Interstate Shellfish Sanitation Conference

Techniques and Practices for Vibrio Reduction and Techniques and Tools for Toxin Management
Virginia Department of Health: Division of Shellfish Safety & Waterborne Hazards

Biotoxin monitoring and management using flow-through real-time sampling and toxin tracking.

October 31, 2021

1. Executive Summary:

- A multi-parameter platform was developed and utilized for the measurement of harmful algal bloom metrics (algal cells, fluorescence, molecular signatures, and biotoxins) along with real-time paired environmental water quality parameters over a broad spatial scale that can be deployed on state Shellfish Authority vessels during routine growing area classification runs.

- Molecular techniques were developed to analyze for potential azaspiracid (AZA) producing species in the region, along with molecular and microscopic screening tools for potential amnesic shellfish poisoning (ASP) and diarrhetic shellfish positioning (DSP) taxa within shellfish growing areas.

- ASP, DSP, and AZA associated taxa were identified using molecular analyses, but at relatively low densities. Toxin analyses for okadaic acid (OA) and domoic acid (DA) of both flow-through spatially integrated samples and discrete water samples all were below detection limits.

- Water quality and algal metrics were paired to characterize environmental niches where biotoxin producing species may be most prevalent to inform future monitoring activities. There was a low correlation between chlorophyll fluorescence measurements and the biotoxin producing species targeted in this study. Preliminary work in higher biomass blooms appears more promising.

- Additional work is necessary to refine toxin tracking, including testing of the system in areas of higher biotoxin concentration. This platform may prove useful in fresh and transitional waters for cyanobacterial monitoring in response to increasing concerns about microcystin and other cyanotoxins in shellfish growing areas and recreational waters.

- The flow-through water monitoring platform provides an enhancement to the growing area classification, and can be integrated relatively easily. The toxin-tracking component can be one part of a larger biotoxin monitoring program, but does not replace the need for tools that better integrate temporal variability like testing of deployed passive samplers and sentinel shellfish.

2. Background:

Algal blooms are annual events in Chesapeake Bay and Virginia tributaries, occurring seasonally throughout the year in a succession of dominant species, including multiple taxa that can potentially produce toxins associated with shellfish poisoning, including *Pseudo-nitzschia* (Amnesic Shellfish Poisoning: ASP), *Dinophysis* (Diarrhetic Shellfish Poisoning: DSP) and other Harmful Algal Bloom (HAB) species of economic and ecological concern (Marshall 1995, Anderson et al. 2010, Mulholland et al. 2018, Wolny et al. 2020). In addition to the identification of HAB species, multiple marine biotoxins, including those associated with ASP and DSP along with azaspiracids (azaspiracid shellfish poisoning: AZP) have also been identified in Chesapeake Bay and coastal Virginia waters recently using a passive toxin sampling technique (Onofrio et al. 2021).

In Virginia, the Virginia Department of Health's Division of Shellfish Safety and Waterborne Hazards (DSSWH) is the Authority responsible for carrying out the National Shellfish Sanitation Program in the protection of public health of shellfish consumers. As such, DSSWH conducts an extensive shellfish growing area classification program, including year round seawater sampling of over 24,000 stations for microbiological standards. To accomplish the required sampling, DSSWH operates and maintains a fleet of small boats and devotes considerable resources to seawater sampling. As part of the VDH Marine Biotoxin Control Plan (VDH 2017), a widely distributed subset of stations are also sampled for phytoplankton and toxin analyses using grab samples. While these stations are meant to represent significant segments of the growing area, blooms are notoriously patchy and may be missed by fixed collection locations (Morse et al. 2011).

Microscopic analyses of water samples is one of the first screening mechanisms within the DSSWH plan, and can quickly identify regional biotoxin producing species including *Pseudo-nitzschia* and *Dinophysis*. Commercial toxin testing kits that use an enzyme linked immunoassay (ELISA) method can also be used on shellfish and water samples to test for DA and OA (along with other biotoxins). However, the taxa associated with AZP are notoriously small and problematic to identify using microscopy, and AZA toxin kits are not yet commercially available. As a component of this study, a molecular screening was developed and utilized to target potential AZA producing dinoflagellates. Quantitative PCR (qPCR) was also used to monitor for *Pseudo-nitzschia* and *Dinophysis* species, as it has the potential of detecting these species at lower concentrations than using traditional methods and when they may otherwise not be identified via microscopy.

In recent years, the use of solid phase adsorption toxin tracking (SPATT) has been shown as a successful way to expand beyond grab samples, and monitor algal toxins in both fresh and marine waters (Lane et al. 2010, Wood et al. 2011, Onofrio et al. 2020). These passive samplers have the ability to be deployed for a length of time and continue to adsorb toxins that may be present in the water. This allows for the integration of a longer period of time, and the detection of temporally heterogeneous blooms and toxins that might otherwise be missed with traditional grab samples. To a much lesser degree, SPATTs have also been used to integrate spatial variability, with limited use in continuous underway flow through systems to identify marine and freshwater toxins (Peacock et al. 2018). Recent SPATT deployments in Virginia waters, at ~1-2 week durations, identified a number of toxins, including azaspiracids, domoic acid and okadaic acid (Onofrio et al. 2021) using a combination of UPLC-MS/MS and ELISA analyses.

However, there continues to be a data-gap in the relationship between the identification and concentration of the HAB species and the toxin concentrations present in the water column as well as the environmental parameters associated with these blooms. This project attempts to utilize molecular and microscopic analysis of HAB species, commercially available ELISA toxin kits with SPATTs in conjunction with a flow-through underway system and continuous monitoring of water quality parameters, to generate a better understanding of how these metrics relate. This approach is aimed at the development of an early warning system of marine biotoxins in the environment, and provide the Authority with the data needed to quickly and efficiently manage potential closures at the appropriate spatial scale.

3. Materials and Methods:

Water quality sondes-

In-Situ (In-Situ Inc. Fort Collins, CO) Aqua TROLL 600 Multi-parameter sondes were procured and utilized for this project. These sondes can be customized and allow for up to 4 interchangeable sensors to be added. The sensors (and their part numbers) used for this project were 1) Conductivity/Temperature (0063460), 2) RDO (0063450), 3) Turbidity (0063480) and 4) Chlorophyll a (0038900). Unlike some other sondes, the Aqua TROLL does not use a visual display to show the data. Instead, the sonde is connected through a twist lock cable to a device, Wireless TROLL Com (0031240), which can connect to an Android or iOS device through Bluetooth. These devices use In-Situ's VuSitu mobile App to display and store the data collected by the sonde. For this project, multiple mobile phones and tablets were utilized successfully. Following the sampling runs, these data were exported from the mobile devices as text files that could be easily manipulated within Microsoft Excel, R, or other statistical software.

Flow-through system-

A transom mounted intake and flow through chamber system was constructed based on similar systems used regionally (Moore et al. 2003, Morse et al. 2013) to bring water onboard the vessel, then have it pass through the water quality sonde and SPATT and be discharged back overboard. The goal was to have a portable and adaptable system to be used on existing state research vessels used for growing area classification sampling that could be deployed by field staff with minimal setup required on a routine basis. The system also was designed to operate relatively autonomously, with minimal hands on requirements by field staff, and not affect the vessel operational speed throughout the collection run, allowing a range from idle to full-throttle (~25-30kt).

Following several revisions and earlier prototypes, the system below was used during this study and constructed using largely off the shelf electrical and plumbing components (Table).

A. The water intake pipe (Fig 1 A) (PVC ID 3/4") extends approximately 8 inches below the water line and 4" below the hull, and is housed in an aluminum bracket mounted to the transom. The bottom of the pipe is cut at a 45 degree angle with the opening facing the stern to facilitate water intake. To reduce excessive water from splashing into the boat and onto the outboard motor, splash guards were mounted both below and above the mounting bracket.

B. A 12V 1100 GPH livewell pump (Rule Industries: 405FC) was attached to the top of the intake pipe and wired to a supplemental 12V marine battery. The pump is not self-priming and requires the boat to be underway to start pumping. Once underway and primed, the pump continues to pull water even when the boat itself has stopped moving.

C. 3/4"ID clear vinyl tubing was attached to the pump using hose barb adapters and stainless steel hose clamps. The clear tubing allows for visual confirmation of water movement, air bubbles, and uptake of potential debris or foreign material

D. The tubing enters the flow through chamber which is constructed of a number of 2" PVC plumbing fixtures (shown in detail with parts list in Appendix). The top of the chamber includes a 2" x 1 1/2" flexible reducing coupling (Fernco) that seals tightly around the Aqua-Troll sonde when tightened. The bottom of the chamber is a 2" flexible rubber cap that allows for the bottom of the sonde to rest against, and prevents breakage compared to earlier attempts using rigid PVC.

E. Part of the flow through chamber, constructed of a 2" PVC cleanout Tee, with removable plug, allows for a space where the SPATT can be inserted and held. This Tee includes inline plastic mesh on both the inlet and outlet that holds the SPATT, which itself is held within a plastic mesh envelope (Figure 3), within the Tee. The plug can be removed and replaced in the field to exchange SPATTs if needed during the cruise.

F. Water leaving the flow through chamber goes through another length of 3/4" ID clear vinyl tubing, which is routed through a gunnel mounted rod-holder and emptied overboard. This allows for visual confirmation that the system is pumping and for determination if any modifications to the plumbing or electrical system is necessary.

G. The water quality sonde used is the In-Situ AquaTROLL 600, which is inserted into the chamber and secured using the Fernco coupling. The setup allows for the manufacture's protective Restrictor and Endcap to remain in place, but requires the rubber Bumper to be removed. A similar chamber can be used for the slightly shorter AquaTROLL 500, but requires a shorter standpipe to seal correctly. The sonde must be inserted and sealed prior to the boat being underway to ensure a closed loop system, otherwise incoming seawater will escape from the top port.

H. The In-Situ AquaTROLL 600 sonde is connected to the Wireless TROLL com, which houses the rechargeable battery and Bluetooth data transfer through a Rugged Twist Lock Cable. This cable is long enough to allow for a vertical depth profile if necessary. The sonde can easily be removed and replaced from the chamber while on station by loosening the Fernco coupling hose clamp.

I. A dedicated removable 12 V marine battery is used to power the livewell pump (B). This is used to allow for the system to be removable, but can be replaced by wiring into the boat's 12V system if desired.

J. The Wireless TROLL com requires a Bluetooth enabled device for data storage. Multiple devices can be utilized to run the In-Situ VuSitu Mobile App (including Android and Apple mobile phones and tablets). Cellular data is not required during data collection, however the App relies on the device GPS sensor for the paired location data. A protective water resistant cover is recommended for whichever device is used. The tablet records and displays the real-time data, and can be located so that the field staff can make collection decisions based on the measured parameters (i.e. elevated chlorophyll a concentrations).

