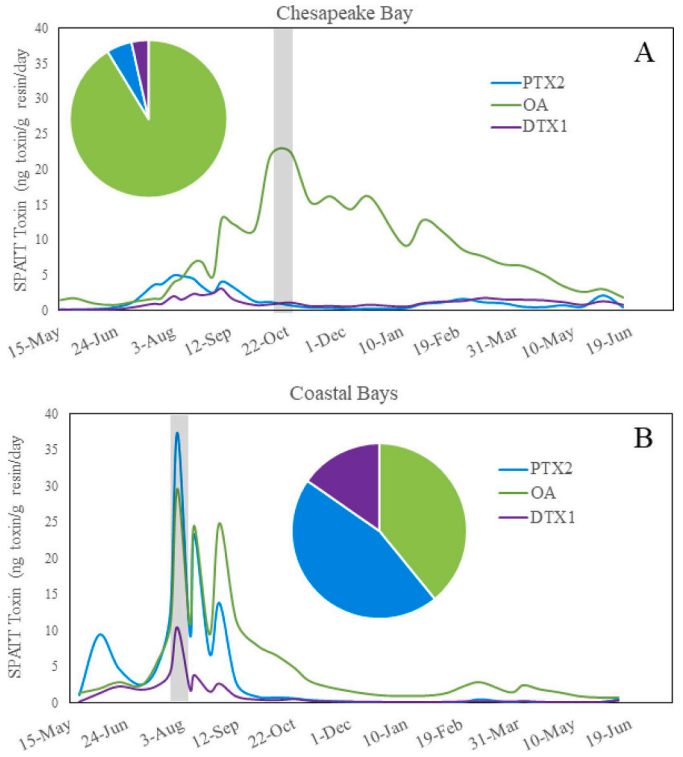

Fig. 6. Toxin profiles of OA, DTX1, and PTX2 in SPATT extracts from representative sites **(A)** within the Chesapeake Bay (site 4) and **(B)** the coastal bays (site 12). Pie charts present the toxin profile in SPATT extract corresponding with the time when total toxin amounts peaked in each area, represented by the gray shading.

contrast, GDA was detectable year-round in SPATT extracts from the southern tributary region, with elevated levels notable as early as May. Site 10, near the Bay mouth, was the only site in the coastal bay region to contain GDA on SPATT and had a lower percentage of SPATT extracts positive for GDA, 22%, as compared to the three other Chesapeake Bay regions.

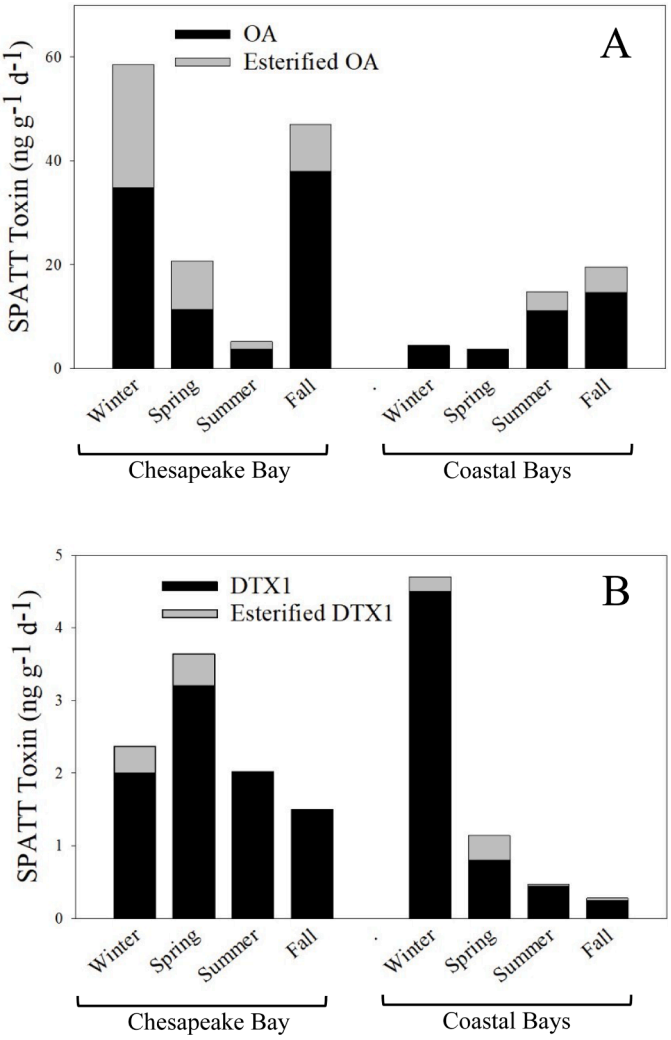

Fig. 7. Composition of total (black) and esterified forms (gray) of **(A)** OA and **(B)** DTX1 present in representative SPATT extracts from within the Chesapeake Bay (site 4), and the coastal bays (site 12). One SPATT extract per season from winter (January), spring (April), summer (July), and fall (October) was chosen for alkaline hydrolysis from each site.

