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Detection of toxins and harmful algal bloom cells in shellfish hatcheries and efforts toward removal

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ABSTRACT

As the start of the supply chain for the aquaculture industry, hatcheries are a crucial component in the success of oyster and northern quahog (hard clam) aquaculture on the East Coast of the US. Intermittent failures in hatchery production slow industry growth and reduce profits. To begin investigations into the possible role of algal toxins in hatchery production failure, post-treatment hatchery water from one research and four commercial hatcheries in lower Chesapeake Bay, USA, was sampled for (1) toxin presence and (2) harmful algal bloom (HAB) cell enumeration. Overall, seven toxin classes, likely produced by six different HAB species, were detected in post-treatment hatchery water, despite a lack of visually identifiable HAB cells within the facility. Toxins detected include pectenotoxin-2, goniodomin A, karlotoxin-1 and karlotoxin-3, okadaic acid and dinophysistoxin-1, azaspiracid-1 and azaspiracid-2, brevetoxin-2, and microcystin-LR. In a second, more targeted study, two batches of source water were followed and sampled at each step of a water-treatment process in the VIMS Aquaculture Genetics and Breeding Technology Center research hatchery in Gloucester Point, Virginia, USA. Two treatment steps showed particular promise for decreasing the concentrations of the three toxins detected in the source water, 24-h circulation through sand filters and activated charcoal filtration. Toxin concentrations of pectenotoxin-2, 3.53 ± 0.56 pg mL^{-1}, okadaic acid, 6.14 ± 0.69 pg mL^{-1}, and dinophysistoxin-1, 1.88 ± 0.0 pg mL^{-1}, were low in the source water. The sand filtration step decreased these concentrations by 49–62%. Activated charcoal filtration subsequently brought the concentrations down to <0.5 pg mL^{-1}, successfully removing another 87–99% of toxins from incoming water. With toxin breakthrough now documented in commercial hatchery facilities during non-bloom conditions, future studies are needed to investigate breakthrough and water-treatment options during more-intense bloom conditions, as well as the potential interactions of algal toxins with other stressors in a potentially multifactorial etiology underlying hatchery production failures.

1. Introduction

Aquaculture in the United States (US) is an important industry; total sales of aquaculture products in 2018 was worth $1.5 billion (2018 Census of Aquaculture, USDA, 2019). Molluscan aquaculture (abalone, clams, mussels, oysters) accounts for approximately 30% of the total sales ($441.8 million) with 64% or $284.9 million of that representing oyster production. The primary species in culture on the East Coast of the US are the eastern oyster, *Crassostrea virginica*, and northern quahog or hard clam, *Mercenaria mercenaria,* with aquaculture of both reliant on the production and distribution of seed (and in oysters, eyed larvae) from shellfish hatcheries for subsequent production of marketable products by aquaculture farms (Hudson and Virginia Sea Grant Marine Advisory Program, 2019). It is, therefore, important to understand

Abbreviations: Azaspiracids, AZAs; Azaspiracid-1, AZA1; Azaspiracid-2, AZA2; Brevetoxin, PbTx; Brevetoxin-2, PbTx2; Domoic Acid, DA; Goniodomin A, GDA; Karlotoxins, KmTxs; Karlotoxin-1, KmTx1; Karlotoxin-3, KmTx3; Microcystins, MCs; Microcystin-LR, MC-LR; Microcystin-RR, MC-RR; Microcystin-YR, MC-YR; Okadaic acid, OA; Dinophysistoxin-1, DTX1; Dinophysistoxin-2, DTX2; Pectenotoxins, PTXs; Pectenotoxin-2, PTX2; Yessotoxin, YTX; Acetonitrile, ACN; Diatomaceous earth, DE; Methanol, MeOH; Solid-phase adsorption toxin tracking, SPATT; Ultra performance liquid chromatography-tandem mass spectrometry, UPLC-MS/MS; Trapping dimension, trap; At-column dilution, ACD.

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sources of production problems that shellfish hatcheries may encounter as well as potential solutions to those problems. In 2007 and 2008, commercial shellfish hatcheries along the US West Coast experienced major oyster larval mortalities that were eventually linked to decreased aragonite and calcite saturation states due to the upwelling of water with increased concentrations of carbon dioxide, a result of ocean acidification (Barton et al., 2015). The failure of the hatcheries to produce enough seed, in conjunction with low natural recruitment, presented an acute challenge to the shellfish aquaculture industry of that region (Barton et al., 2015). While less dramatic than the events on the US West Coast, hatcheries on the East Coast, as well, experience intermittent difficulties in producing enough seed to meet the demand of their customers (Gray et al., 2022). The causes of many of these failures or crashes, which can range from poor or slowed growth before reaching seed stage to larval mortality events, are often unknown (Gray et al., 2022).

Source water for the hatcheries is one potential cause of hatchery failures. Large volumes of seawater are required to produce oyster or clam seed. It is time and cost prohibitive to make artificial seawater, which in addition is not an ideal medium for shellfish larvae, so most hatcheries are located in coastal areas with easy access to natural seawater. The quality of the source water used is of utmost importance to insure the health and production of the oyster and clam seed, starting with the spawning of adult oysters and clams. There are many factors that can affect the quality of the source water. Water quality can be degraded by altered water chemistry due to environmental changes brought on through climate change as well as by the presence of harmful algae and/or their toxins. In 2009 and 2011, shellfish hatcheries in Virginia, USA, reported water quality issues with no known origin that resulted in a decrease in production (Hudson and Virginia Sea Grant Marine Advisory Program, 2019). More recently, an unusual period of low salinity was also reported to have negative effects on shellfish hatchery production in 2018 (Hudson and Virginia Sea Grant Marine Advisory Program, 2019).

The spawning season for aquacultured oysters and clams now spans from late winter through the spring and summer months in the lower Chesapeake Bay, USA, along the Mid-Atlantic region of the East Coast, where our intensive analyses have been focused. Early-season hatchery production coincides with seasonal abundance peaks of multiple toxigenic harmful algal bloom (HAB) taxa: *Dinophysis* spp., responsible for the production of okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxins (PTXs); *Pseudo-nitzschia* spp., responsible for the production of domoic acid (DA), and *Karlodinium veneficum*, responsible for the production of karlotoxins (KmTxs) (Glibert et al., 2007; Marshall et al., 2008; Wolny et al., 2020). Although early-season hatchery production in the Chesapeake Bay region does not coincide with the late-summer bloom season for *Alexandrium monilatum* (Wolny et al., 2020), it has been shown that the associated toxin, goniodomin A (GDA), can persist in the water through winter, spring, and early summer in the absence of detectable cells (Onofrio et al., 2021). Onofrio et al. (2021) also documented the presence of other HAB toxins in the lower Chesapeake Bay during the hatchery season, microcystins (MCs) and azaspiracids (AZAs).