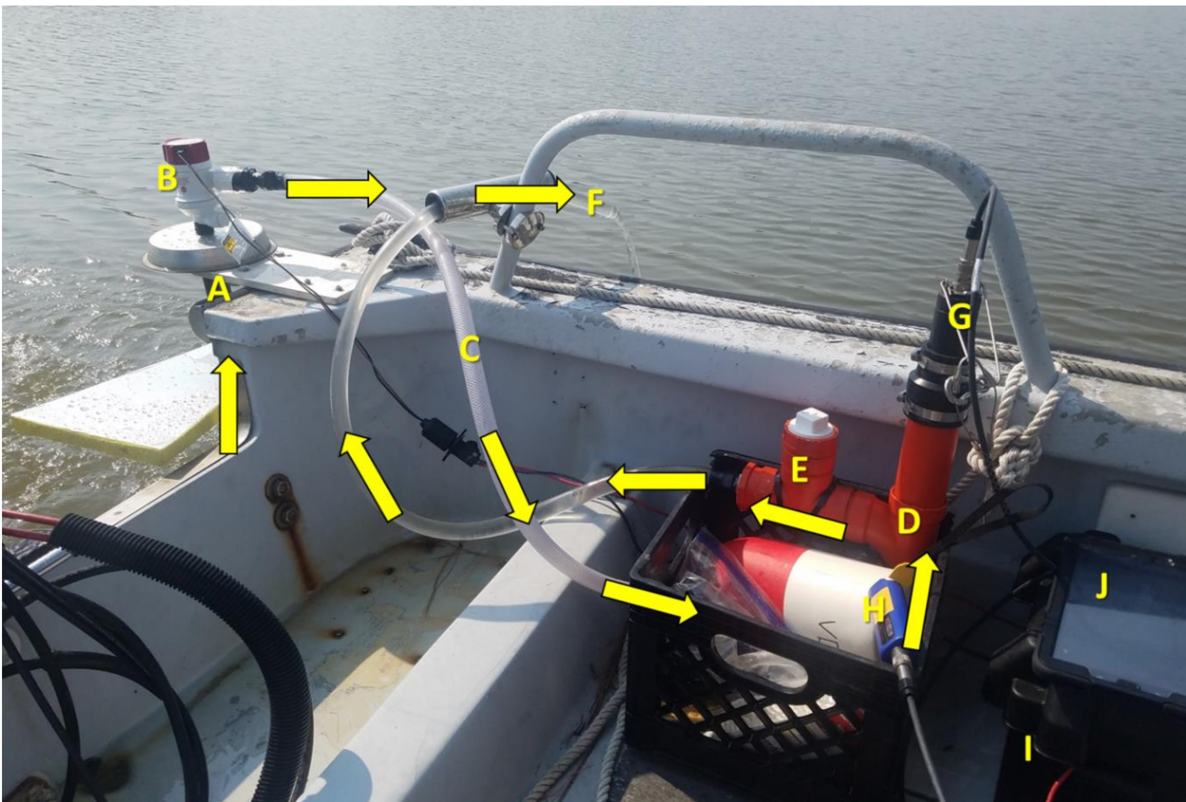

Figure 1: Portable transom mounted flow-through chamber system in use during a VDH sampling cruise.

The transom mounted system was the primary system used during this study, as it is able to be retrofitted to a number of existing small boats utilized for routine shellfish growing area classification. In addition, preliminary work exploring the use of an integrated livewell system was conducted which may provide a simplified approach in vessels where a through-hull is available. In this system, the sonde was placed directly in the livewell which received a constant intake of seawater while underway. This allowed for a continuous flow of seawater from the through-hull, and eliminated the need for additional intakes, plumbing, and electrical systems. Three supplemental cruises were conducted using this setup. While additional work is needed to refine this approach to develop a more stable system to hold the sonde and evaluate the effectiveness of the different sensors, this system is promising in its simplicity in vessels that have an integrated livewell.

Figure 2: Integrated livewell application for continuous monitoring and toxin tracking during VDH sampling cruise.

SPATTs and toxin assays-

SPATTs were constructed following minor modifications of prior studies (Lane et al. 2010, Peacock et al. 2018, Onofrio et al. 2021). Briefly, 3g of Alfa Aesar Diaion HP20 resin beads (Thermo Fisher Scientific 46488) were held in pouches constructed of Nitex bolting cloth (100µm mesh) (WildCo 3-24-C34). These pouches (~2x3") were formed by folding the cloth in half and sealing the 3 remaining edges with an impulse sealer (Clamco 210-12E) (Figure 3). SPATTs were activated following Lane et al. (2010) with 100% MeOH, rinsed with de-ionized water, and stored in de-ionized water in refrigeration prior to use. SPATTs were placed in an additional protective holder and placed in the water stream within the flow-through system for the duration of the sampling run (approximately 2 hours). Following the run, SPATTs were removed from the flow through, rinsed with DI water and stored at -20C prior to extraction. SPATT extractions were conducted using a modified approach of Onofrio et al. (2021) for analysis using Eurofins Abraxis ELISA toxin kits. The SPATTs in this study were extracted using a 75% methanol concentration. As per the ELISA manufacturer's instructions, the extractions were diluted to 5% MeOH prior to analysis to avoid matrix effects.

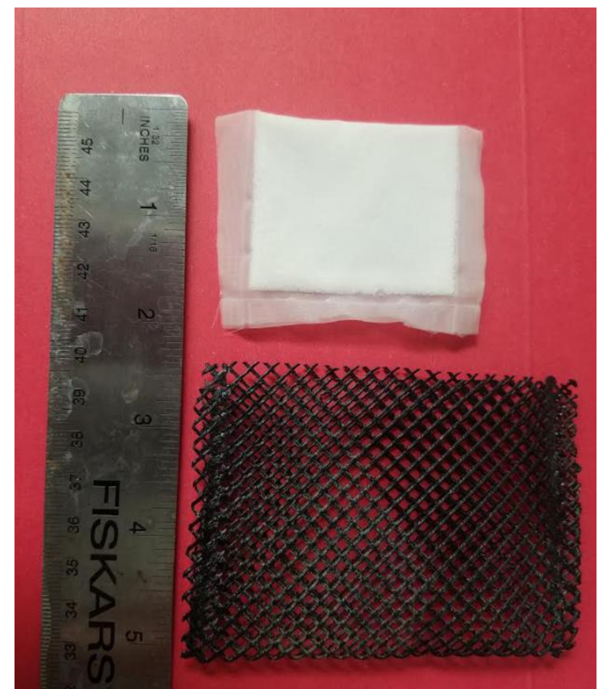

Figure 3: Nitex SPATT pouch (top) and SPATT holder (bottom)

Whole water unpreserved samples were collected in 50ml centrifuge tubes from the monitoring stations (0.5m) and stored at -80C prior to analyses. Whole water samples were lysed using a 3x freeze thaw method prior to toxin analyses. Abraxis ELISA kits (Eurofins Abraxis) were utilized to analyze both SPATT extracts as well as whole water seawater samples. This included the Domoic Acid (PN ON0021) and Okadaic Acid (PN 520021) ELISA kits. Analysis was conducted following the manufacturer's instructions, with evaluation conducted using microplate photometer at 450nm (Abraxis 4303).

Microscopy-

Whole water seawater samples (250ml) were collected from the surface (0.5m) at each sampling station and preserved with Lugol's solution (1% final concentration). Samples were analyzed using inverted light microscopy (Olympus CKX41) at 100-400x with glass bottom well plates (Cellvis P12 1.5H-N). All algal cells were quantified and identified to the lowest taxonomic unit, and recorded as cells/ml (Egerton et al. 2014). Algal biomass estimates were calculated based on cell densities and species specific biovolume and biomass estimates (Smayda 1978, Egerton et al. 2012).

Molecular analyses-

Samples to be analyzed by qPCR were collected in parallel to the microscopy and toxin samples, and consisted of 250ml of unpreserved whole water that was held on ice during transport to the lab. Following sample homogenization (shaking), 100mls of sample were filtered onto 47mm 3.0µm PC membrane filters (Isopore TST04700), placed in 5.0mL DNA LoBind tubes (Eppendorf 003018310) and stored at -20C prior to analysis. DNA was extracted using the QIAamp Fast Stool Mini Kit (QIAGEN) according to the manufacturer's protocol with modifications (Pease et al. 2021).

Pseudo-nitzschia spp.

Standard PCR primers developed by Penna et al. (2007) were used in a SYBR green qPCR assay (Table 1). Each 10µl reaction consisted of 1X PowerUp SYBR Green Master Mix (Applied Biosystems), 900nM of each primer and 1µl of DNA template. The samples were amplified using a 7500 Fast Real-Time PCR System (Applied Biosystems). Cycling parameters included a denaturing step of 20 seconds at 95°C and then 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Melt curve analysis was performed at the end of the qPCR run to check for non-specific amplicons.

AZA2 Producers

All the 2017 samples where AZA2 was detected in the Onofrio et al. 2021 study were initially screened with the *A. spinosum* and *A. poporum* TaqMan® qPCR assays (Toebe, et al. 2012) but they were all negative. Next, an Amphidomataceae family-level SYBR green assay (Smith et al. 2016) (Table 1) was used to identify samples with possible AZA2 producers present. This assay targets the DNA of *Azadinium* and *Amphidoma* species. Note that the DNA from both toxic and non-toxic species in these genera are amplified by these primers. SYBR green qPCR analysis using these primers was done using the same amplification parameter as above.

Dinophysis spp.

A genus level standard PCR primer set (Penna et al. 2007) was used to screen samples from a previous study where *Dinophysis* species cells had been observed by microscopy. The 360 bp amplicons from the internal transcribed spacer (ITS) of the rRNA gene region were cloned and sequenced as previously reported (Moss et al. 2008). Sequences were quality scored and visually checked for sequencing errors and then aligned with control *Dinophysis* spp. sequences from GenBank using the MUSCLE algorithm (Edgar 2004) in MacVector 18.1.5 (MacVector Inc.). The National Center for Biotechnology Information (NCBI) Primer-Blast tool (Ye et al. 2012) was used to design primers and a TaqMan® probe specific to *Dinophysis* spp. (Table 1). Each 10µl reaction consisted of 1X TaqMan® Fast Advanced Master Mix (Applied Biosystems), 900nM of each primer, 250 nM probe, 0.4 mg/ml BSA, and 1µl of DNA template. The samples were amplified using a 7500 Fast Real-Time PCR System (Applied Biosystems). Cycling parameters included a denaturing step of 20 seconds at 95°C and then 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C.

Table 1: Primers and probe sequences for the PCR assays used in this study to target the DNA of *Pseudo-nitzschia* spp., Amphidomataceae species and *Dinophysis* spp.