3.4. Azaspiracids

Azaspiracid-2 was detected in SPATT extracts in every region, but the amounts of AZA2 were always 2-3 orders of magnitude lower than other phycotoxins detected in this study (Fig. 9, Table 3). Azaspiracid-2 was present in summer, fall, and winter, but generally absent in spring, except for two sites near the mouth of the Chesapeake Bay (sites 6 and 10) where AZA2 was observed during every season. Two of the southern tributary sites (sites 4 and 6) and one coastal bay site (site 10) had the highest relative amounts of AZA2 compared to all sites. Seasonally, AZA2 peaked during the fall across the three Chesapeake Bay regions, with the highest recorded amounts of 0.043 ng AZA2/g resin/day from a SPATT collected in the southern tributary region (site 4). For the coastal bay site 10, however, toxin amounts peaked earlier, at 0.030 ng AZA2/g resin/day from the SPATT collected in summer.

A second azaspiracid, AZA1, was much less prevalent and abundant in the Chesapeake Bay and coastal bays when compared to AZA2. AZA1 was only detected in SPATT extracts from one region: the southern tributary region (sites 4 and 6, Table 3) in fall and winter (September 11, 2017 – February 5, 2018). Azaspiracid-1 occurrence in SPATT extracts

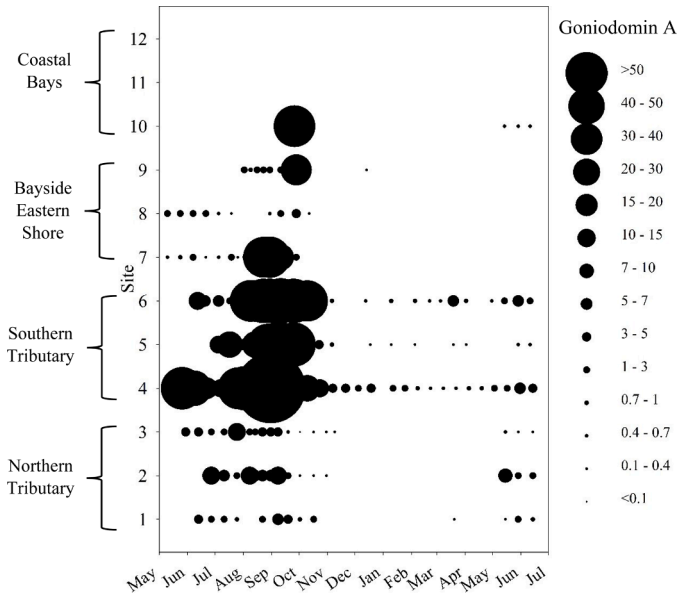

Fig. 8. SPATT toxin data (ng GDA/g resin/day) for goniodomin A (GDA) across 12 sites within the lower Chesapeake Bay and the coastal bays from May 2017 – June 2018.

was rare (4%) compared to AZA2 (55%). All extracts positive for AZA1 were also positive for AZA2, showing co-occurrence. Only trace, non-quantifiable, amounts of AZA1, were detected in extracts.

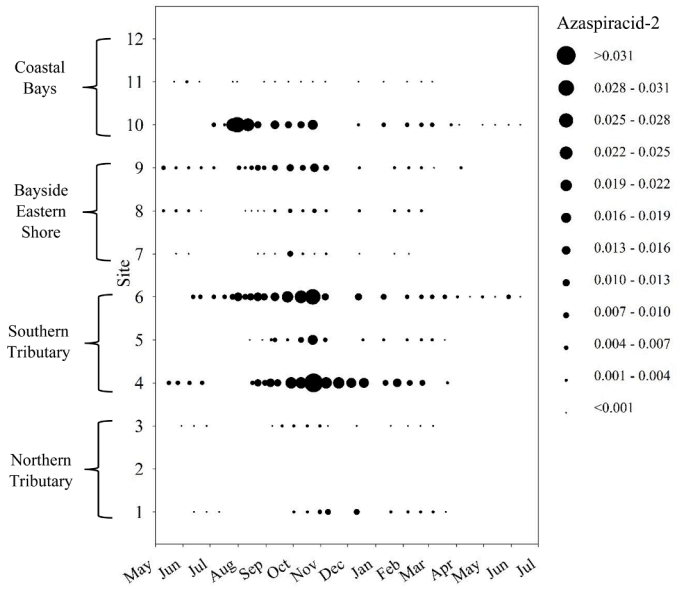

Fig. 9. SPATT toxin data (ng AZA2/g resin/day) for azaspiracid-2 (AZA2) across 12 sites within the lower Chesapeake Bay and the coastal bays from May 2017 – June 2018.