The possible breakthrough of these marine and freshwater toxins into hatcheries has not yet been studied. Historically, there have been anecdotal observations of HAB cells breaching hatchery defenses (S. Shumway, pers. comm.). Only two studies, however, have published data on these breakthrough events. Deeds et al. (2002) documented breakthrough of live *K. veneficum* cells into a finfish hatchery in the Maryland portion of the Chesapeake Bay while Pease et al. (2021) documented live *K. veneficum* and *Prorocentrum cordatum* (previously *P. minimum*) cells in an oyster hatchery in the Virginia portion of the Bay. Given the co-occurrence of multiple toxins in the Bay during the hatchery season, and the deleterious effects observed in oyster larvae in laboratory studies (KmTxs: Glibert et al., 2007; Stoecker et al., 2008; Lin et al., 2017; Pease et al., 2021; GDA: May et al., 2010; Pectenotoxin-2 (PTX2): Gaillard et al., 2020; Pease et al., 2022), the breakthrough of toxins into hatcheries must be investigated if the role of toxins as a co-stressor to production is to be elucidated and solutions realized. Furthermore, the bioactivity of other toxins found in the Bay, OA and DTX1, AZAs, MCs, and DA (Onofrio et al., 2021), have not yet been studied in local bivalves, and so their discovery in hatcheries would highlight the need for toxicological studies in the near future. The objectives of this work were, therefore, to (1) determine the breakthrough of HAB toxins and cells into shellfish hatchery water through analyses at five commercial and research hatcheries, and (2) identify water-treatment practices that have the potential to reduce HAB cells and/or associated toxins within facility water.

2. Material and methods

2.1. Studies

Two studies were conducted as part of this work: (1) a hatchery survey of HAB cells and toxin breakthrough using quasi-structured sampling within five hatcheries, and (2) a focused time-series in one of those hatcheries where two batches of source water were sampled throughout the water-treatment process.

2.1.1. Study 1: hatchery survey

Water sampling, roughly once per month, was conducted in five shellfish hatcheries in Virginia, USA, during one production season (April – August 2018). Four of the hatcheries were commercial hatcheries and one was a research hatchery, the Virginia Institute of Marine Science Aquaculture Genetics and Breeding Technology Center (VIMS ABC) hatchery at Gloucester Point, Virginia. Diverse geographical representation was captured across these five hatcheries, including along both the western and eastern shores of lower Chesapeake Bay as well as in a coastal bay on the seaside of the Eastern Shore of Virginia. Hatcheries were included in the survey that produce *C. virginica* eyed larvae and seed, and/or seed of *M. mercenaria*. Each hatchery uses some combination of media and size-fraction filtration (1–50 μm nominal pore size) to treat, i.e., clean up, source water to be used for larval production and algal feed within the facility. More detail will not be presented herein regarding the hatchery locations or specific water-treatment methods to respect proprietary rights of the hatchery owners. Hatchery data will remain anonymous through their assignment of a randomized number; the exception is the VIMS ABC research hatchery, which is identified in this study as hatchery #2 (37.24769, −76.50466).

At each sampling, discrete whole-water samples (200 mL) of post-treatment hatchery water were collected. Water was stored in 250-mL HDPE bottles and frozen (−20 °C) for total toxin analysis, i.e., including both extracellular and intracellular toxins in the water. Post-treatment water was also collected for HAB species identification and enumeration using microscopy (whole water preserved with Lugol's Solution; Carolina Biological Supply) and DNA (i.e., quantitative or qPCR) analysis (100 mL water sample filtered onto a 3-μm Isopore membrane, frozen at −20 °C until analyzed). Solid-phase adsorption toxin tracking (SPATT) devices containing 3 mg of activated Diaion® HP-20 resin were constructed according to Onofrio et al. (2021) and deployed in a holding tank of post-treatment water for 17–51 days, averaging 28 ± 9 (mean ± STD) days. Time-integrated SPATTs were used to passively sample for extracellular toxins within the treated hatchery water over time for each facility, i.e., between discrete samplings. Diaion® HP-20 resin was chosen for this study as it has been used for a variety of marine and freshwater toxins of differing polarities and sizes (MacKenzie et al., 2004; Lane et al., 2010; Kudela, 2011; McCarthy et al., 2014; Roué et al., 2018). Three of the five hatcheries had holding tanks where the treated water was held until needed; more treated water was added periodically to replenish supply. Two hatcheries did not have a holding tank, but instead produced treated water on demand. At these two hatcheries, SPATTs were deployed in a bucket that was refilled

anytime new source water was treated (to match what occurs in the holding tanks of the other hatcheries). The amount of time each SPATT was deployed was recorded and the SPATT was rinsed with DI water before being frozen (−20 °C) until toxin extraction.

2.1.2. Study 2: sampling along the hatchery water-treatment process

In a second study, the source water for the VIMS ABC hatchery was tracked and sampled along the treatment process twice in May 2020 (Fig. 1). Discrete samples were collected as (1) whole water for total toxin analysis (1-L HDPE, frozen at −20 °C until extraction), (2) filters for intracellular toxin analysis (200 mL whole water filtered over a 47-mm GFF, frozen at −20 °C until extraction), and (3) whole water for HAB species identification and enumeration (as described in Section 2.1.1. Study 1: Hatchery survey). Sampling occurred within the hatchery after each of the following treatment steps: source water (step 1), 40-μm multicyclone filter (step 2), 24-h circulation through sand filter (step 3), 20-μm cartridge filter (step 4), 24-h circulation through diatomaceous earth (DE) filter (step 5), and 24-h circulation through ultraviolet (UV) sterilization (step 6), followed by either 1-μm sock filtration (step 7A; for use in larval tanks for seed production) or activated charcoal filtration (step 7B; for use in algal feed culturing with additional steps not investigated herein; Fig. 1).

2.2. Toxin extraction

Whole water, filters, and SPATTs were extracted for total, intracellular, and extracellular toxins, respectively. Samples of whole water for total toxin analysis were thawed and sonicated for 30 mins in a bath sonicator at <20 °C. Whole water (45–900 mL) was then loaded onto equilibrated Waters Oasis HLB (3 cc, 60 mg) solid phase extraction cartridges (Waters Inc., Milford, MA, USA) at a flow rate of <10 mL min^{-1}. Toxins were eluted off of the HLB using two 0.75-mL aliquots of 100% methanol (MeOH) that were pooled. Intracellular toxins were extracted from filters (Whatman 47-mm GF/F) in 100% MeOH using bath sonication at <20 °C, for 30 mins. Samples were centrifuged at 3200 rcf at 4 °C for 10 mins to separate the supernatant from the filter and cellular debris. The methanolic supernatant was collected and the remaining pellet discarded. SPATTs were sequentially extracted with 35% and 100% MeOH using 0.45-μm PVDF spin-filter centrifuge tubes (Thermo Fisher Scientific, Waltham, MA, USA) as described in Onofrio et al. (2021). An additional extraction step, using 10 mL of 100% acetonitrile (ACN), followed the SPATT extraction procedure to capture remaining PTX2 and brevetoxin-2 (PbTx2). All methanolic and ACN extracts were passed through a 0.22-μm, 13-mm PVDF syringe filter (Millipore Sigma, Burlington, MA, USA), and frozen until toxin analysis. For Study 2, subsamples of the whole-water and SPATT extracts underwent alkaline hydrolysis (Villar-González et al., 2008) to convert derivatives of OA, DTX1, and dinophysistoxin-2 (DTX2) into their parent toxins before analysis.