Target	**Type**	**Sequence**	**Reference**
Pseudo-nitzschia			
Pseudo-5’	Forward	CGATACGTAATGCGAATTGCAA	Penna et al. 2007
Pseudo-3’	Reverse	GTGGGATCCRCAGACACTCAGA	
Amphidomataceae			
Amp240F	Forward	CAACTTTCAGCGACGGATGTCTC	Smith et al. 2016
Amp418R	Reverse	AAGCYRCWGGCATKAGAAGGTAGWGGC	
Dinophysis			
DinoITS_52F	Forward	CATGTGGAAGCTCGAGGGTA	This study
DinoITS_130R	Reverse	GTGAGCCAAGCAGACGGTAG	
DinoITS_82Pr	Probe	AGCAGTGTGGTCTTGCTGTT	

4: Results and Discussion:

Exposure time laboratory SPATT test

As our prior experience using SPATTs involved extended duration deployments of 1-2 week, additional work was needed to confirm whether toxins would bind to the HP20 resin and be detectable with a much shorter exposure time. Neither toxin producing *Dinophysis* or *Pseudo-nitzschia* cultures were available commercially or through regional HAB partners, so the study was conducted using available toxic cyanobacteria as a proxy for marine biotoxins.

Briefly, 3g SPATTs were submerged in a microcystin producing *Microcystis aeruginosa* culture and exposed for a range of times from 15 mins to 96 hrs. At each time period, the SPATT was removed and a sample of the culture was also taken. SPATTs were rinsed with DI water and stored at -20C prior to extraction. Culture samples were lysed using a 3x freeze thaw method prior to analysis using an Abraxis microcystin kit (PN 520011).

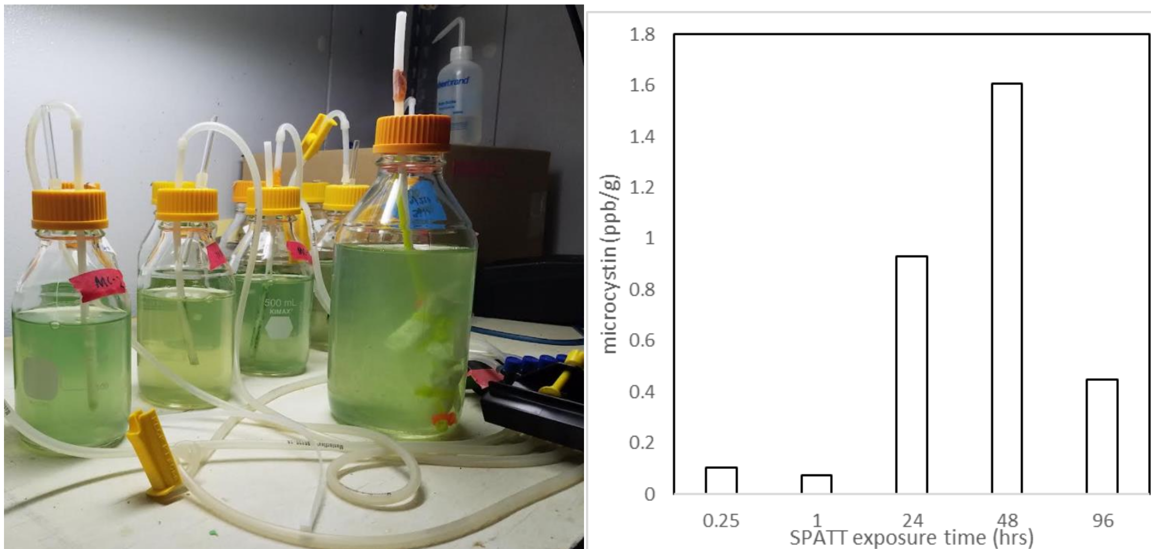

Figure 4: Left: *Microcystis* culture with submerged SPATTs. Right: microcystin concentrations of the 5 SPATT extracts from exposures to the culture for times ranging from 15 mins (0.25hr) to 4 days (96hrs).

The culture of *Microcystis* tested positive for microcystin at each time point (Time 0- 96hrs), above the upper detection limit of the test (>5ppb). Subsequent analyses of diluted culture indicated a microcystin concentration of approximately 221 ng/ml (ppb). SPATT extracts from each of the time periods also tested positive for microcystin above the lower detection of the assay (0.15ppb or 0.05 ppb/g using a 3g SPATT) (Figure 4). This included detectable toxin measurements even given a 15 minute exposure to the culture. As the expected duration of the sampling runs were 2 hrs, this indicated that the method had potential of detecting toxins, even at relatively low periods of exposure. It should be noted however that the toxin concentrations in the extracts were significantly higher given longer (>1hr) exposure periods, and that this study utilized a culture with a high toxin concentration. Both of these support what has been the primary application of SPATTs in longer-term deployments, but do indicate at least the potential application using shorter durations.

Sampling Cruises

Sampling cruises as well as other fieldwork, labwork and procurement were significantly delayed and impacted by the COVID-19 pandemic. However, developmental work into the flow-through system and SPATT analyses was conducted in 2020, with the sampling cruises occurring in spring-summer 2021 (Table 2). Four cruises were conducted in May-June 2021 using SPATTs and included the full suite of sample collection and analyses for a total of 18 sets of grab samples (Table 3). In addition, to test the flow-through system in a wider range of environmental conditions, 5 additional supplemental sampling cruises were conducted in July-September 2021 (Figure 5).

Table 2: Sampling cruises and flow-through environmental parameters

Cruise	Date	Duration (hrs)	Temp (ºC)			Salinity (psu)			Chl a (µg/L)			Turbidity (ntu)			DO (mg/L)		
			average	max	min	average	max	min	average	max	min	average	max	min	average	max	min
James River	5/13/2021	2:05	17.6	22.1	16.9	17.3	20.9	8.8	0.2	2.5	0.1	98.3	5018.9	1.5	7.6	8.6	5.6
York River	5/17/2021	2:09	19.5	20.5	18.5	15.4	19.5	9.3	4.0	19.8	0.1	45.5	1581.4	3.8	7.1	8.5	5.8
Rappahannock River	5/24/2021	2:31	23.0	25.0	21.6	11.4	12.4	8.4	10.3	27.8	2.4	32.7	304.0	5.3	6.1	8.0	4.5
Chesapeake Bay mouth	6/2/2021	2:12	20.5	22.6	19.8	23.3	29.3	0.4	0.3	4.6	0.1	16.1	1021.2	2.1	7.6	8.2	6.7
Upper Rappahannock River	7/9/2021	1:39	29.1	31.0	27.0	7.1	10.7	0.0	9.1	524.0	0.0	130.4	21950.6	8.4	6.9	7.2	4.7
Piankaktank River	7/21/2021	1:10	28.6	29.6	28.2	11.5	16.5	1.1	0.4	9.0	0.0	514.3	3411.6	22.8	6.7	7.2	6.3
Corrotoman River	7/26/2021	2:24	29.2	30.3	27.8	12.3	14.6	0.0	1.8	26.7	0.0	211.8	952.5	0.8	6.4	7.1	5.9
Lower Chesapeake Bay	8/27/2021	2:00	28.9	29.6	26.3	23.9	29.3	5.0	4.3	59.3	0.0	25.0	2997.3	0.0	6.6	8.1	5.4
Nansemond River	9/13/21	2:16	25.4	26.8	24.3	13.8	19.9	4.8	10.1	27.4	0.1	29.7	862.8	12.5	5.7	8.6	4.1

Table 3: Grab sample locations and environmental parameters

Cruise	Date	Station ID	Time	Temp °C	Salinity (psu)	Secchi depth (m)	Chl a (µg/L)	Turbidity (ntu)	DO (mg/L)	DO (% sat.)	Latitude	Longitude
James River	5/13/2021	62-1	11:20	16.98	16.21	0.6	0.27	61.11	8.05	91.18	36.933081	-76.482786
	5/13/2021	64-KK30	12:38	17.58	15.57	0.9	0.24	42.3	7.74	88.72	36.904335	-76.342014
	5/13/2021	56-V40	12:16	17.48	20.42	1.1	0.17	22.41	7.66	88.99	36.966287	-76.395918
	5/13/2021	57-E61	11:46	17.63	15.41	0.5	0.28	34.92	7.11	80.67	37.041012	-76.514425
York River	5/17/2021	51-18	10:32	18.88	18.05	1.2	0.64	27.24	7.31	86.47	37.260421	-76.541794
	5/17/2021	52-2	10:18	18.83	18.45	1.4	1.38	19.64	7.46	88.1	37.229163	-76.491527
	5/17/2021	46-9	10:00	19.37	19.42	1	0.16	7.31	7.6	91.24	37.261326	-76.420582
	5/17/2021	49-103	11:25	20.37	9.44	0.4	7.33	16.33	6.7	77.21	37.480453	-76.74346
	5/17/2021	50-18	10:59	20.47	15.52	0.3	15.56	49.86	6.12	73.32	37.343431	-76.645975
Rappahannock River	5/24/2021	29-1	11:04	23.08	12.15	-	5.09	102.4	6.93	85.73	37.641572	-76.56386
	5/24/2021	28-12	11:24	24.11	11.6	0.4	8.54	35.95	5.29	66.48	37.672576	-76.601316
	5/24/2021	23-1	12:00	23.87	11.81	0.5	14.21	17.96	6.15	77.05	37.771762	-76.584173
	5/24/2021	26-1	12:33	24.88	8.43	0.3	26.46	30.92	5.82	71.75	37.810881	-76.744109
Chesapeake Bay mouth	6/2/2021	CBFP	11:58	21.98	21.96	0.7	0.99	10.07	6.94	89.04	36.903483	-76.095461
	6/2/2021	BM1	11:39	20.56	21.17	1.7	0.25	30.57	7.96	99.15	36.974276	-76.109637
	6/2/2021	BM2	11:20	20.4	20.94	2	0.21	32.04	7.96	98.15	37.039124	-76.072951
	6/2/2021	Wise Pt	10:40	20.16	29.24	0.9	0.26	17.87	7.06	98.63	37.127567	-75.949548
	6/2/2021	BM3	10:57	20.24	23.72	1.5	0.19	5.24	7.68	91.45	37.089529	-75.987847