3.5. Domoic acid

Domoic acid was distributed across the Bay and coastal bays, with detection in all four regions, but amounts of DA on SPATT were relatively low when compared to OA, DTX1, PTX2, and GDA (Table 3). Temporally, DA was sparse, only 7% of the 321 extracts analyzed were positive for DA by ELISA. These positive detects came from 7 of the 12 sites, at various times throughout the year (Fig. 10). With most sites having a limited number of extracts that tested positive for DA with no obvious temporal pattern (Fig. 10), seasonal distribution of DA will not be discussed. The highest amounts of DA were seen in the coastal bays at site 12 (0.25 ng DA/g resin/day), and in the southern tributary region at site 4 (0.22 ng DA/g resin/day). These amounts, however, were only slightly elevated compared to the rest of the positive samples; concentrations of positive samples ranged from 0.05 to 0.25 ng DA/g resin/day with a mean of 0.13 ng DA/g resin/day.

3.6. Microcystins

The freshwater phycotoxin, MC-LR, was only detected in one region, the southern tributary region (August 26 - September 11, 2017) at sites 5 and 6. Overall only 3% of the total extracts analyzed across all sites and time points contained MC-LR. The two other microcystins evaluated, MC-RR and MC-YR, were never detected in field SPATT extracts during this study. The presence of MC-LR was, therefore, not distributed throughout the Chesapeake Bay and coastal bays in space or time. When detected, MC-LR was below the limit of quantification of the method, but above the limit of detection (LOD; Onofrio et al. 2020); this phycotoxin is, therefore, reported as presence/absence data. The

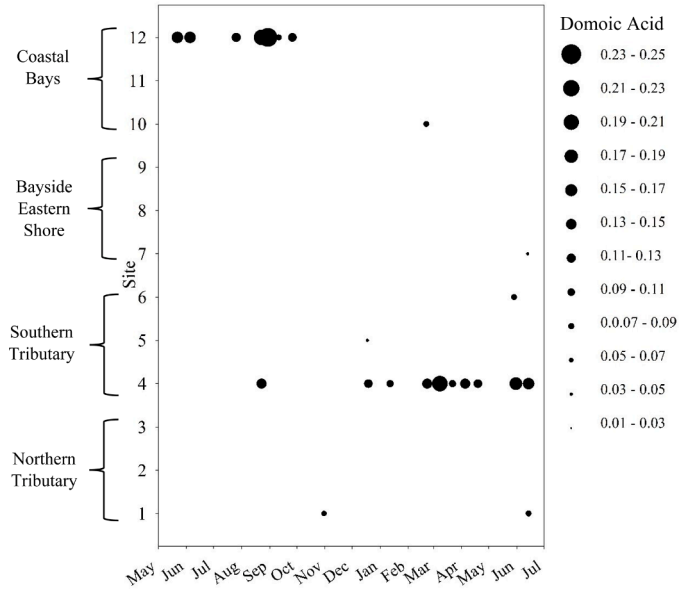

Fig. 10. SPATT toxin data (ng DA/g resin/day) for domoic acid (DA) across 12 sites within the lower Chesapeake Bay and the coastal bays from May 2017 – June 2018.

Table 3
Spatial distribution of eight phycotoxins and HAB cells over four regions of the Chesapeake Bay and coastal bays. Amounts of toxin on SPATT were summed over all time points within each site, three sites were averaged per region, and the results presented as annual cumulative toxins on SPATT in each region.

Regions	Annual cumulative toxins on SPATT (ng/g resin/d)						Sum of all toxins	Presence (+)/Absence (-) of toxins or cells		
	OA	DTX1	PTX2	GDA	AZA2	DA		AZA1	MC-LR	HAB cells observed
N. Tributary	93.66	10.96	11.83	48.70	0.02	0.07	165.24	-	-	*K. veneficum; Dinophysis* spp.
S. Tributary	221.54	36.68	89.99	15229.08	0.19	0.63	15578.11	+	+	*A. monilatum; K. veneficum; Dinophysis* spp.;
Bayside ES	124.67	15.34	30.69	81.70	0.05	0.02	252.46	-	-	*A. monilatum; K. veneficum; Pseudo-nitzschia* spp.; *Dinophysis* spp.
Coastal Bays	211.79	40.71	180.86	66.07	0.07	0.49	499.99	-	-	*A. monilatum*

instrumental LOD for MC-LR was 12 pg on-column, which would correspond to 0.06 ng of MC-LR per gram of resin per day in SPATT extract.