2.3. Toxin analysis

A suite of 15 different toxins, representing nine classes (Table 1), were investigated in whole-water, filter, and SPATT extracts. Analysis was carried out using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) equipped with a trapping dimension (trap) and at-column dilution (ACD) using the conditions described in detail in Onofrio et al. (2020). Instrumentation included a Waters Xevo TQ (tandem quadrupole) mass spectrometer (Waters, Milford, MA, USA) attached to a Waters Acquity UPLC, which consisted of an Acquity FTN Sample Manager, Acquity Column Manger and three I-Class Acquity Binary Solvent Managers (Waters, Milford, MA, USA). MRM transitions and mass spectrometry conditions listed in Onofrio et al. (2020) were used, including the additional MRM parent-daughter transitions listed in Onofrio et al. (2021). Domoic acid was analyzed in the 35% methanolic extracts using UPLC-MS/MS with trap/ACD by adding two additional transitions: *m/z* 311.99 - > 311.99, 30 V, 2 eV and *m/z* 311.99 - > 266.11, 30 V, 15 eV. As with all toxins in this study, the parent-daughter transition was used for quantification of DA.

Certified reference material was purchased from the National Research Council Canada (Halifax, Nova Scotia, Canada) for the following toxins: DA, azaspiracid-1 (AZA1), azaspiracid-2 (AZA2), OA, DTX1, DTX2, PTX2 and yessotoxin (YTX). The three MCs (microcystin-RR, MC-RR; microcystin-LR, MC-LR; and microcystin-YR, MC-YR) were purchased as a mixed standard from Sigma Aldrich. Brevetoxin-2 was purchased from Abcam. Karlotoxins, purified from *K. veneficum*, were provided by Dr. Allen Place (IMET, UMCES), and GDA, purified from *A. monilatum*, was provided by Drs. Constance and Thomas Harris (VIMS). Injection volumes ranged from 100 to 150 μL for Study 1, and toxins are presented as detected/not detected. Injection volumes for Study 2 were 150 μL for whole-water and filter extracts, and 200 μL for all SPATT extracts; quantitative results are reported for Study 2. All standard curves, check standards and blanks were prepared and run as described in Onofrio et al. (2020), with the exception that the standards used in creating standard curves were matched to the extraction solvent in the current study: standards were prepared in 35% MeOH, 100% MeOH, or 100% ACN. Samples with values less than the limit of detection (LOD) were represented as blank (or zero); concentrations that were less than the limit of quantification (LOQ) were represented as ½ LOD. See Onofrio et al. (2020) for information on LODs; all were <0.25 μg L^{-1} in vial, except karlotoxin-3 (KmTx3) which was 0.64 μg L^{-1}. Extracellular toxin (whole-water) data are presented as pg toxin mL^{-1} and SPATT toxin data is presented as pg toxin/g resin/day. Intracellular toxins (on filters) were below detection limits therefore cellular toxin data are not reported herein.

2.4. Cell enumeration – microscopy and qPCR

Source and treatment water were monitored for several toxigenic

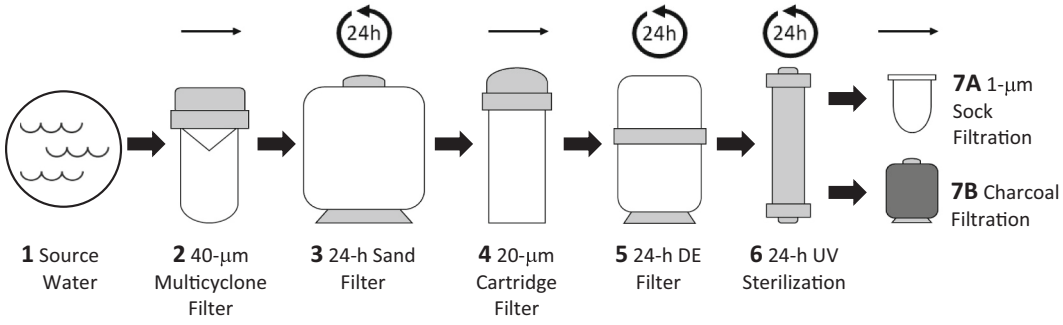

Fig. 1. Schematic of the water-treatment process at the Virginia Institute of Marine Science Aquaculture Genetics and Breeding Technology Center hatchery. Samples were collected in Study 2 at each numbered step for the analysis of toxins in whole-water samples (extracellular and intracellular) and filters (intracellular toxins), as well as for enumeration of HAB cells.

Table 1
Summary of the nine toxin classes that encompass the fifteen toxins analyzed and the suspected causative organisms from Chesapeake Bay (Marshall et al., 2008 and 2009; Wolny et al., 2020) that were targeted for identification in these studies. Results from study 1, the hatchery survey, are included for the presence/absence of toxins detected in whole-water samples and SPATTs, as well as cells detected either visually or with qPCR within hatchery-treated water.

Toxin Class	Toxins Analyzed	Toxins Detected		Potential HAB Species Responsible in Chesapeake Bay	Cells Detected	
		Total Toxin	SPATT		Visual	qPCR
Pectenotoxins (PTXs)	PTX2	+	+	*Dinophysis* spp.	–	
Goniodomins	GDA	+	+	*Alexandrium monilatum*	–	–
Karlotoxins (KmTxs)	KmTx1	+	–	*Karlodinium veneficum*	–	+
	KmTx3	+	–			
Yessotoxins (YTXs)	YTX	–	–	*Protoceratium reticulatum*, *Lingulodinium polyedrum*, *Gonyaulax* spp.	–	
Azaspiracids (AZAs)	AZA1	+	+	*Azadinium* spp., *Amphidoma* spp.		
	AZA2	+	+			
Brevetoxins	PbTx2	+	–	*Chattonella subsalsa*, *Chloromorum toxicum*	–	
Domoic Acids	DA	–	–	*Pseudo-nitzschia* spp.	–	
Okadaic Acid and derivatives	OA	+	+	*Dinophysis* spp., *Prorocentrum lima*	–	
	DTX1	+	+			
	DTX2	–	–			
Microcystins (MCs)*	MC-LR	+	+	*Microcystis* spp., *Dolichospermum* spp., *Oscillatoria* spp.	–	
	MC-RR	–	–			
	MC-YR	–	–			
				Prorocentrum cordatum^	+	+
				Margalefidinium polykrikoides^	–	–
				Gyrodinium instriatum^	–	
				Phaeopolykrikos hartmannii^	–	

+ indicates the presence of a toxin or causative organism; – indicates "not detected" but tested or monitored; gray shading indicates "not applicable;" * freshwater toxin class; ^ HAB species lacking a characterized bioactive chemical.

and otherwise harmful algae (Table 1) that have been reported in Chesapeake Bay (Marshall et al., 2008 and 2009; Wolny et al., 2020). HAB cells were enumerated using either microscopy or qPCR. For the former, a 1-mL Sedgewick Rafter counting chamber and light microscopy at 100× magnification using an Olympus 1 × 51 with Olympus DP73 digital camera (Center Valley, PA, USA) were used. During the hatchery survey, live samples when possible were used for initial observation and identification, e.g. based on swimming pattern. Quantification of *A. monilatum* (Vandersea et al., 2017), *K. veneficum* and *P. cordatum* (Pease et al., 2021), as well as *M. polykrikoides* (Wolny et al., 2020), was also conducted using qPCR analysis of extracted DNA as previously described. Samples were considered positive for DNA of the targeted species if the threshold cycle (C_t) of duplicate qPCR assays for a DNA sample fell within the standard curve, with the lower detection limits of the curves ranging from 0.05 cells ml^{-1} for *K. veneficum* to 0.90 cells ml^{-1} for *P. cordatum*. Molecular methods for the other HAB species have not yet been optimized for the Bay.