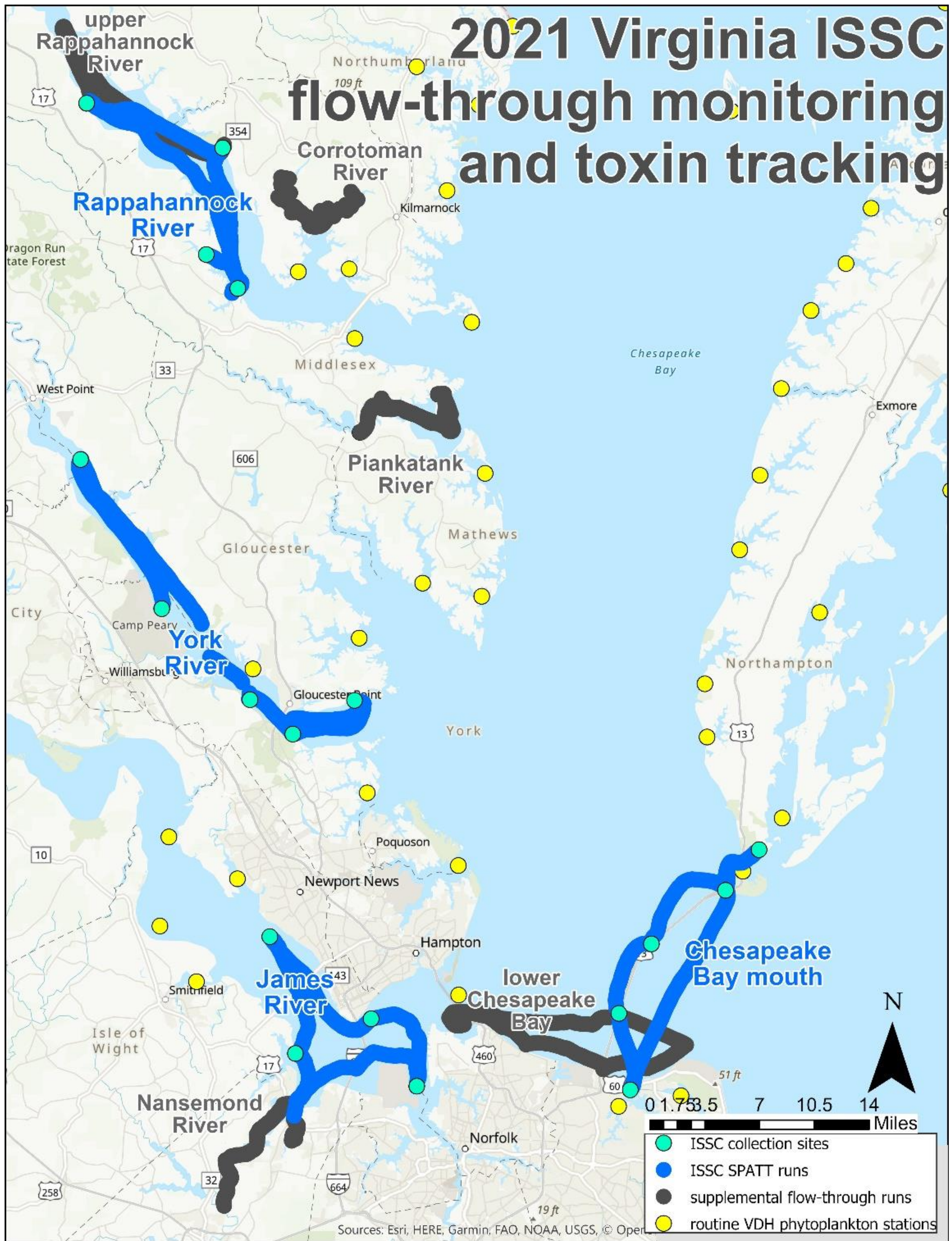

Figure 5. Station and cruise location map

Sampling cruises with SPATTs and sample collections were conducted in both the Hampton Roads and Middle Peninsula/ Northern Neck vicinities, regions of both significant shellfish industry importance and historical harmful algal bloom activity (Figure 5). The mouth of Chesapeake Bay, spanning from Lynnhaven River to Wise Point on the eastern shore also represents a region where potential biotoxin producers have been identified in the past, including marine taxa from the Atlantic (Marshall and Egerton 2009, Onofrio et al. 2021).

Environmental water quality data

The sampling cruises were conducted over a range of environments throughout the region in mesohaline and polyhaline waters (Table 2). This included ranging from waters where there was minimal chlorophyll/ phytoplankton (ie. James River), to areas with active algal blooms present (ie. upper Rappahannock River and lower Chesapeake Bay). Each cruise, with measurements recorded by the AquaTROLL sonde every 2 seconds, resulted in between approximately 2000-4000 data points. Each data point representing a location, date/time and multiple environmental parameters, including water temperature, salinity, chlorophyll a, turbidity and dissolved oxygen. A total of 32,237 rows of data were generated over the 9 cruises. These data were exported to Excel and Arc GIS Pro for statistical and spatial analyses. The averages and ranges of these parameters are summarized in Table 2.

To visualize the spatial distribution of these parameters, maps were generated of the salinity, chlorophyll, turbidity and dissolved oxygen for each of the cruises in ArcGIS Pro using color-coded symbology corresponding to different ranges of the parameters (Figures 6-9). These same attributes from the collection sites (grab sample locations) were also mapped to compare and contrast the range of these parameters measured by traditional sampling methods to those collected using the flow-through system. As may have been expected, the range of data captured by the continuous sampling approach was greater than that from what was measured at just the collection sites. More importantly, the flow-through data and maps better identified gradients or distinct patchiness that would otherwise by under-represented or missed using only a traditional station based approach.

In the case of Chlorophyll a, the sensor data collected in the field was paired with the National Oceanic and Atmospheric Administration's National Centers for Coastal Ocean Science Algal Bloom remote sensing data (https://coastwatch.noaa.gov/cw_html/NCCOS.html). Chlorophyll-a (Gilerson et al. 2010) imagery was obtained for the same day as the sampling cruise when possible, or within 1-2 days if necessary due to cloud cover. The satellite imagery was paired with the sensor data in ArcGIS Pro, and the sensor data symbology was changed to match the colors and ranges used in the NOAA imagery to allow for more direct visual comparison.

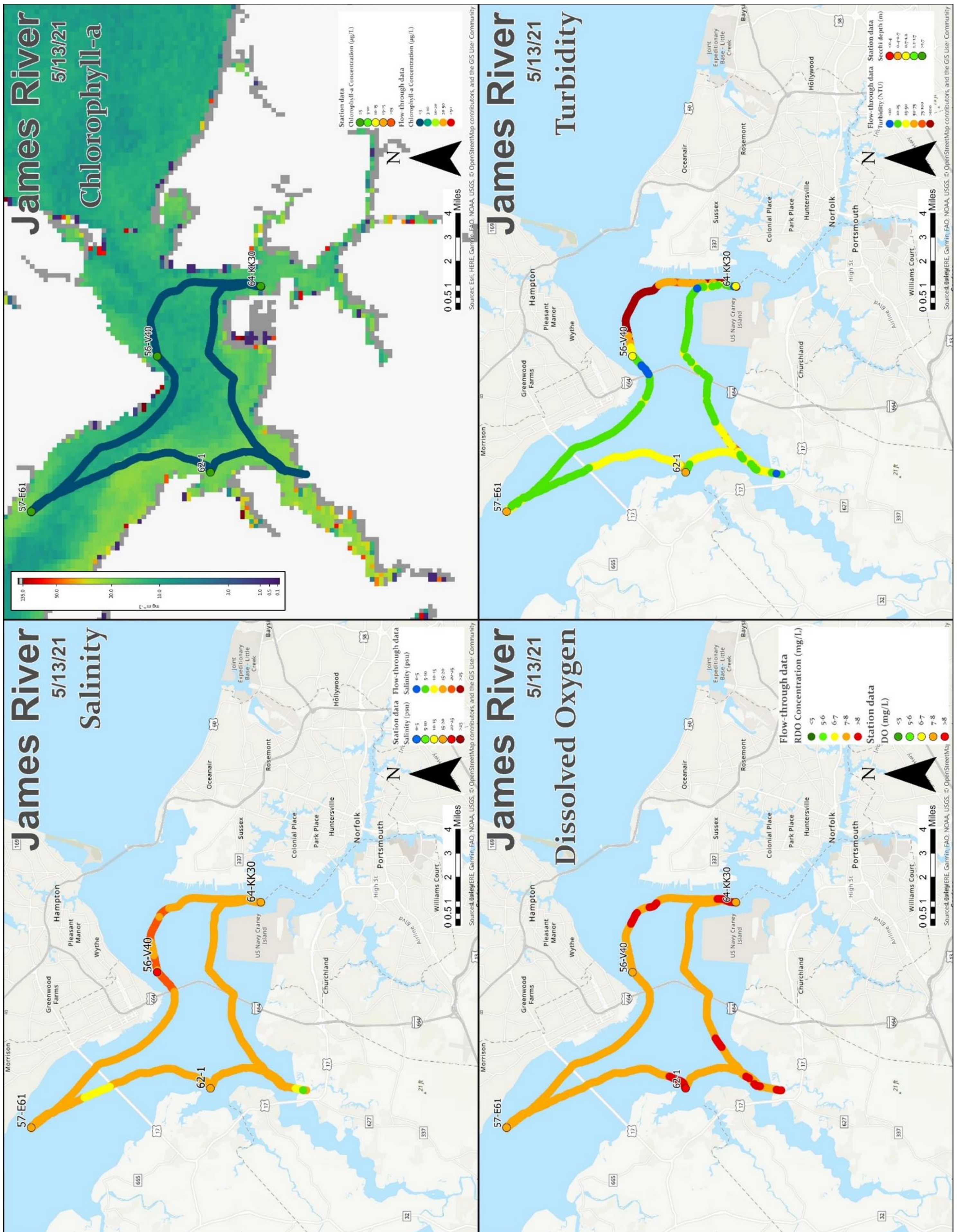

Figure 6: Composite maps of flow-through and grab sample environmental parameters collected in the lower James River on 5/13/21.