3.7. Karlotoxins and Yessotoxins

Karlotoxins (KmTx 1 and KmTx 3) and yessotoxin (YTX) were not found in any SPATT extracts across all sites and time points evaluated.

3.8. Microscopic analyses for HAB cells

Two potentially toxigenic HAB species, *Alexandrium monilatum* and *Karlodinium veneficum*, were successfully enumerated during the study, i.e. cell concentrations were above the detection limit of 1 cell/mL for quantitative analysis. The chain-forming dinoflagellate *A. monilatum* ranged in cell concentration from 5 – 1500 cells/mL, and was observed in 2.4% of all surface water samples. *A. monilatum* was present in summer (August 3 - September 11, 2017) in samples from the southern tributaries (site 4), the bayside Eastern Shore (sites 7, 8, and 9), and the coastal bays region, near the mouth of the Chesapeake Bay (site 10; Table 3). An *A. monilatum* bloom was observed at site 4 in the late summer of 2017 (August 3 - 31, 2017), as confirmed by high cell counts (>1000 cells/mL). *Alexandrium* was not observed in the northern tributary region (Table 3).

Karlodinium veneficum was observed in 4% of all surface water samples, and concentrations ranged from 12 – 455 cells/mL in samples from the northern tributary region (sites 1 and 2), the southern tributary region (sites 4 and 5), and the bayside Eastern Shore (site 7; Table 3). This HAB species was well distributed in time and space, with the only exceptions being its absence from the study from August – December, and the lack of cells in the higher salinity coastal bays region (Table 3).

Qualitative microscopic analysis rarely detected the presence of other harmful algal species in high-volume subsamples (Table 3). *Dinophysis* spp. were observed in five of 321 total samples (2%) across three regions, and *Pseudo-nitzschia* spp. were only observed in two samples (1%) in one region. At the beginning of the sampling period, in May 2017, *Dinophysis* spp. were observed in one sample from the northern tributary region (site 2), one sample from the southern tributaries (site 6), and two samples from the bayside Eastern Shore (site 9). *Dinophysis* spp. were not observed again until almost a year later, in March 2018, when the genus was detected in one sample from the northern tributary region (site 3). *Pseudo-nitzschia* spp. were only observed in summer 2017 in two samples from the bayside Eastern Shore (site 9). Neither genera were observed in the coastal bays region during the study period.

Other HAB species were monitored in surface water samples by microscopy due to their historical occurrence, but were never observed during this study: possible yessotoxin-producers *Protoceratium reticulatum, Lingulodinium polyedrum,* and *Gonyaulax* spp., the DSP toxin-producer *Prorocentrum lima*, and microcystin-producers *Microcystis* spp., *Oscillatoria* spp., *Dolichospermum* (formerly *Anabaena*) spp., and *Planktothrix* spp.. Azaspiracid-producers *Azadinium* spp. and *Amphidoma languida* were considered too small and nondescript to observe by light microcopy (Tillmann et al. 2012).

4. Discussion

This comprehensive field study using solid phase adsorption tracking (SPATT) is the first to show that multiple phycotoxins co-occur over spatial and temporal scales throughout the nearshore waters of the lower Chesapeake Bay and Virginia's coastal bays. At least one toxin group was detected on SPATT resin at each sampling time point, demonstrating the year-round presence of dissolved toxins in the studied waters of this region. Of the 14 toxins that were screened for in the SPATT extracts, eight were detected: OA, DTX1, PTX2, GDA, AZA1, AZA2, MC-LR, and DA. Although amounts of toxin in SPATT extracts cannot be directly related to concentrations in the environment without more studies in uptake kinetics and degradation, the normalized data (ng/g resin/day) can be used to compare relative amounts across regions and over time.

Phycotoxins co-occurred in time and space, with most SPATT extracts testing positive for multiple phycotoxins (Figs. 3-5, 8-10). DSP toxins, OA and DTX1, were present in every extract, and PTX2 was present in all but one extract, showing their ubiquitous distribution in the lower Chesapeake Bay and coastal bays. Co-occurrence of multiple phycotoxin groups was also common; out of 321 total SPATT extracts analyzed, 244 (76%) contained phycotoxins from more than one toxin group, and 105 (33%) contained toxins from three or more classes.

While OA, DTX1 and PTX2 were the most ubiquitous in the Bay and coastal bays, GDA had the greatest toxin maximum across the study. Phycotoxins PTX2, OA, DTX1 had the next highest toxin maximums, followed by DA and AZAs in maximum amount on resin. More specifically, GDA (Fig. 8), ranged from 0 – 102,050 ng/g resin/day, while PTX2 (Fig. 4) ranged from 0 – 70 ng/g resin/day, OA (Fig. 3) ranged from 0.22 – 61 ng/g resin/day, and DTX1 (Fig. 5) from 0.04 – 15 ng/g resin/day. Relatively lower amounts were observed for DA (Fig. 10) and AZA2 (Fig. 9), from 0 – 0.25 ng/g resin/day and from 0 – 0.043 ng/g resin/day, respectively. Trace amounts of MC-LR and AZA1 were also detected in SPATT extracts, but amounts were below limits of quantification.