3. Results

3.1. Study 1: hatchery survey

The total toxin and SPATT toxin data are reported as presence/absence in Study 1 due to the nature of the sampling: scheduling and methods adjusted to fit within the daily routine and system of each hatchery, and the variability in individual hatchery season commencement and duration. These data, are therefore, meant to be a survey of five hatcheries, and not a robustly quantitative assessment or inter-hatchery comparison. During the hatchery survey, none of the five hatcheries reported larval mortality events or other problems with production.

In the whole-water samples, 10 of the 15 toxins that were analyzed were detectable during some part of the hatchery season in at least one of the five hatcheries (Fig. 2 and Table 1). Pectenotoxin-2, OA, and DTX1 were ubiquitous (Fig. 2A and D), being detected in every sampled month at all five hatcheries. Karlotoxins were the next most prevalent class, with detection in 66% of the samples (Fig. 2C), and detected at least once at every hatchery; two hatcheries had KmTxs detected in every month sampled. In three of the five hatcheries, GDA was detected, representing 33% of the samples (Fig. 2B). Azaspiracid-1 and AZA2 (Fig. 2E) and PbTx2 (Fig. 2F) were present at several hatcheries, but only in May. One hatchery had detectable MC-LR later in the season, in June and July (Fig. 2G). There was no detection of YTX, DTX2, DA, MC-RR or MC-YR in whole-water samples from Study 1.

Results from SPATT deployed in post-treatment water in the five hatcheries showed similar patterns to the whole-water samples (Fig. 3 and Table 1). Once again, PTX2 (Fig. 3A), OA and DTX1 (Fig. 3C) were detected in every SPATT sample at every hatchery. Goniodomin A, detected in 56% of the samples (Fig. 3B), and AZAs, detected in 44% of the samples (Fig. 3D), were present in three of the five hatcheries. Microcystin-LR was only detected in one hatchery in SPATTs deployed during June and July (Fig. 3E). Of note, there were no KmTxs or PbTx2 detected on the SPATT samples although these toxins were detected in the whole-water samples. In agreement with whole-water samples, there was no detection of YTX, DTX2, DA, MC-RR or MC-YR in SPATT samples from Study 1.

Microscopic analysis for HAB species in the treated hatchery water resulted in visual confirmation for only one species, *P. cordatum*, in one hatchery during April and May at a concentration of 25 cells mL^{-1} (Table 1). The qPCR analysis, however, was more sensitive, detecting the DNA of *K. veneficum* and *P. cordatum* in almost every sample of treated hatchery water, across three of the five hatcheries (Table 1). The estimated concentration ranged from <1 to 330.8 cells mL^{-1} for *K. veneficum* and from 2.1 to 22.8 cells mL^{-1} for *P. cordatum*.

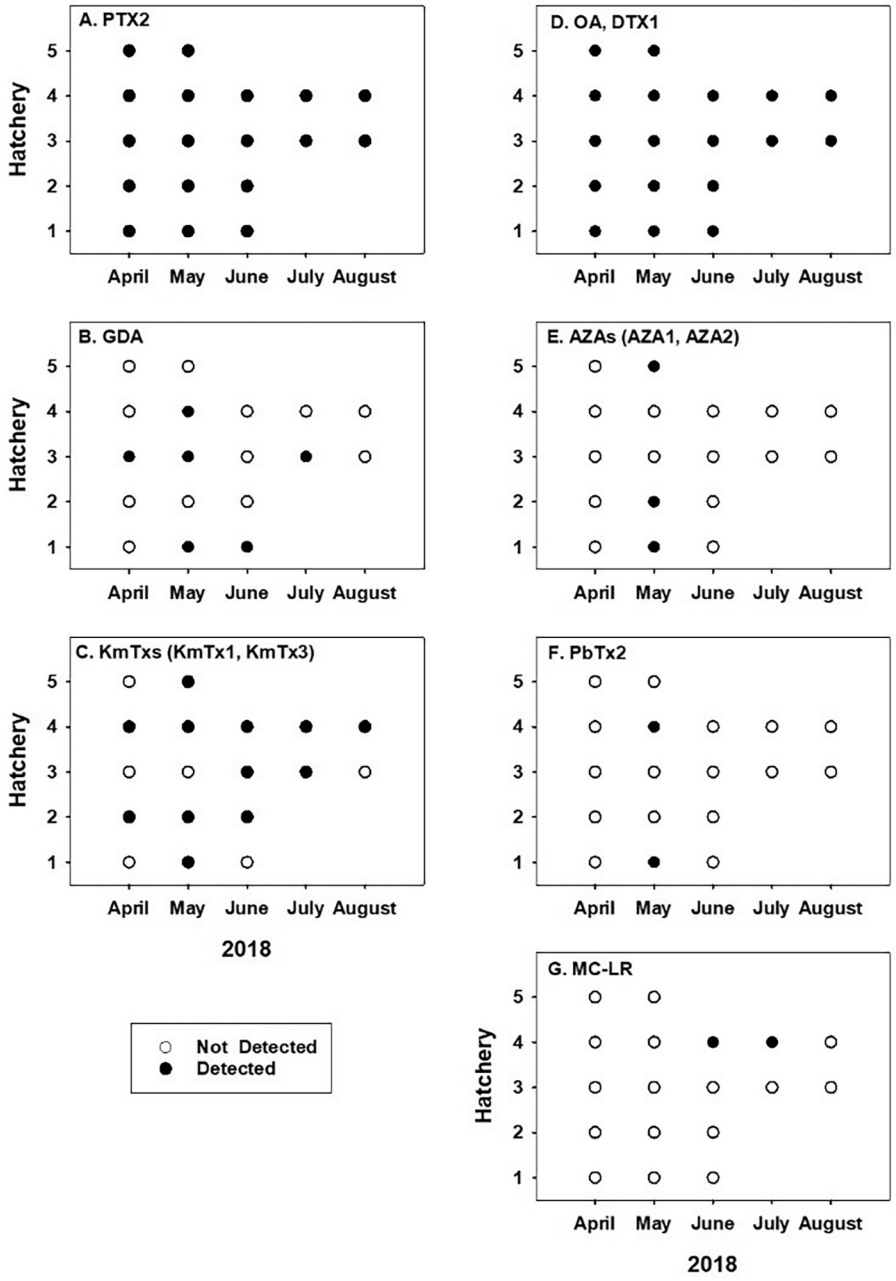

Fig. 2. Study 1 - Presence (solid circle) or absence (open circle) of the detected toxins from whole-water samples (intracellular + extracellular) during a survey of treated water from within five hatcheries (randomly assigned a hatchery number 1 through 5) during their main shellfish production season of 2018 (April–August). The lack of a circle indicates no sample was collected.