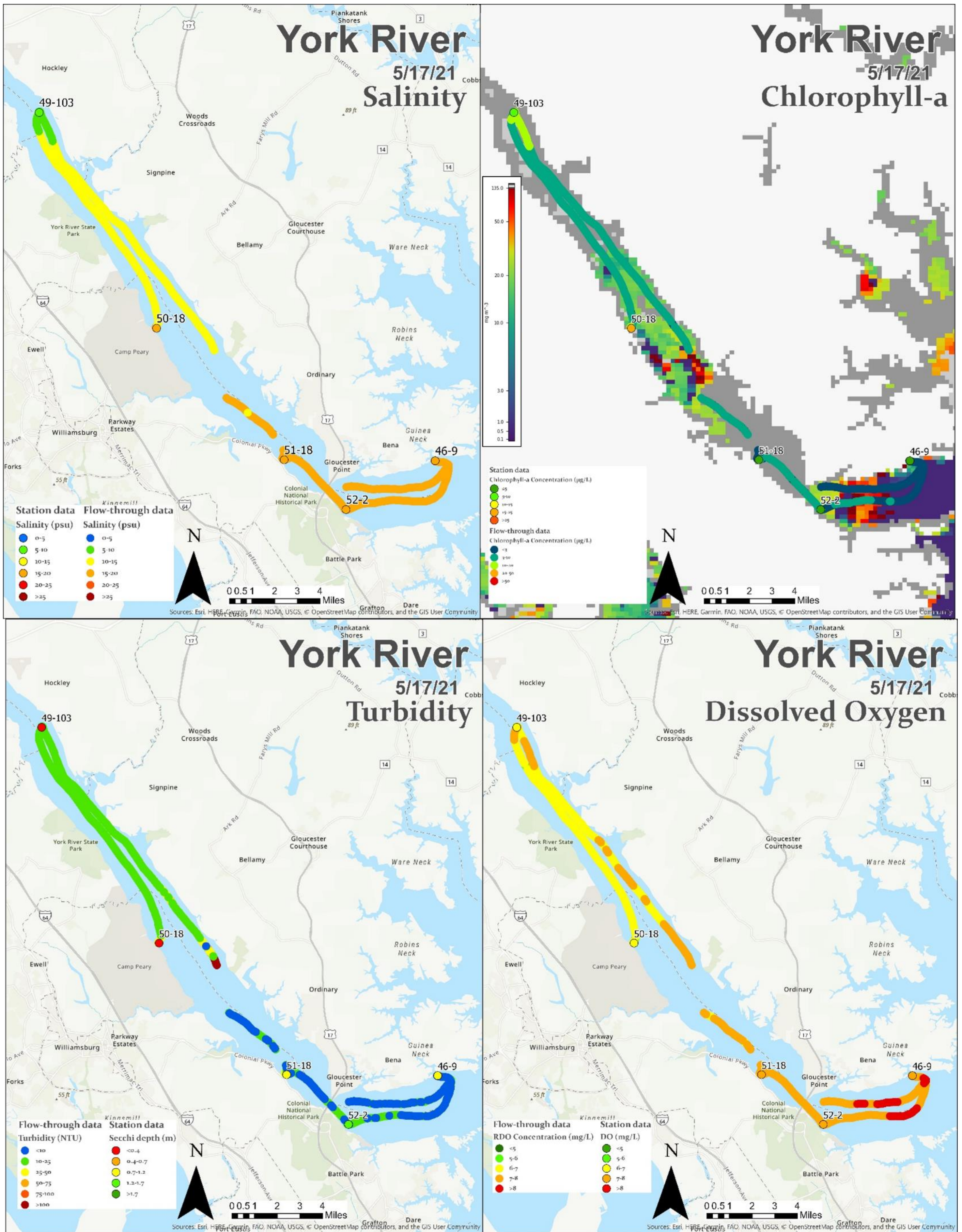

Figure 7: Composite maps of flow-through and grab sample environmental parameters collected in the York River on 5/17/21.

Figure 8: Composite maps of flow-through and grab sample environmental parameters collected in the Rappahannock River on 5/24/21.

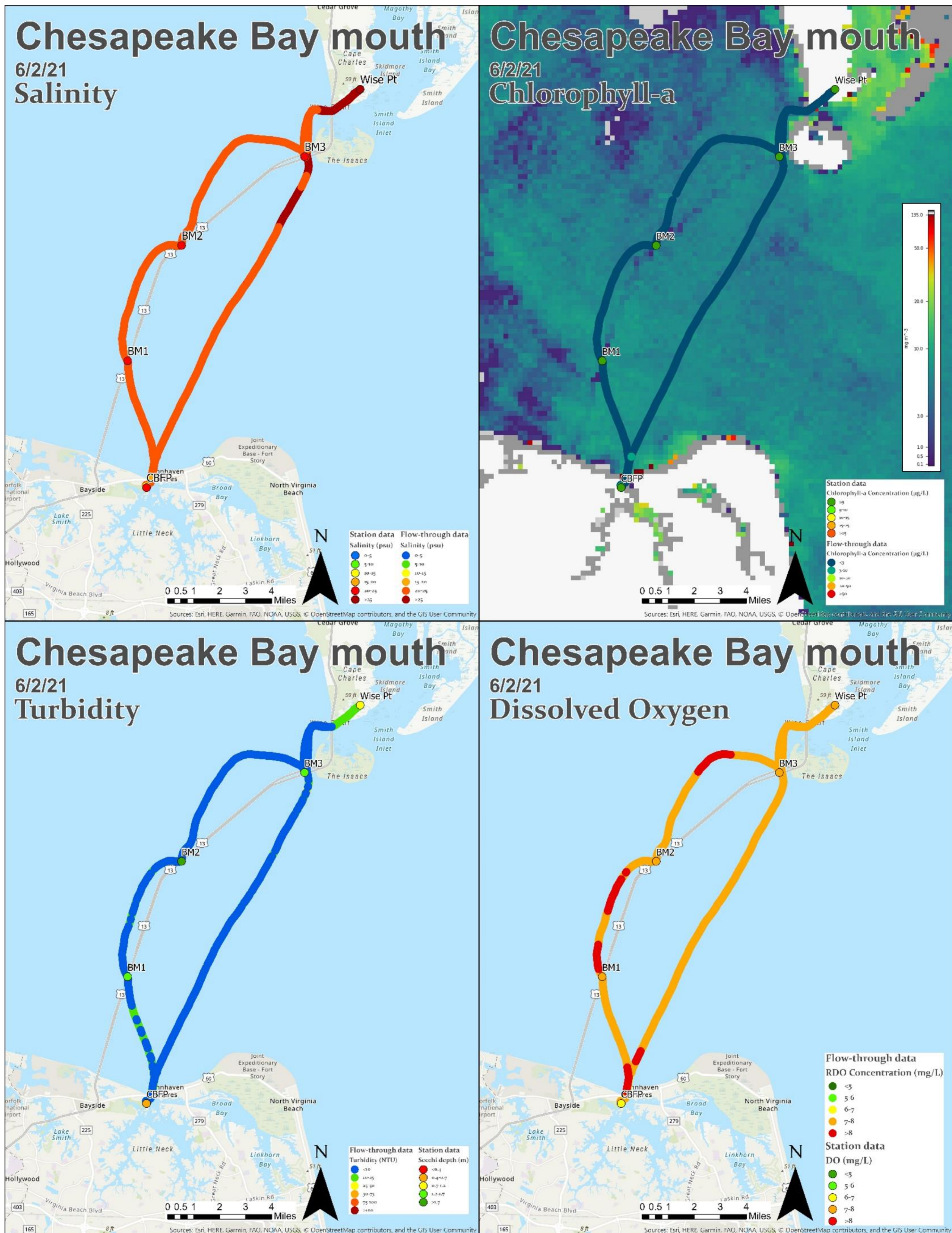

Figure 9: Composite maps of flow-through and grab sample environmental parameters collected at the mouth of Chesapeake Bay on 6/2/21.

Phytoplankton data and harmful algal bloom species

Overall, there was relatively little in the way of significant marine biotoxin events during the course of the study. Of the 18 phytoplankton samples analyzed, only 1 resulted in an identification of potential marine biotoxin producing taxa (*Dinophysis*, *Pseudo-nitzschia, Azadinium* spp.) by visual/microscopic analyses (Table 4). By contrast, the qPCR analyses did identify the molecular signatures of these species in a significant number of samples, including some level of *Pseudo-nitzschia* spp. signal at every site (Table 4). These concentrations (genome equivalents/ml) can be interpreted as comparable to cells/ml and are generally low, with the vast majority less than 1/ml. While the exact relationship between cell density and toxin concentration has not yet been fully determined for these species, these densities are well below what have been considered high by state shellfish authorities. For example, states within the Gulf of Mexico Alliance have related Domoic Acid with *Pseudo-nitzschia* concentrations between 100->10,000 cells/mL, and propose utilize densities of >1000/ml as a threshold to collect additional samples and analyses and progress within the marine biotoxin response plan (Lewitus et al. 2014). For Okadaic Acid, densities of *Dinophysis* >1/ml are suggested as a threshold criteria to conduct additional sampling and response (Lewitus et al. 2014). Less work has been done on azaspiracid producing taxa belonging to the family Amphidomataceae (including *Azadinium* and *Amphidoma*). These species were not identified in the current study nor other phytoplankton monitoring in the region by light microscopy. Their identification here by qPCR, albeit at low concentrations, is the first observation of these taxa within these waters. The molecular analyses were conducted on 100ml filtered samples, compared to 5ml settled samples used in the visual microscopic analysis.

Table 4: Cell densities (cells/mL and equivalent) of potential marine biotoxin producing species

Cruise	Date Collected	Station ID	*Dinophysis* spp.		*Pseudo-nitzschia* spp.		Amphidomataceae	
			qPCR	visual	qPCR	visual	qPCR	visual
James River	5/13/2021	62-1	0	0	8.9	0	0	0
		64-KK30	0.03	0	2.3	0	0	0
		56-V40	0.03	0	1.4	0	0	0
		57-E61	0	0	1.8	0	0	0
York River	5/17/2021	51-18	0.07	0	0.61	0	0	0
		52-2	0.09	0	0.1	0	0	0
		46-9	0.45	0	0.59	0	0.13	0
		49-103	0	0	0.7	0	0	0
		50-18	0	0	0.77	0	0.14	0
Rappahannock River	5/24/2021	29-1	0	0	0.17	0	0.25	0
		28-12	0	0	0.01	0	0.67	0
		23-1	0	0	0.05	0	0.25	0
		26-1	0	0	0.23	0	0.03	0
Chesapeake Bay mouth	6/2/2021	CBFP	0.01	0	0.57	0	0	0
		BM1	0	0	0.04	0	0	0
		BM2	0	0.4	0.1	0	0	0
		Wise Pt	0	0	2.75	0	0	0
		BM3	0	0	2.75	0	0	0

Outside of the taxa recognized as potentially producing marine biotoxins by the NSSP, multiple other HAB species are present in these waters, including the dinoflagellates *Karlodinium veneficum, Margalefidinium polykrikoides* and *Prorocentrum minimum.* These species can result in fish kills and other ecological impacts in Chesapeake Bay, including those to shellfish aquaculture (Stoecker et al. 2008, Mulholland et al. 2009). Molecular and/or microscopic techniques identified these taxa within a majority of samples (Table 5), although there was relatively low agreement in the results between the two methods. It should be noted that the water samples for microscopy and molecular analyses although collected in parallel were separate samples and therefore can have different cell counts due to the variable nature of plankton heterogeneity.