Examining across seasons, all phycotoxins reached their peak in summer and fall, between June and November; however, the progression of phycotoxin dominance varied between the Chesapeake Bay and coastal bays. Within the Chesapeake Bay regions, PTX2 was the most abundant phycotoxin on SPATT in early summer (July – August), followed by GDA that dominated through late summer into fall (August – October), and OA and AZA2 that peaked in early fall into winter, and then persisted into spring (October – May). Trace amounts of MC-LR were also present in late summer-early fall (July – September) in the southern tributary region. In the coastal bays region, the progression of elevated phycotoxins flipped: phycotoxin maximums began with OA, AZA2, and PTX2 in early summer (July), which carried through to fall (September/October), followed by a delayed peak in GDA in mid fall (October). The late winter and spring seasons marked lower phycotoxin amounts on SPATT overall, but many of the dominant phycotoxins (i.e. OA, DTX1, PTX2, AZA2) persisted and co-occurred in the Chesapeake Bay and coastal bays throughout the colder months.

Spatially, the southern tributary region and coastal bays region exhibited the highest amount of total phycotoxins on SPATT when compared to the other two regions: northern tributary and bayside Eastern Shore (Table 3). All regions contained the dominant phycotoxins (i.e. OA, DTX1, PTX2, AZA2, GDA, DA) at some point over the year, however, two additional phycotoxins, AZA1 and MC-LR, were detected at a subset of sites within the southern tributary region, marking this region as having the greatest diversity of phycotoxins. Okadaic acid was the dominant, or most abundant, phycotoxin in all regions, except the southern tributary region where GDA was the most concentrated on SPATT over the year (Table 3). Taking both the spatial and temporal trends into consideration, aquatic biota within the southern tributary and coastal bays during the summer and fall months experienced the highest amounts and most diverse set of extracellular, bioactive compounds.

4.1. DSP toxins and pectenotoxin-2

Okadaic acid, DTX1, and PTX2 were ubiquitous across all spatial and temporal scales (Figs. 3 – 5). In contrast, *Dinophysis* spp. cells were rarely observed, being present in only 2% of surface samples observed by microscopy, and *Prorocentrum lima* was not detected. Furthermore, there was a disparity between when *Dinophysis* cells were detected, March - May, and when maximum DSP toxin and PTXs were found in SPATT extracts, July - October. The persistent, year-round presence of DSP

toxins and PTX2 in the system could be due to low, background cell abundances of the causative organism, *Dinophysis* spp. in the Chesapeake Bay. Wolny and co-authors (2020a) reported *Dinophysis acuminata* at a mean cell concentration of 0.4 cells/mL in the lower Chesapeake Bay, a value below the current study's detection limit of 1 cell/mL. *Dinophysis* spp. were assumed to be the causative organism due to this taxa's history in the Chesapeake Bay and coastal bays and the presence of PTX2 in the profile, however, *Prorocentrum lima* is an epiphytic/epibenthic dinoflagellate that produces OA and DTX1 (Barbier et al. 1999). This species could have contributed to the presence of DSP toxins, and its absence from surface samples could be explained by its preference for benthos as habitat. The decoupling of cell presence and toxin peaks was likely due to chemical persistence in the aqueous environment after the release from cells (Blanco et al. 2018), however long-term persistence is yet unexplained. More work is needed, therefore, using benthic sampling for *P. lima*, cell-concentration techniques for *Dinophysis* spp., and chemical stability experiments to explain the continuity of DSP toxins and PTX2 in the system.

Differences were observed in both toxin profile and the peak timing when comparing between the Chesapeake Bay and coastal bays region. Phycotoxin peaks were observed in the summer (August) in the coastal bays, and OA (39%) and PTX2 (46%) were equally represented in the toxin profile, with DTX1 representing only 15% of the total profile (Fig. 6B). Within the Chesapeake Bay, however, the phycotoxins (OA + DTX1 + PTX2) peaked later in the fall (October), and the toxin profile was dominated solely by OA (91%; Fig. 6A). Throughout the rest of the year, toxin profiles within the Bay and in the coastal bays were more comparable (Fig. 6). These differences in toxin profile (Fux et al. 2011) and timing can be indicative of distinct *Dinophysis* species or strains, highlighting the need for a paired toxin-molecular study to compare the populations within the Chesapeake Bay and coastal bays and the environmental parameters that drive these dynamics.