3.2. Study 2: sampling along the hatchery water-treatment process

During the two sampling events at the VIMS ABC hatchery in Study 2, three toxins were quantifiable in the total toxin samples: PTX2 (Fig. 4A), OA (Fig. 4B), and DTX1 (Fig. 4C). Source water had the highest concentration of OA, 6.14 ± 0.69 pg mL^{-1} (here and elsewhere, mean ± STD), followed by PTX2, 3.53 ± 0.56 pg mL^{-1}, and DTX1, 1.88 ± 0.0 pg mL^{-1}. These concentrations dropped to 2.57 ± 0.13, 0.66 ± 0.09, and 0.33 ± 0.03 pg mL^{-1}, respectively, after the 1-μm sock filtration (step 7A). When the treatment concluded with activated charcoal filtration (step 7B), the concentrations decreased to 0.42 ± 0.60, 0.02 ± 0.03, and 0.07 ± 0.09 pg mL^{-1}, for OA, PTX2, and DTX1, respectively. The first treatment in the sequence that exhibited the greatest removal of toxins was step 3, 24-h circulation through sand filters, where concentrations were roughly halved from the source values. Activated charcoal filtration, step 7B, was the second most effective treatment step in the sequence (Fig. 4). There were no measurable intracellular toxins in the filter samples for either sampling event, including the source water.

Visually, no HAB cells were detected within the VIMS ABC hatchery in Study 2. Based on quantitative real-time PCR data, *P. cordatum* DNA was present at steps 1–4, before being fully removed by step 5, the DE filter, whereas, *K. veneficum* DNA was detected further in the sequence, to step 6, before being eliminated in either of the final two filtration steps, the 1-μm sock or activated charcoal. Excluding the source water, cell concentrations based on qPCR were all <1 cell mL^{-1} with one exception, when *P. cordatum* was measured at 4.2 cells mL^{-1} after step 2, the 40-μm multicyclone filter, during the second sampling event

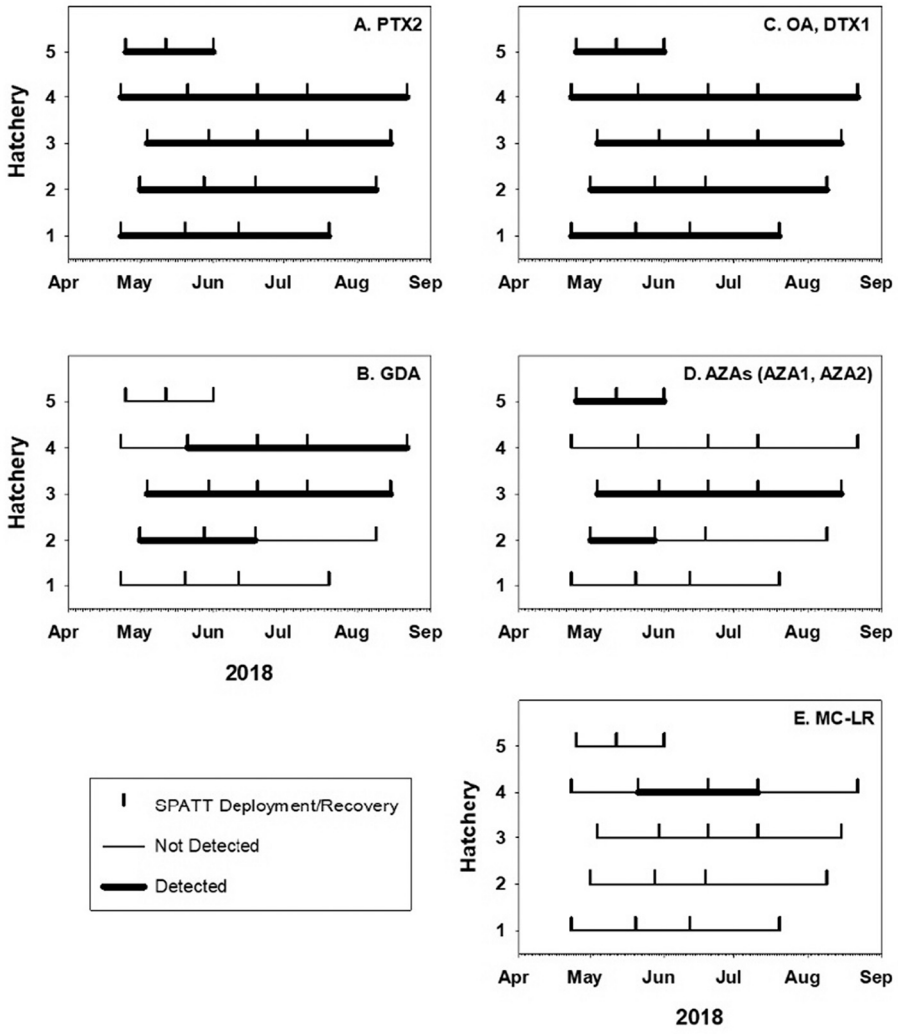

Fig. 3. Study 1 - Presence (thick horizontal line) or absence (thin horizontal line) of the detected toxins in SPATT samples during a survey of treated water from within five hatcheries (randomly assigned a hatchery number 1 through 5) during their main shellfish production season of 2018 (April–August). Vertical lines represent the deployment and/or recovery date of the SPATT deployment.

(Table 2).

4. Discussion

This study is the first to report the breakthrough of HAB toxins, and one of two studies to confirm the breakthrough of HAB cells, into oyster and clam hatcheries. Importantly with regard to mitigation of potential HAB impacts, it is also the first study to examine the efficacy of individual water-treatment steps to remove HABs and toxins from water used for shellfish and algal feed production. Breakthrough of ten toxins, representing seven toxin classes, and two HAB species was observed in five shellfish hatcheries during the 2018 production season. A more targeted study of the water-treatment steps in one hatchery during 2020 found two steps, 24-h circulation through sand filters and activated charcoal filtration, to be the most effective at removal of the toxins detected. While many toxins were present and co-occurring in hatchery water, no significant larval crashes were reported during the study. Hatcheries appeared to have sufficient tools available, therefore, to remove the majority of algal toxins within source water during at least low-bloom years (<1000 cells mL^{-1}), with 58–99% overall removal in the research hatchery.