Table 5: Cell densities (cells/mL and equivalent) of additional HAB species of shellfish and ecological concern

Cruise	Date Collected	Station ID	*Karlodinium veneficum*		*Margalefidinium polykrikoides*		*Prorocentrum minimum*	
			qPCR	visual	qPCR	visual	qPCR	visual
James River	5/13/2021	62-1	0.08	0.0	0.46	0.0	2.9	10.1
		64-KK30	0.7	0.0	0.35	0.0	3.87	0.0
		56-V40	0.58	10.1	0.25	0.0	2.85	0.0
		57-E61	0.04	0.0	0.15	0.0	10.45	0.0
York River	5/17/2021	51-18	3.31	20.3	2.15	0.0	49.29	20.5
		52-2	0.81	81.1	0.93	0.0	20.85	0.2
		46-9	9.81	0.0	2.11	0.0	14.38	0.4
		49-103	1.56	0.0	2.39	0.0	2.29	0.0
		50-18	15.86	43.5	6.57	0.0	71.09	29.0
Rappahannock River	5/24/2021	29-1	0	50.7	0	0.0	64.01	101.4
		28-12	0	0.0	0	0.0	12.2	0.0
		23-1	0	67.6	0	0.0	9.56	33.8
		26-1	0	0.0	0	0.0	33.86	0.6
Chesapeake Bay mouth	6/2/2021	CBFP	0	0.0	0	0.0	0	0.0
		BM1	0	0.0	0	0.0	0	0.0
		BM2	0	0.0	0	0.0	0	0.0
		Wise Pt	0	0.0	0	0.0	0	0.0
		BM3	0	0.0	0	0.0	12	0.0

To further investigate the linkage or disconnect between the molecular and microscopic results, a number of additional samples collected in 2021 (n>250) were compared outside of those directly collected for this study. These results also identified considerable uncertainty between the two approaches, in part due to the relatively low and narrow range of densities observed. Additional work is needed and ongoing in this area, including controlled laboratory culture based approaches to refine standard curves and compare the assays to a number of different species across a range of densities.

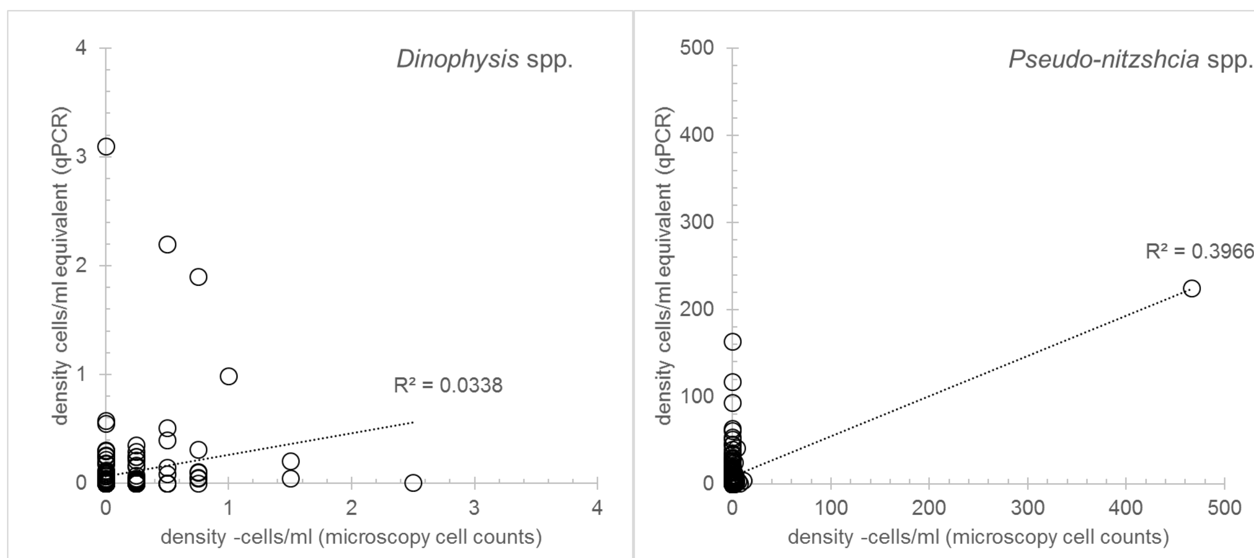

Figure 10: Regression analyses of *Dinophysis* spp. and *Pseudo-nitzschia* spp. densities as calculated by qPCR (DNA concentration cell equivalents) and microscopic cell counts on paired samples. This includes the collections made as part of the Flow-through cruises, as well as additional collections in 2021 (*Dinophysis* spp. n=254) (*Pseudo-nitzschia* spp. n=261).

Phytoplankton data and Chlorophyll measurements

One of goals of the project was to utilize the real-time measurements of Chl a fluorescence to target potentially harmful blooms in the field and direct sampling as necessary. Besides potential HAB species, a diverse assemblage of other algal taxa were observed within the samples analyzed. An average of 19 taxa were identified in each sample (13-25). The species identified were typical for the region and season (Marshall et al. 2006). Taxa biomass estimates were grouped by major taxonomic group, with diatoms and dinoflagellates generally being the dominant taxa (Figure 11). Chlorophyll a measurements from the AquaTROLL were paired to the total algal biomass estimates. There was no significant correlation with samples between total algal biomass, or the biomass of the dominant groups with the chlorophyll measurements (Figure 11). Prior similar work in the region had identified significant positive correlations between these metrics (Marshall and Egerton 2013) over a much larger range than what was measured in these samples. These samples included chlorophyll measurements from <1- 26μg/L and algal biomass estimates from <50-~1000 μgC/L. In comparison, prior work identifying a significant correlation had chlorophyll measurements >500 μg/L, and biomass estimates >100,000 μgC/L (Marshall and Egerton 2013).

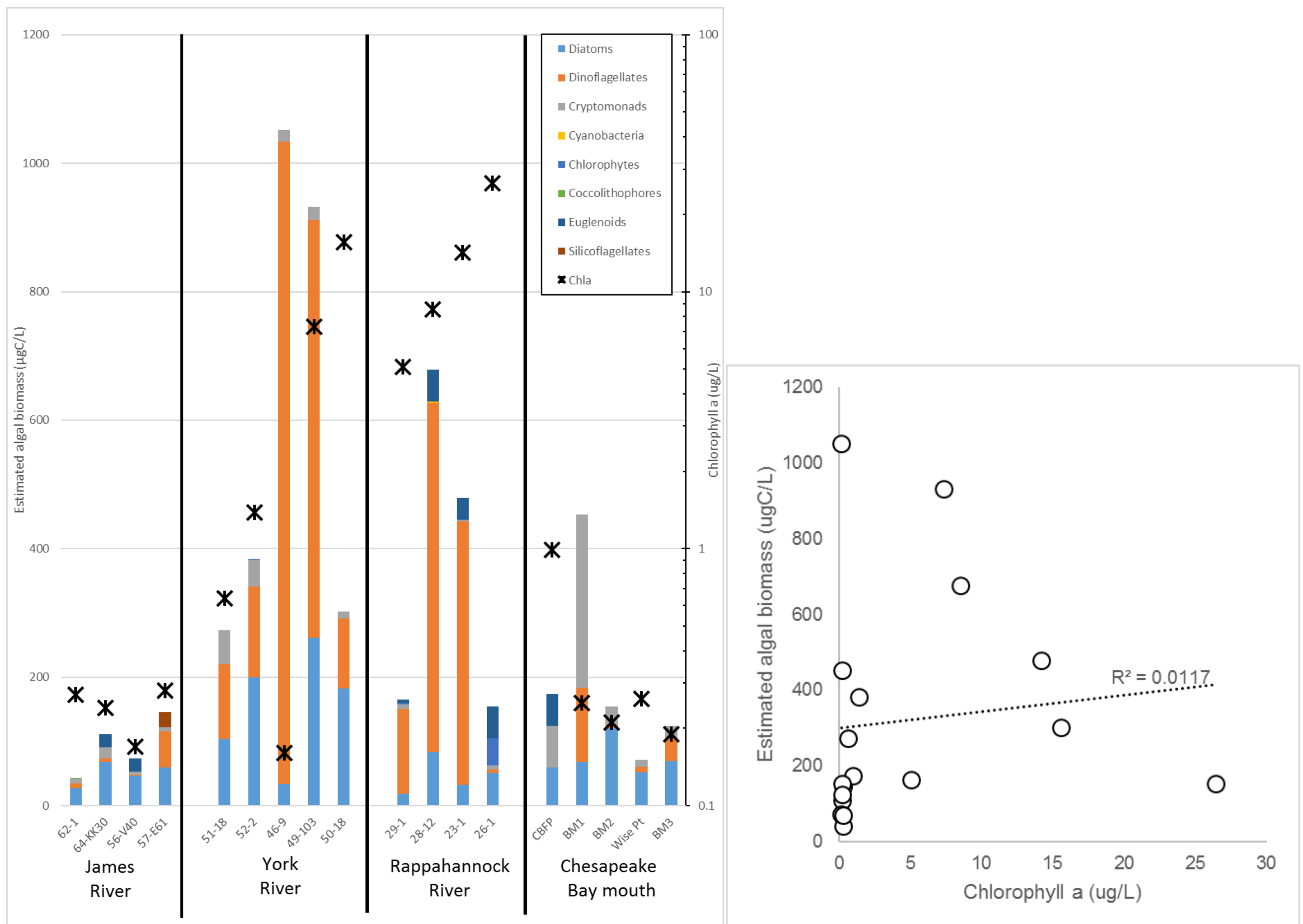

Figure 11: Left: Algal Biomass of major taxonomic groups. Chla for each station shown with * (log scale on right axis). Right: No significant relationship between measured in-situ Chla fluorescence and total algal biomass.

Phytoplankton data and environmental parameters

To better characterize the environmental niche and possible identify or narrow water quality ranges of the potential marine biotoxin producers (*Dinophysis, Pseudo-nitzschia, Azadinium*), the qPCR densities were related to water temperature and salinity. This was done both using the samples collected during this study as well as a larger set of samples collected during 2021.

Table 6: Environmental parameters and ranges for biotoxin producing species detected by qPCR in 2021 (both in the flow through cruises, and additional collections).

		n	Water temperature (°C)			Salinity (psu)			Secchi (m)			Dissolved Oxygen (mg/L)		
			avg.	max	min	avg.	max	min	avg.	max	min	avg.	max	min
Dinophysis	detected (qPCR)	152	12.5	26.4	1.2	16.6	32.6	1.9	1.0	4.5	0.1	8.2	18.2	3.9
	>0.5/ml	11	10.9	22.7	2.9	18.5	31.2	8.2	0.8	1.4	0.1	7.5	8.9	6.7
Pseudo-nitzschia	detected (qPCR)	246	14.7	30.5	1.2	15.3	32.6	1.8	0.9	4.5	0.1	7.9	18.2	3.9
	>50/ml	12	19.5	24.6	9.9	21.7	32.0	2.3	0.6	0.9	0.4	6.9	9.3	4.3
Amphidomataceae	detected (qPCR)	76	18.4	29.7	1.2	17.2	32.0	6.6	0.9	4.5	0.3	7.3	18.2	4.7
	>1/ml	9	18.5	23.5	13.9	13.6	31.2	8.1	0.9	1.4	0.3	6.7	6.7	6.7

- <u>*Dinophysis* spp.</u>

Dinophysis concentrations based on qPCR data ranged from the detection limit (below 0.05 cells/ml) to 3 cells/ml, with the vast majority (93%) of positive samples analyzed less than 0.5 cell/ml (Table 6). Water temperature where concentrations exceeded 0.5 cells/ml ranged from 3-23 C, spanning collections in February to June. Likewise, at these sample locations, there was a wide range of salinities from 8-31, with the majority of positive samples in mesohaline cool to moderate (<20C) waters (Figure 12).