In addition to OA and DTX1, the esterified forms of DSP toxins were quantified to allow for comparison of pools between regions and seasons. In all cases, more parent toxins, or "free" OA and DTX1, were detected on SPATT than the esterified forms (Fig. 7). The percent composition of esterified OA and DTX1 ranged from 0% – 45% and 0% – 29% of the total DSP toxin amount on SPATT, respectively, with mean values (± standard deviation) of 22% (± 16%) esterified OA and 10% (± 9%) esterified DTX1. Similar percentages of esterified OA (19%) and esterified DTX1 (8%), were found in SPATT extracts in Long Island Sound (Hattenrath-Lehmann et al. 2018). Previous studies indicate that the esterified compounds were either produced by *Dinophysis* spp., (MacKenzie et al. 2005; Hackett et al. 2005; Fux et al. 2011) or *P. lima* (Wu et al. 2020) and/or released into seawater after biotransformation and excretion by shellfish (Torgersen et al. 2008). As esterified forms can be present in high amounts and may contribute to shellfish toxicity, i.e. directly or through conversion back into parent structures (Van Egmond et al. 2004), these analyses provide a more comprehensive understanding of the total DSP toxins present.

4.2. Goniodomin A

In the warmer months, both GDA and the abundance of its producer, *Alexandrium monilatum,* peaked in the Chesapeake Bay (August – October, Fig. 8). Interestingly, GDA then persisted in the system through the cooler seasons, winter and spring, in the southern tributary region (Fig. 8). This persistence of GDA was unexpected because these two seasons are outside of when *A. monilatum* was observed in the Chesapeake Bay during this study and historically (Wolny et al., 2020b), and GDA rapidly degrades in seawater (Onofrio 2020). Water collections for cell enumeration were consistently conducted during peak irradiance, i.e., when this migratory species is typically found in the surface waters of the Bay. As such, the decoupling of cells and GDA in the system during the cooler seasons cannot be explained by seasonal alterations to sampling technique. Instead these results indicate more research is needed to understand reversible physicochemical interactions that stabilize the compound in the environment and allow it to persist year-round: complexation with potassium (Tainter et al. 2020), or sorption to particulate organic matter, a process observed with the structurally similar PTX2 (Kuuppo et al. 2006).

In addition to dissolved GDA that was available for SPATT sorption, particulate GDA may be present in cysts of *A. monilatum*, providing another potential source of this toxin to aquatic organisms outside of this species' peak bloom season. Cysts of *A. monilatum* have been documented within the southern tributary region (Van Hauwaert, 2016); Pease 2016), but research, like that conducted on another *Alexandrium* species (Oshima et al. 1992), is needed to determine if cysts of this species contain toxins.

The spatiotemporal distribution of GDA suggests that the phycotoxin, and possibly *A. monilatum* cells, are susceptible to southern transport along the western portion of the Chesapeake Bay, following water circulation (Tyler and Seliger, 1978). The delayed and ephemeral presence of GDA at the mouth of the Chesapeake Bay relative to the southern tributary region indicates a fleeting pulse of the phycotoxin and/or cells to this site due to the flushing of water seaward, toward the Bay mouth, from the southern tributary region. This pattern of transport agrees with previous findings by Wolny et al., (2020b), which found that *A. monilatum* blooms have primarily been observed at the mouths of the southern tributaries and southward from there towards the mouth of the Bay. GDA was never detected in the more northern coastal bays (sites 11 and 12; Fig. 1).

4.3. Azaspiracids

This study marks the first report of azaspiracids (AZA1 and AZA2) on the east coast of the U.S.: in Chesapeake Bay and the VA coastal bays. The only other report of AZAs nationally has been in Puget Sound, WA on the west coast (Trainer et al. 2013; Kim et al. 2017). Spatiotemporally, AZA2 was well distributed; the phycotoxin was detected in SPATT extracts from every region studied, in every season except spring (Fig. 9, Table 3). Of the two congeners included in toxin analyses, AZA2 was the predominant azaspiracid found, with AZA1 present in relatively lower amounts and frequency, and only when AZA2 was at its highest. Although AZA2 (Fig. 9) was often observed in SPATT extracts from this study, relative amounts of AZAs were extremely low across all sites in which they were observed compared to other phycotoxins quantified (Figs. 3 – 5, 8). As acceptable recovery was obtained for both AZA1 and AZA2 in SPATT extractions (Table 2), these low concentrations in SPATT extracts are likely reflective of low concentrations of dissolved compounds in the water column. In Ireland, AZA2 amounts in SPATT extracts were consistently 1 – 2 orders of magnitude higher than observed in this study, and in contrast to the observed toxin profile, AZA1 was always found in higher amounts than AZA2 (Fux et al. 2009). As the causative species is currently unknown in this system, it is premature to discuss the source, transport, or persistence of these chemicals in the system. The discovery of these compounds within the Chesapeake Bay and coastal bays, however, raises the awareness that this region does indeed harbor these compounds, thus guiding local monitoring programs to consider AZAs in local biotoxin contingency plans.