4.1. Breakthrough of toxins and HAB cells into shellfish hatcheries

4.1.1. Toxin breakthrough

Five of the seven toxin classes (i.e., six of the ten toxins) detected in treated hatchery water (Table 1) have been previously reported to have negative effects on shellfish larvae or oocytes: PTX2 (Gaillard et al., 2020; Pease et al., 2022), GDA (May et al., 2010), KmTxs (Glibert et al., 2007; Stoecker et al., 2008; Lin et al., 2017; Pease et al., 2021), OA (De Rijcke et al., 2015), and brevetoxin (PbTx; Leverone et al., 2006). The remaining two toxin classes, AZAs and MCs, have not been fully investigated for impacts to bivalves, especially in their early life stages. There have, however, been reports of freshwater MC transport into marine systems, resulting in detrimental effects for marine animals through trophic transfer (Miller et al., 2010), and adult blue mussels (*Mytilus edulis*) displayed increased mortality, reduced motility, decreased filtration rate, and increased pseudofeces production upon exposure to azaspiracid-producing *Azadinium spinosum* (Jauffrais et al., 2012).

In the two hatchery studies, the most prevalent or abundant toxins detected were PTX2, OA, and DTX1. These toxins were present in 100% of whole-water and SPATT samples during each month sampled at all five hatcheries in Study 1, as well as throughout the water-treatment process in the VIMS ABC hatchery in Study 2. To begin investigations into any contribution these toxins may have toward unexplained

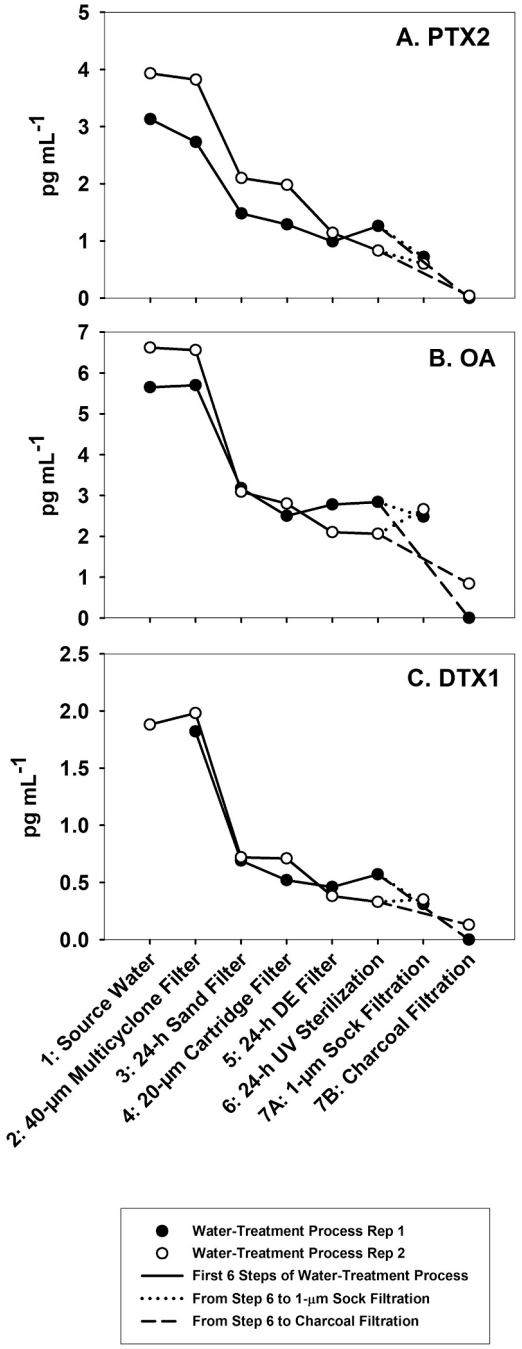

Fig. 4. Study 2 - Concentrations (pg mL^{-1}) of toxins (A) PTX2, (B) OA, and (C) DTX1 in whole-water samples (intracellular + extracellular) collected at each step of the VIMS ABC hatchery water-treatment process. Solid circles represent the first batch of water sampled along the water-treatment process (Rep 1), the open circles represent the second batch of water (Rep 2). The solid line represents the first six steps of the water-treatment process. The dotted line represents the divergence of water to the last steps, taken in parallel: (step 7A) a 1-µm sock filter (water to be used for larval production), and (step 7B) activated charcoal filtration (water to be used for feed algae culturing).

hatchery failures, we must turn to the literature. Gaillard et al. (2020) demonstrated that exposure to PTX2 concentrations as low as 5 nM (equivalent to 4295 pg mL^{-1} PTX2) decreased fertilization success of Pacific oyster *Crassostrea gigas* oocytes. Additionally, Pease et al. (2022) demonstrated increased mortality and reduced motility in *C. virginica* larvae due to PTX2; however, they observed no effects due to OA exposure (toxin concentrations were equivalent to a range up to 10,000

Table 2
Study 2 - Concentrations (cells mL^{-1}) of *Karlodinium veneficum* and *Prorocentrum cordatum,* based on qPCR analysis, in two batches of water along the water-treatment process at the VIMS ABC hatchery.

qPCR (cells mL^{-1})			
		Karlodinium veneficum	*Prorocentrum cordatum*
May 11–14, 2020	**1** Source Water	0.49	2.0
	2 40- µm Multicyclone Filter	0.45	0.95
	3 24-h Sand Filter	0.58	0.99
	4 20- µm Cartridge Filter	0.48	0.81
	5 24-h DE Filter	0.02	0
	6 24-h UV Sterilization	0	0
	7A 1- µm Sock Filtration	0	0
	7B Charcoal Filtration	0	0
May 25–28, 2020	**1** Source Water	0.50	1.5
	2 40- µm Multicyclone Filter	1.0	4.2
	3 24-h Sand Filter	0.29	1.1
	4 20- µm Cartridge Filter	0.11	0.42
	5 24-h DE Filter	0	0
	6 24-h UV Sterilization	0.01	0
	7A 1- µm Sock Filtration	0	0
	7B Charcoal Filtration	0	0

cells mL^{-1} of *Dinophysis acuminata*). Larval viability of the blue mussel, *M. edulis*, however, was significantly reduced by exposure to OA concentrations of 37.8 µg L^{-1} (equivalent to 37,800 pg mL^{-1} OA; De Rijcke et al., 2015). There have been no studies thus far on the effects of DTX1 on shellfish larvae. Toxin concentrations in hatchery-treated water for use in larval production (Study 2, treatment through step 7A) were very low, 0.66 ± 0.09, 2.57 ± 0.13, and 0.33 ± 0.03 pg mL^{-1} for PTX2, OA, and DTX1, respectively. The concentrations for PTX2 and OA in this level of hatchery-treated water were roughly four orders of magnitude less than the concentrations used by Gaillard et al. (2020), 5nM = 4295 pg mL^{-1} PTX2, and De Rijcke et al. (2015), 37.8 µg L^{-1} = 37,800 pg mL^{-1} OA, in their studies demonstrating harmful effects on shellfish larvae. While some impacts of these toxins have been confirmed in laboratory settings, their extrapolation to hatchery-level production effects is still uncertain.