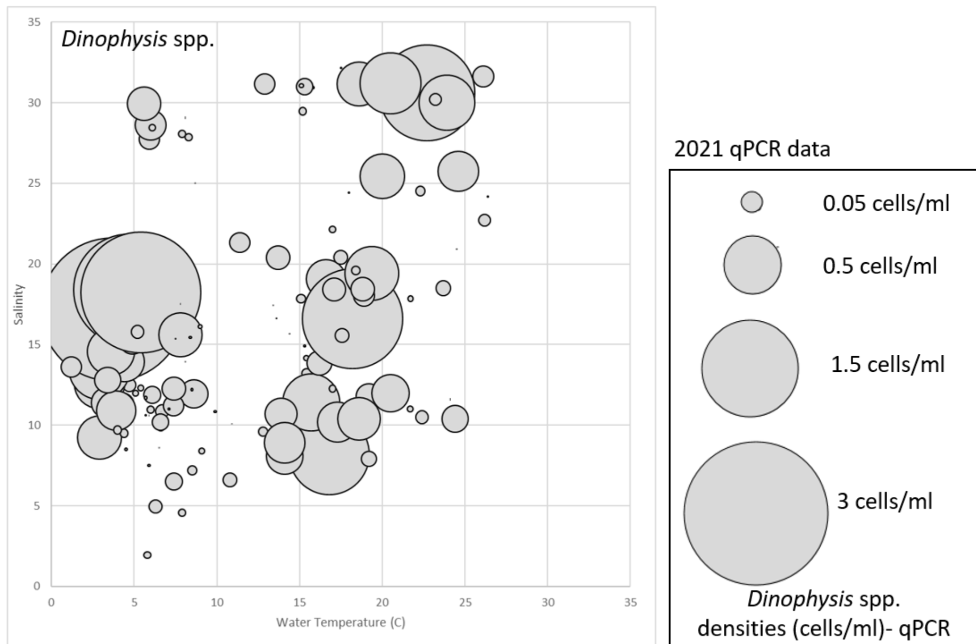

Figure 12: Temperature and salinity parameters where *Dinophysis* spp. DNA was detected by qPCR in 2021 (both in the flow through cruises, and additional collections) n=152.

- *Pseudo-nitzschia* spp.

Pseudo-nitzschia concentrations based on qPCR data ranged from the detection limit (well below 1 cells/ml) to 524 cells/ml, with 95% of positive samples analyzed less than 50 cell/ml and 37% less than 1 cell/ml. Water temperature where concentrations exceeded 50 cells/ml ranged from 10-25 C, spanning collections from January to July. Likewise, at these sample locations, there was a wide range of salinities from <5->30, with the majority of higher density positive samples in polyhaline moderate to warm waters (Figure 13).

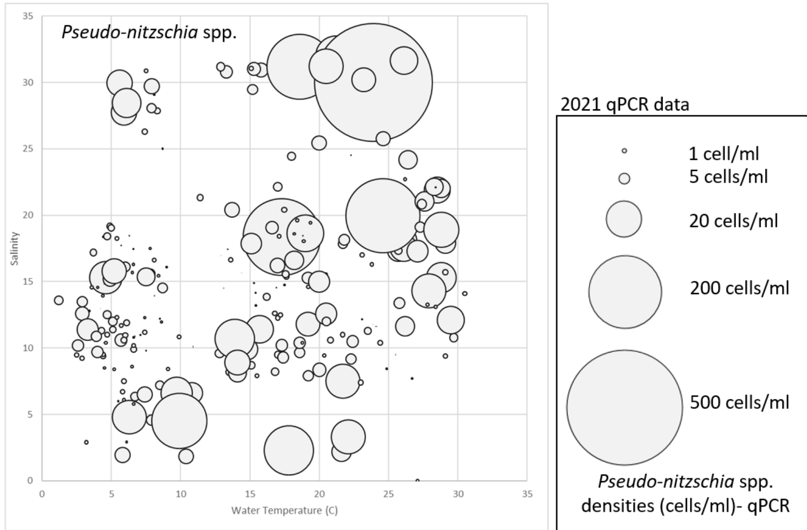

Figure 13: Temperature and salinity parameters where *Pseudo-nitzschia* spp. DNA was detected by qPCR in 2021 (both in the flow through cruises, and additional collections) n=246.

- Amphidomataceae

Amphidomataceae (which includes *Azadinium* and *Amphidoma* spp.) concentrations based on qPCR data ranged from the detection limit (well below 1 cells/ml) to 5.3 cells/ml, with 88% of positive samples analyzed less than 1 cell/ml (Table 6). Water temperature where concentrations exceeded 1 cell/ml ranged from 14-24 C, spanning collections from April to June. Likewise, at these sample locations, there was a wide range of salinities from 8->30, with the majority of higher density positive samples in mesohaline moderate to warm waters (Figure 14).

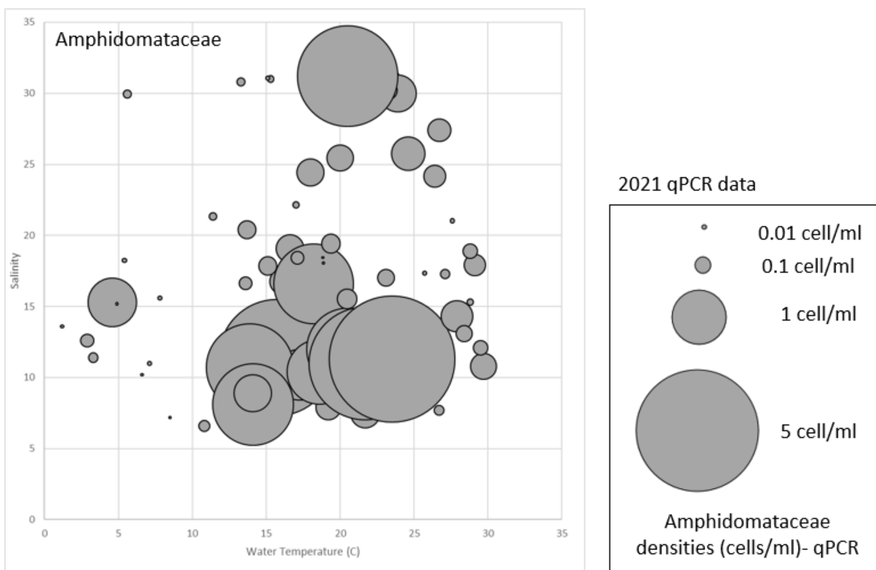

Figure 14: Temperature and salinity parameters where Amphidomataceae DNA was detected by qPCR in 2021 (both in the flow through cruises, and additional collections) n=78.

Toxin data

All samples analyzed, both the SPATT extracts as well as the wholewater grab samples were negative for both Domoic Acid (ASP) and Okadaic Acid (DSP). There is no toxin kit for Azaspiracids commercially available, so there are no AZP results. The lack of positive results from the wholewater samples is consistent with testing conducted as part of Virginia's marine biotoxin control plan, particularly given the low concentrations of cells identified within the samples. Water samples analyzed for ASP or DSP toxin, including those where *Pseudo-nitzschia* or *Dinophysis* have been identified, have historically failed to have detectable toxin concentrations in the vast majority of cases. In 2020, none of the samples analyzed had detectable DSP, despite *Dinophysis* densities of 0.25-4.5 cells/ml. Of water samples analyzed for ASP where *Pseudo-nitzschia* was present, approximately 18% of samples did measure detectable Domoic Acid (0.6-1.0 ppb). These came from samples where *Pseudo-nitzschia* densities ranged from 1-24 cells/ml. All shellfish meat samples tested by VDH have been negative for both ASP and DSP toxins (<0.5 ppb).

Table 7: Toxin Results

Cruise	Date	SPATT exposure time (hrs)	SPATT ASP ELISA	SPATT DSP ELISA	Station ID	ASP test result (ppb)	DSP test result (ppb)
James River	5/13/2021	2:06	BDL	BDL	62-1	-	-
					64-KK30	<0.5ng/mL	<0.1 ng/mL
					56-V40	-	-
					57-E61	-	-
York River	5/17/2021	2:10	BDL	BDL	51-18	<0.5ng/mL	<0.1 ng/mL
					52-2	-	-
					46-9	<0.5ng/mL	<0.1 ng/mL
					49-103	<0.5ng/mL	<0.1 ng/mL
					50-18	-	-
Rappahannock River	5/24/2021	2:31	BDL	BDL	29-1	<0.5ng/mL	<0.1 ng/mL
					28-12	<0.5ng/mL	<0.1 ng/mL
					23-1	<0.5ng/mL	<0.1 ng/mL
					26-1	<0.5ng/mL	<0.1 ng/mL
Chesapeake Bay mouth	6/2/2021	2:05	BDL	BDL	CBFP	<0.5ng/mL	<0.1 ng/mL
					BM1	<0.5ng/mL	<0.1 ng/mL
					BM2	<0.5ng/mL	<0.1 ng/mL
					Wise Pt	<0.5ng/mL	<0.1 ng/mL
					BM3	<0.5ng/mL	<0.1 ng/mL

In contrast, SPATT samples have been used over the last few years in the region to detect ASP, DSP, AZP and microcystin among other compounds (Onofrio et al. 2021). The areas and seasons monitored in this study overlap with where these toxins were detected in this recent work. In that study however, SPATTs were deployed at stationary positions for 1-2 weeks at a time. While the laboratory culture study conducted indicated that the method should theoretically be possible to measure toxins at a short (minutes to hours) exposure period, it utilized high concentration of microcystin toxin. The relatively low or background concentration of marine biotoxins that may be present in Chesapeake Bay may not be suitable for such an approach that relies on a lower exposure time to the SPATT resin. In addition, the current study used utilized commercially available ELISA toxin kits instead of an ULMC MS/MS method. These kits, have a much lower initial equipment cost and training requirements can be employed within a state shellfish laboratory, but have some limitations compared to a mass spectrometry method. Toxins from SPATTs are extracted using methanol, which must be diluted to <5% prior to the ELISA to avoid a matrix effect. This dilution has the potential of raising the detection limit where low concentrations of toxins may not be identified, particularly if there was minimal initial exposure.

Additional work is necessary to refine this method and better characterize detection limits with this method explicit for the marine biotoxins. This will require laboratory cultures and dilutions of toxic *Pseudo-nitzschia* and *Dinophysis* that can be analyzed with different exposure times as was done using *Microcystis*. Work is ongoing with state partners to isolate and establish cultures of these phytoplankton, and VDH plans to continue collaborations in these efforts including toxin testing and cell enumeration, both microscopically and molecularly.