4.4. Domoic acid

Low amounts of DA were previously reported in phytoplankton and water samples from the upper Chesapeake Bay, MD, and from one site within the lower Chesapeake Bay: York River, VA (Thessen and Stoecker 2008). The current study therefore expands upon these data, describing DA distribution across the lower Chesapeake Bay and coastal bays. Domoic acid was present in SPATT extracts from 7 of the 12 sites (Fig. 10), spanning all four regions. While this shows wide-spread distribution of DA in the Chesapeake Bay and coastal bays, the overall presence of DA was sporadic with multiple sites only having one sample

with detectable amounts (Fig. 10). Amounts of DA on SPATT were relatively low as compared to other phycotoxins quantified; the highest recorded amount of DA was 0.25 ng DA/g resin/day from the coastal bays region (site 12). This study's maximum DA amount was 2 – 3 orders of magnitude lower than the highest amounts observed in a field study using SPATT on the U.S. west coast (Lane et al. 2010). As a hydrophilic compound, DA is susceptible to loss during water rinses before extraction from Diaion® HP-20 resin (Lane et al. 2010), resulting in artificially low amounts in SPATT extracts. These results, therefore, may underestimate the amount of DA present throughout the lower Chesapeake Bay and coastal bays, but demonstrate the need for monitoring of the causative species in the system.

4.5. Microcystin-LR

Microcystin-LR was found in SPATT extracts from the southern tributary region (sites 5 and 6) only in late summer, confirming that trace amounts of cyanotoxins can be present in meso- and polyhaline waters of Chesapeake Bay. The high recovery of MC-LR (Table 2) from SPATT demonstrates that results are accurate and reflect a low presence in the system. The limited presence of MC-LR suggests that this compound is not widespread throughout the Chesapeake Bay, but is more likely associated with episodic bloom events in the upstream, fresher reaches of the tributaries being brought downstream with flow. Microcystins have previously been reported in upstream, tidal waters of the Chesapeake Bay, including the oligohaline portion of the James River (Tango and Butler 2008; Bukaveckas et al. 2018) and in the aquatic and terrestrial food webs of this system (Wood et al. 2014, Bukaveckas et al. 2017). The detection of MC-LR in SPATT extracts from meso- and polyhaline regions of the lower Bay (sites 5 and 6) was a novel finding, and parallels reports of freshwater phycotoxins in estuarine and marine environments in other areas (Miller et al. 2010; Gibble et al. 2014; Peacock et al. 2018).

4.6. Additional phycotoxins to consider

SPATT sampling, extraction, and detection methods used in this study were appropriate to screen for the 14 phycotoxins investigated. The 6 toxins included in analyses, but not detected in any samples were DTX2, YTX, MC-RR, MC-YR, KmTx 1, and KmTx 3. *Dinophysis* spp. produces the DSP toxins OA and DTXs, but DTX2 has not been reported in Atlantic strains of *Dinophysis* spp. (Fux et al. 2011; Tong et al. 2015; Wolny et al. 2020a), and similarly was not detected in any samples from this study. DTX2, however, has been documented in Monterey Bay, CA on the U.S. West Coast (Shultz et al. 2019). Yessotoxin was not found in this study despite acceptable percent recovery (Table 2) and previous reports of the potential toxin producers *Gonyaulax* spp. in the Chesapeake Bay (Marshall et al. 2005). While low amounts of MC-LR, the most commonly found and abundant microcystin (Wu et al. 2019), were detected in this study, MC-RR and MC-YR were not observed in any SPATT extracts. This is not surprising as microcystin profiles can vary for this large toxin class (Wu et al. 2019).

Karlodinium veneficum frequently blooms in the Chesapeake Bay and is associated with the production of karlotoxins KmTx 1 and KmTx 3 (Li et al. 2015; Bachvaroff et al. 2008). *Karlodinium veneficum* cells were present in water samples from 5 of the 12 sites of the current study, with the highest cell concentrations in the northern tributary region, at 455 cells/mL (site 2). Karlotoxins, however, were not detected in this study. The phenomena of cell presence but phycotoxin absence indicates that either the cells were of a non-toxic strain (Adolf et al. 2009) or were below concentrations needed to produce a detectable amount of karlotoxin (Adolf et al. 2015). Alternatively, the phycotoxins were degraded or precipitated in the environment (Brownlee et al. 2008), rendering karlotoxin amounts on the SPATT too low for detection. The SPATT extraction and detection method used was not responsible for the non-detect, as recovery for KmTx 3 using the described extraction method was sufficient, 90%. The absence of karlotoxin but presence of causative cells emphasizes that SPATT should not replace traditional sampling strategies, but instead remains useful as a complementary tool for monitoring and management purposes.