Goniodomin A was also detected in every month from April through August, although not in every hatchery (Study 1), with detection in 33% and 56% of the whole water and SPATT samples, respectively. A study of the GDA-producing dinoflagellate, *A. monilatum*, impacts on *C. virginica* and *M. mercenaria* larvae showed that while larvae were tolerant of whole cells, exposure to lysed cells (GDA presumably present in the lysate) resulted in 62% mortality of clam larvae, and 10% mortality of oyster larvae (May et al., 2010). The breakthrough of the toxin GDA into shellfish hatcheries has the potential to cause larval mortality. It is important to point out, however, that the concentration of GDA breakthrough into hatcheries remains unknown, as these data were collected as presence/absence, and therefore cannot be used to estimate impacts to production at hatchery level.

In this study, AZAs were detected in whole-water samples of hatchery-treated water at three hatcheries during May (Study 1); SPATTs had detectable AZAs in two of the same hatcheries, and the third positive SPATT sample was from a different hatchery. The occasional mismatch of toxin presence can likely be explained by sample collection method. Whole water samples are discrete samples, or "snapshots," that show what toxins were present at the time of collection; whereas SPATT samples integrate toxins over the time of deployment, and therefore represent multiple batches of treated water (28 ± 9 days deployed).

The freshwater toxin, MC-LR, was detected in one hatchery during the summer (June and July of 2018) in both whole-water and SPATT samples. The presence of this toxin is most likely due to the excessive rainfall events in coastal Virginia during the spring and summer of 2018 (National Oceanic and Atmospheric Administration, National Weather Service, NOAA Online Weather Data (NOWData), 2022), flushing the causative freshwater HAB species and/or the toxins from upstream rivers and lakes into the tributaries that flow into the bay. Pruett et al. (2021) demonstrated that MC-LR up to concentrations of 15 μg L^{-1} had no effect on the early life stages of *C. virginica*; therefore, the presence of MC-LR may not be of importance to shellfish hatchery production.

Whole-water samples contained KmTxs and PbTx2, however these toxins were not detected in the SPATT samples. This discrepancy is likely due to the instability of KmTxs on HP-20 resin and the poor recovery of PbTx2 (Onofrio, 2020). Karlotoxins were the second most prevalent toxin class detected in whole-water samples (66%), being detected at least once in all five hatcheries and all sampled months. Exposure of the KmTx-producing HAB species, *K. veneficum*, to larvae of *C. virginica* resulted in significant mortality of the larvae (Glibert et al., 2007; Stoecker et al., 2008; Lin et al., 2017; Pease et al., 2021). Stoecker et al. (2008) reported the concentration of KmTxs in the *K. veneficum* exposed to the oyster larvae to be approximately 5 ng mL^{-1}. Brevetoxin-2 was only present in two hatcheries during May. Larval mortality of *C. virginica* and *M. mercenaria* was demonstrated by Leverone et al. (2006) with exposure to *Karenia brevis* containing concentrations of PbTx from 53.8 μg L^{-1} in cells to 68.9 μg L^{-1} in lysate. *Karenia brevis* is not found in the lower Chesapeake Bay, however, other PbTx producing HABs are found in the bay, i.e. *Chatonella subsalsa* and *Chloromorum toxicum,* thereby suggesting that the breakthrough of PbTx may be of potential concern to shellfish hatcheries.

4.1.2. HAB cell breakthrough

The causative HAB species for six of the toxins detected in the hatchery treated water (Study 1) have been reported as common bloom forming species in the lower Chesapeake Bay during the seasons of this study, late spring and early summer: dinoflagellates *Dinophysis* spp. (PTX2, OA, and DTX1) and *K. veneficum* (KmTx1 and KmTx3), as well as cyanobacterium *Microcystis* spp. (MC-LR) in the less saline tributaries of the bay (Marshall et al., 2008). Raphidophytes *C. subsalsa* and *C. toxicum* (PbTx2) are also reported in the lower Chesapeake Bay during these months (Marshall et al., 2009). However, with the exception of *K. veneficum* (<1–331 cells mL^{-1}, well below typical bloom concentrations of >1000 cells mL^{-1}), none of these HAB species were detected in the hatchery-treated water. Similarly, there were no reported toxigenic HAB blooms during April – August of 2018 in the lower Chesapeake Bay and the coastal bays of Virginia, with the exception of three short-lived, sporadic bloom patches of *K. veneficum* in the York River during May, June, and July (K. Reece, unpublished data). It is therefore uncertain if the lack of cells within hatchery water was due to efficient water-treatment or reduced HAB abundance.

There was no visual observation or qPCR detection of the late summer species *A. monilatum*, however, GDA was detected. This HAB species typically does not bloom until mid- to late August (Wolny et al., 2020), therefore the timing of GDA in April–August in the first study is offset from the normal bloom season. These data support earlier findings of the chemical persistence of GDA in the Bay (Onofrio et al., 2021). The presence of AZAs cannot be associated with the presence of a particular HAB species because the causative organisms have not yet been identified in the Bay, and the potential producers, *Azadinium* spp. and *Amphidoma* spp., are too small and non-descript to be visually identified using traditional microscopy. Work is currently underway to develop a qPCR method for the detection of these taxa in the Bay based on earlier work by Kim et al. (2017).

The only HAB species detected in the treated hatchery water beside *K. veneficum* was *P. cordatum*, which does not produce a known toxin. Cells of *P. cordatum* were visually detected in one hatchery (25 cells mL^{-1}) and by qPCR in three hatcheries, representing 66% of the samples, with cell concentrations based on qPCR amplification ranging from 2.1 to 22.8 cells mL^{-1}, also well below typical bloom concentrations. There were, however, two small blooms of *P. cordatum* observed in early April 2018 in the lower Chesapeake Bay with cell concentrations ranging from 1000 to 2000 cells mL^{-1} (K. Reece, unpublished data). These are the same two HAB species previously reported inside an oyster hatchery (Pease et al., 2021); however, in that study, cell concentrations were sometimes found at bloom levels (≥ 1000 cells mL^{-1}). The previous study also investigated the effect of a range of cell densities on *C. virginica* larvae, finding that at the lowest densities tested for *K. veneficum* (1000 cells mL^{-1}) and *P. cordatum* (100 cells mL^{-1}), larval motility was impaired. No larval mortality was seen with *P. cordatum* densities up to 50,000 cells mL^{-1}, however, oyster larvae mortality ranged from 21%, at *K. veneficum* concentrations of 1000 cells mL^{-1}, to >83% at concentrations from 5000 to 50,000 cells mL^{-1} (Pease et al., 2021). Although hatcheries use size exclusion filters and/or media to treat their source water, some of these small cells, <20 μm in length, appear to make it through the treatment steps. Likewise, it is possible that other HAB species with cell sizes <20 μm, e.g. *Azadinium* spp. and *Amphidoma* spp., could also be making it past the treatment steps but are undetectable using current methods.

These results, showing the presence of toxins in the absence of the causative HAB species, with the exception of KmTxs, suggest that these toxins were extracellular and either entered the hatchery in dissolved form, or HAB cells were lysed in the water-treatment process, thereby releasing their toxins into the water. Extracellular, or dissolved, PTX2, OA, DTX1, GDA, and AZA2 have been reported to persist year-round in the lower Chesapeake Bay and coastal bays of Virginia (Onofrio et al., 2021) in the absence of detectable cells. Microcystin-LR was also detected in both Chesapeake Bay (Onofrio et al., 2021) and in one of the hatcheries in 2018, but only in the late summer, presumably the result of episodic bloom events in the freshwater reaches of tributaries (Onofrio et al., 2021).