5: Feasibility, efficiency and summary:

The project was successful at developing and implementing a flow-through water monitoring platform that can be integrated into a state Shellfish Authority's growing area classification program. The portable transom mounted system can be constructed with minimal costs and adapted to a variety of vessels with little impact on the routine use once the staff becomes familiar with the setup and operation of the sonde. Regarding the long term reliability of the system, we are still within the first full year of testing the current configuration. Expected maintenance and replacement of components such as the pump, gaskets and hoses is expected, and will continue to be monitored moving forward as we continue to use the system. In vessels that have an integrated livewell, this system seems to be even easier to adapt and requires almost no additional work while providing a significant increase in data and capability.

The Aqua-TROLL sonde itself proved to be largely a user friendly and reliable system. The sonde is sturdy and performed well both in the flow-through chamber, or deployed directly to perform surface water sampling or depth profiles. The data generated was easy to export in a consistent tabular format that can be integrated into any existing database or data management platform. The lack of an integrated visual screen on the handheld/sonde does require some additional time and equipment in the field, including syncing the Bluetooth with a tablet or phone. This would require some additional training with field staff that are more use to a typical integrated system (ie. YSI PRO instruments), and could be implemented with a field checklist for the setup and operation of the method. One aspect of the system that was concerning was the Wireless TROLL Com that contained the batteries and Bluetooth communications. The USB Micro-B charging port, and cover was fragile compared to the rest of the sonde, and on an initial device was a point of failure. Discussions with the manufacturer indicated that this was a recognized concern and that future models were planned with a more sturdy connection. Since the study, there has also been a telemetry system released (In-Situ VuLink) that eliminates a wired connection entirely and would allow for more remote monitoring over mobile 4G network. We have not tested this system, but it may be promising where continuous monitoring and real-time reporting is necessary.

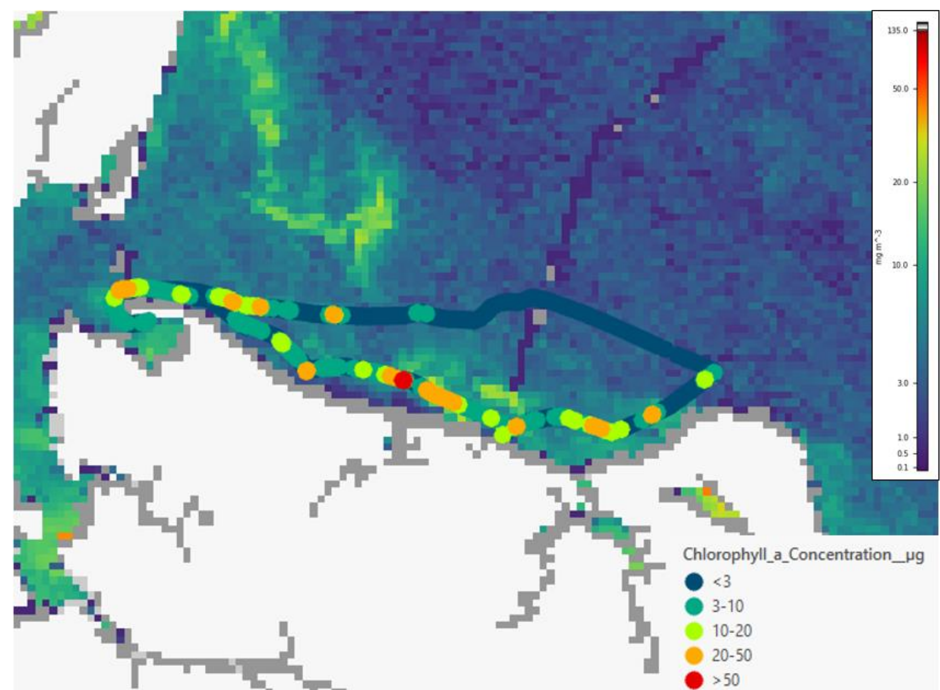

Figure 15: Satellite Chlorophyll imagery overlaid with flow-through chla concentrations from August 24 cruise during ongoing high biomass bloom of Margalefidinium polykrikoides.

The ability to collect and visualize water quality parameters, particularly chlorophyll while underway will be most useful when evaluating and responding to high algal biomass events. While the concentrations were relatively low in the main four SPATT runs of the study, the August sampling cruise in lower Chesapeake Bay (Figure 15) included a dense and very patchy bloom of *Margalefidinium polykrikoides*. During this cruise, the sonde was successful at capturing a wide range of chlorophyll (Table 2) including high concentrations when the vessel was within the bloom patches. Having the ability to use the data to

spatially define the extent of a bloom, and target sampling at high chlorophyll site that will be very helpful in events where the Authority may have to determine closure size or other management decisions. These tools also provide data to ground truth and refine remote sensing models that can monitor and predict algal bloom distributions within growing areas (Wolny et al. 2020, O'Shea et al. 2021). By gathering a growing database of chlorophyll and other environmental data and collaborating with state, federal and academic partners, these algorithms can be refined to provide more accurate estimates of HABs and associated health risks. Additional work to compare cell counts and chlorophyll concentrations of target HAB taxa is necessary to determine sampling criteria or other numeric thresholds that could be used as part of a response plan or monitoring program. Outside of the biotoxin needs discussed here, similar work utilizing available Rhodamine sensors could be used for dye studies of wastewater outfalls or other pollution assessments. Additional measurements of salinity, temperature, dissolved oxygen and turbidity will support the growing area classification far beyond measurements made at a small number of fixed sites. These measurements can be used to further refine the environmental factors that favor certain algal taxa, including those that may produce marine biotoxins. These data can be used to target certain regions or seasons for additional sampling, toxin testing or other monitoring and management actions.

While the current study didn't identify marine biotoxins using the flow-through system, it still does show possible utility as a component within a monitoring program network, but not a replacement for additional sampling methods. In regions where toxin concentrations are much higher, as in the ASP blooms experienced on the US West Coast and elsewhere, this approach would have a much greater chance of measuring toxins, and could aid in establishing the spatial extent of biotoxins in the water. Temporally integrated methods, including longer-term SPATT or shellfish deployments have a greater chance of identifying biotoxins that may be present at low concentrations or only sporadically. In addition to the marine biotoxins discussed here, SPATTs and shellfish deployments can and are also being used to monitor for cyanobacteria toxins within the growing areas (Gibble et al. 2016, Onofrio et al 2021). This is an area of growing concern, and these tools are well suited in those monitoring efforts, including measuring possible toxin concentrations across an estuarine gradient (Peacock et al. 2018).

Moving forward, VDH plans to continue to use the lessons learned as part of this study to expand on its monitoring network across multiple platforms, including molecular analyses of ASP, DSP and AZP taxa, toxin testing of multiple matrices (water, SPATT, shellfish) and increased characterization of the growing areas using the flow-through system. Across the combination of the strengths and weaknesses of the individual components, the network as a whole is best equipped for detecting biotoxin concerns that may be present and responding as necessary.

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Appendix 1: Phytoplankton species List: Phytoplankton species identified during visual counts from study, and presence (X) within samples from the 4 cruises. Marine biotoxin producing species **highlighted in red.**

	James River	York River	Rappahannock River	Chesapeake Bay mouth
Diatoms				
Actinoptychus senarius		X		X
Amphiprora sp.	X	X		X
Aulacoseira granulata	X		X	
Cerataulina pelagica		X		
Chaetoceros sp.		X		
Cocconeis sp.		X		X
Coscinodiscus sp.	X	X	X	X
Cyclotella caspia			X	
Cyclotella spp. > 30 um	X	X	X	
Cylindrotheca closterium	X	X	X	X
Cymbella sp.			X	X
Dactyliosolen fragilissimus	X	X	X	X
Diploneis sp.	X		X	X
Gyrosigma fasciola		X		X
Gyrosigma sp.				X
Leptocylindrus danicus				X
Leptocylindrus minimus	X	X	X	
Navicula sp.	X	X	X	X
Nitzschia sp.	X	X		
Odontella mobiliensis	X		X	
Odontella sinensis				X
Paralia sulcata	X	X		X
Pleurosigma angulatum	X	X	X	
Pleurosigma elongatum			X	X
Pleurosigma sp.	X	X	X	X
Skeletonema costatum		X	X	X
Surirella sp.			X	
Synedra sp.			X	X
Thalassionema nitzschioides	X	X		X
Thalassiosira sp.	X	X		
Unid Centric Diatom < 10um	X	X	X	X
Unid. Centric Diatom Diam >60 um		X	X	X
Unid. Centric Diatom Diam 10-30 um	X	X	X	X
Unid. Centric Diatom Diam 31-60 um		X	X	X
Unid. Pennate Diatom Length >60 um	X	X	X	X
Unid. Pennate Diatom Length 10-30 um	X	X	X	X
Unid. Pennate Diatom Length 31-60 um	X	X	X	X
Dinoflagellates				
Ceratium furca				X
Ceratium longinum		X		
Dinoflagellates (Unid.)	X	X	X	X
Dinophysis acuminata				**X**
Diplopsalis lenticula		X		X
Gymnodinium sp.			X	X
Gyrodinium aureolum		X	X	
Gyrodinium fusiforme		X		

Heterocapsa rotundata	X	X	X	X
Heterocapsa triquetra		X		
Karlodinium veneficum	X	X	X	
Levanderina fissa			X	
Peridinium quinquecorne		X		X
Polykrikos kofoidii		X	X	
Prorocentrum gracile				X
Prorocentrum micans	X	X	X	X
Prorocentrum minimum	X	X	X	X
Prorocentrum scutellum				X
Protoperidinium bipes		X		
Protoperidinium conicum			X	X
Protoperidinium sp.		X		
Scrippsiella trochoidea			X	X
Chlorophytes				
Ankistrodesmus falcatus			X	
Pyramimonas sp.		X		
Coccolithophores				
Rhabdosphaera hispida	X			
Cryptomonads				
Cryptomonas sp.	X	X	X	X
Cyanobacteria				
Anabaena sp.	X			
Dactylococcopsis raphidioides			X	
Euglenoids				
Euglena sp.	X		X	X
Silicoflagellates				
Ebria tripartita	X			
Ciliate (non algal)				
Myrionecta rubra	X	X	X	X

Appendix 2:

Detailed build sheet and parts list of flow-through chamber

- 2” diameter Schedule 40 PVC pipe
    - PVC cement (clear) and primer (purple) for all PVC fittings
- 2x2x2” diameter 45-degree schedule wye (2)
- 2”x3/4” PVC bushing fitting (2)
- ¾” x ½” PVC hose barn male adapter (2)
- ¾” inside diameter clear vinyl tubing
- 2” PVC cleanout adapter with plug
- 2” PVC cleanout Tee
- 2” PVC flexible Fernco end cap with SS clamp
- 2x 1 ½” PVC flexible Fernco reducing coupling with SS clamps
- Stainless steel hose clamps
- 3mm black vinyl mesh