4.7. HAB abundance

Cells were observed less frequently during this study than was expected based upon previous studies (Marshall et al. 2005, Wolny et al., 2020a). *Karlodinium veneficum* and *A. monilatum* were the only two HAB species found above the current study's detection limit, with maximum abundances of 455 and 1500 cells/mL, and were detected in 4% and 2.5% of all the samples collected, respectively. *Dinophysis* and *Pseudo-nitzschia* were below the detection limit, and so were only qualitatively observed (i.e., in concentrates) in 1.2% and 0.6% of the samples, respectively. While this made for interesting results demonstrating the persistence of numerous phycotoxins in the absence of high biomass, it also made any investigation into linkages between phycotoxins on SPATT and cell abundances impossible. The enumeration technique utilized for this study had a detection limit of 1 cell/mL and should have been able to capture moderate to high blooms of *Pseudo-nitzschia* spp., 100 – 1000 cells/mL (Thessen and Stoecker 2008); *A. monilatum,* 100 – 10,000 cells/mL (Wolny et al., 2020b); *K. veneficum,* 1,000 – 100,000 cells/mL (Marshall and Egerton 2009); and *Microcystis* spp., 100,000 cells/mL (Tango and Butler 2008). *Dinophysis* spp. is typically found in the system below 1 cell/mL, the current study's detection limit (Wolny et al., 2020a). Current efforts are being placed on incorporating cell-concentration techniques and molecular methods to improve cell enumeration so that relationships between cells, intracellular toxins, extracellular toxins, and SPATT toxins can be explored.

4.8. Relevance and management considerations

Many of the phycotoxins found in SPATT extracts from the lower Chesapeake Bay and coastal bays are regulated in edible shellfish meat in the U.S. The current regulatory limits for DSP, ASP, and AZP toxins are 160 μg OA equivalents/kg shellfish meat, 20 mg DA/kg shellfish meat, and 160 μg AZAs/kg shellfish meat, respectively (U.S. FDA 2019). While the EU also has regulatory limits of 160 μg PTX equivalents/kg shellfish meat and 3.75 mg YTX equivalents/kg shellfish meat, the U.S. does not regulate either toxin group [EFSA 2009; European Community 2013)No 786/2013; (U.S. FDA 2011)]. Despite the presence of DSP, ASP, and AZP toxins in SPATT extracts all year round and in all four regions, the Chesapeake Bay has had no reported human illnesses due to the presence of harmful algae or associated toxins in seafood, and this region is not subject to recurring shellfish harvest closures due to phycotoxin contamination.

This presence of phycotoxins, but absence of human illness is likely due to the relatively lower amounts of phycotoxins in Chesapeake Bay and coastal bay waters, as according to SPATT. DSP toxin amounts in this study were much lower than those observed in other regions that experience frequent shellfish harvesting closures, such as in Ireland (Fux et al. 2008; Fux et al. 2009; McCarthy et al. 2014) and Spain (Pizarro et al. 2013). AZA2 amounts detected on SPATTs from the current study were much lower than amounts detected on SPATTs deployed in Ireland (Fux et al. 2008; Fux et al. 2009) and Norway (Rundberget et al. 2009). Similarly, DA amounts on SPATT were lower than those found on the U.S. west coast where *Pseudo-nitzschia* spp. blooms and associated closures are common (Lane et al. 2010). The correlation between toxin accumulation in shellfish meat and toxin amounts on SPATTs is currently unknown for the Chesapeake Bay and coastal bays.

While further studies are needed in the region to clarify relationships between SPATT toxins, intracellular toxins, and toxins in shellfish meat, the results from this study demonstrate that SPATT is already an immediate, beneficial, and complementary tool for monitoring and management of phycotoxins in the protection of human health. This work

was conducted through a collaboration between VIMS, a research and advisory institute, and the VDH Division of Shellfish Safety, a state regulatory management agency. Such partnerships ensure that study design meets management needs for new information, that results are rapidly disseminated to end-users, and that management plans are rapidly adapted to include the latest methods and materials. This use-driven, actionable science is at the heart of translational ecology (Enquist et al. 2017). As a result of this study, the VDH monitoring program has begun 1) molecular screening for *Azadinium* spp. in field samples to identify and quantify the suspected producer of AZAs, and 2) monitoring for multiple phycotoxins in co-deployed shellfish and SPATT. Continued partnerships between academic institutions and state departments will ensure a proactive approach for mitigating potential impacts due to phycotoxin contamination.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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