4.2. Efforts toward toxin and HAB cell removal

During Study 2, the focus shifted to identifying specific water-treatment options that may remove toxins and/or cells from source water. The goals were twofold, first to determine if toxins or cells that are present in the source water are subsequently being removed and/or lysed during the hatchery water-treatment process, and second to determine the most effective treatment steps for removal of detected toxins and HAB cells. Tracking a mass of source water through each step of the water-treatment process was completed twice in May 2020.

4.2.1. Toxin removal

The water-treatment process removed the majority of PTX2, OA, and DTX1 from source water (albeit the initial concentrations were quite low), decreasing overall concentrations by 58–99% in the two final water types used for production and feed algae, respectively (Fig. 4). The media cartridges (sand, DE, and activated charcoal; steps 3, 5, and 7B, respectively) appear to be more effective than the size exclusion steps (40-μm multicyclone, 20-μm size filter; steps 2 and 4, respectively) at toxin reduction, evidence that the toxins were introduced into the hatchery extracellularly in dissolved form and not via algal cells. Sand filtration and activated charcoal filtration were the most effective at reducing the toxins detected in source water. Overall, concentrations of PTX2, OA, and DTX1 decreased by 81.3, 58.1, and 82.4%, respectively, from source water (step 1) to a 1-μm sock filtration (step 7A); this level of treated water would be typical for larval production in the research hatchery. These results suggest that while sand filtration is already removing 49–62% of the toxins from water during a low-bloom year, the addition of an activated charcoal filtration step (step 7B) to the water-treatment will further decrease these PTX2, OA, and DTX1 concentrations down to 99.4, 93.2, and 96.3% respectively.

4.2.2. HAB cell removal

No HAB cells were detected microscopically in any of the samples collected at the water-treatment steps, including the source water, limiting inferences into the effects of specific water-treatment steps on HAB cell removal. However, based on quantitative real-time PCR data, *P. cordatum* DNA was able to make it all the way through the 20-μm cartridge (step 4) before being fully removed by the DE filter (step 5). Step 5 also serves as a 2–5 μm size filter, and appears to have captured any remaining *P. cordatum* cells. The unthecated *K. veneficum,* or more likely its DNA, was able to make it through to the second to last step, 24-h recirculating UV sterilization (step 6), before being eliminated in either the 1-μm sock (step 7A) or activated charcoal filtration step (7B). Concentrations for both species via qPCR were < 5 cell mL^{-1} for all steps, including source water, confirming no bloom was present external to the facility. With such low cell concentrations, the microscopic analysis would not have detected any cells due to a detection limit of 1 cell mL^{-1}, showing the limitations of visual microscopy. The only other study to date on HAB cell breakthrough in a shellfish hatchery, Pease et al. (2021), measured cell concentrations of *P. cordatum* in different types of treated hatchery water (mixed-media, 10 μm, and feed algae) ranging from <1 to 3630 cells mL^{-1}; however, they did not report the cell concentrations in the source water. The previous study also reported *K. veneficum* ranging from <1 to 1094 cells mL^{-1} in the hatchery treated water, showing that the species could reach bloom levels within the facility.

4.3. Need for future studies to help improve shellfish hatchery production

These studies were conducted in two years without significant HAB blooms, and therefore, cell densities of HAB species, as well as toxin concentrations, were very low. Even so, breakthrough of HAB toxins and cells was observed into shellfish hatcheries. The following toxins were detected in five hatcheries across Virginia during the 2018 production season: PTX2, OA, DTX1, GDA, KmTx1 & 3, AZA1 & 2, PbTx2, and MC-LR. Water treatment steps at the VIMS ABC hatchery effectively reduced the concentrations of PTX2, OA, and DTX1 in water, the only quantifiable toxins during this part of the 2nd study in 2020. Two water-treatment steps in particular proved to be the most effective, sand and activated charcoal filtration; however, the latter treatment is considered to be relatively costly and is not a commonly-used step in most small to moderate-scale facilities. More needs to be known regarding the breakthrough and removal of these toxins and HAB cells under moderate to high bloom conditions, and under a variety of treatment regimes, to determine if commercial treatment systems can withstand higher loads of toxins, debris, and dissolved organic carbon that can accompany blooms.

Also of note, several toxins were detected in hatchery-treated water at the same time, and therefore, the effects of these toxins alone and combined (co-exposure studies) should be considered in order to fully assess their effects on shellfish larvae. Little to no shellfish larval toxicity data are available for AZAs, MCs, and various PTXs analogues, and co-exposure studies are near absent (Pease et al., 2021, 2022). Any additional studies should be conducted at environmentally-relevant levels: low cell densities and toxin concentrations as seen in these studies, as well as concentrations that are relevant during bloom conditions, in order to elucidate any potential effects on the production of shellfish in hatcheries.

5. Conclusion

The production failures that continue to arise as a challenge to expanding shellfish aquaculture production remain enigmatic as to causes (Gray et al., 2022). Altered microbiomes, reduced seawater pH, and the activity of specific microbial pathogens are all prominent on the list of stressors deserving further study as we seek to resolve these vexing phenomena in support of increased aquaculture sustainability. Harmful algal cells and their toxins should be included in this effort to understand the multifactorial etiology driving production failures, as bloom levels have been detected within hatcheries, bloom equivalent densities have been linked to immobility and mortality (Pease et al., 2021), and chronic effects are unknown for all of the toxins detected herein within hatchery waters.

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Author contributions

Marta P. Sanderson: Methodology; Validation, Formal analysis, Investigation, Writing – Original draft, Writing – Review and editing, Visualization, Supervision; Karen L. Hudson: Conceptualization, Methodology, Investigation, Writing – Review and editing, Supervision, Funding acquisition; Lauren S. Gregg: Investigation, Writing – Review and editing, Visualization; Amanda B. Chesler-Poole: Investigation, Writing – Review and editing; Jessica M. Small: Investigation, Writing – Review and editing, Supervision; Kimberly S. Reece: Conceptualization, Methodology, Resources, Writing – Review and editing, Funding acquisition; Ryan B. Carnegie: Conceptualization, Methodology, Writing – Review and editing, Project administration, Funding acquisition; Juliette L. Smith: Conceptualization, Methodology, Resources, Writing – Original draft, Writing – Review and editing, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Highlights of the manuscript

Shellfish hatchery production is the first crucial step in shellfish aquaculture regionally, and therefore, the potential breakthrough of harmful algae and/or toxins into hatcheries was investigated. This is the first study to observe the breakthrough of toxins that are of concern to shellfish health and one of two studies to observe breakthrough of HAB cells into commercial shellfish hatcheries. This is also the first study to investigate the efficacy of a hatchery water-treatment process for removing toxins and/or HAB cells. Two steps, sand filtration and charcoal filtration, successfully reduced low initial toxin concentrations by >49% and > 87%, respectively.

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.

Data availability

Data will be made available on request.

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