An on-farm workflow for predictive management of Paralytic Shellfish Toxin-producing harmful algal blooms for the aquaculture industry

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Abstract

Paralytic Shellfish Toxins (PSTs) produced by marine dinoflagellates significantly impact shellfish industries worldwide. Early detection on-farm and with minimal training would allow additional time for management decisions to minimise economic losses. Here we describe and test a standardised workflow based on the detection of *sxtA4*, an initial gene in the biosynthesis of PSTs. The workflow is simple, inexpensive and does not require a specialised laboratory. It consists of: 1) water collection and filtration using a custom a gravity sampler; 2) buffer selection for sample preservation and cell lysis for DNA; and 3) the Phytoxigene™ DinoDtec lypholised qPCR assay. Water samples spiked with *Alexandrium catenella* showed cell recovery was > 90% when compared to light microscopy counts. The performance of the lysis method (90.3% efficient), Longmire's buffer, and the DinoDtec qPCR assay (tested across a range of *Alexandrium* species (90.7 to 106.9% efficiency; $r^2 > 0.99$)), were found to be specific, sensitive and efficient. We tested the application of this workflow weekly from May 2016 to 30th October 2017 to compare the relationship between *sxtA4* copies L^{-1} in seawater and PSTs in mussel tissue (*Mytilus galloprovincialis)* on farm, and spatially (across multiple sites), effectively demonstrating a ~2-week early warning of two *A. catenella* HABs (r = 0.95). Our tool provides an early, accurate and efficient method for the identification of PST risk in shellfish aquaculture.

Key words: Paralytic Shellfish Toxins (PSTs), Harmful Algal Blooms (HABs), aquaculture industry, *Alexandrium* spp., *sxtA4* gene, molecular detection, on-farm workflow

Synopsis: This study presents a practical, three-step on-farm workflow utilising the *sxtA4* gene for the early, efficient and cost-effective prediction of Paralytic Shellfish Toxin risk in shellfish aquaculture, enhancing industry preparedness and safeguarding economic loss.

1. Introduction

Paralytic Shellfish Toxins (PSTs) comprising saxitoxin and its analogues, are a group of neurotoxic alkaloid compounds responsible for the syndrome Paralytic Shellfish Poisoning (PSP) [1]. PSTs are produced by certain marine dinoflagellates, and their accumulation in shellfish has resulted in severe economic impacts through farm closures and product recalls worldwide [1-4]. In Australia, a leading PST-producing species is *A. catenella* (Group 1). This species was first implicated in a recall of blue mussels (*Mytilus galloprovincialis*) from Tasmania which were contaminated with PSTs, resulting in an estimated $AUD 23 M loss [5]. Since then, *A. catenella* blooms have re-occurred seasonally in Tasmania, with the highest concentration of PST ever recorded in 2017, at 150 mg kg^{-1}, over 150 times above the regulatory level of 0.8 mg kg^{-1} [6].

Shellfish safety monitoring for harmful algal blooms (HABs) and their toxins as implemented by seafood safety programs worldwide commonly rely on a few main techniques. The first is the identification of harmful algal species in seawater using light microscopy, but discriminating between species requires highly specialised skills, and misidentification can result in unnecessary farm closures [7, 8]. The second technique is the detection of PSTs in shellfish using chemical methods such as liquid chromatography with tandem mass spectrometry (LC-MS/MS and LC-MS) [9]. This method is time consuming, expensive, and not suitable for use on-farm. More rapid, cost-effective, and on-farm testing methods would therefore make on-farm shellfish harvest management simpler, faster and result in fewer closures. Methods to rapidly detect PSTs in shellfish tissue have been developed based on enzyme-linked immunosorbent assays (ELISA), lateral flow approaches and biosensors [10-14]. However, there is known to be a time-lag of at least several days, or longer between the presence of PST-producing species in the water column and a measurable toxin concentration in shellfish tissue [15]. Another technique using molecular probe technology, is the Environmental Sample Processor (ESP), a deployable automated sampling device for molecular analyses *in situ*, yet this is still considered in its developmental infancy and expensive. Overall, there is a need for an inexpensive early warning system, which would allow sufficient time for appropriate shellfish harvesting management decisions [15].

Molecular detection of toxic dinoflagellates can be performed using environmental DNA (eDNA) and quantitative Polymerase Chain Reaction (qPCR) assays based on barcoding markers, for example the rRNA array [16-19]. Using this method, cryptic species such as *A.*

catenella can be quantified and discriminated from other co-occurring species [7, 19]. However, rDNA-based qPCR assays may not always be the most appropriate means of quantification, as copy numbers of rRNA genes can vary by 3-5 orders of magnitude within a species [20-23]. An assay based on a gene involved in PST biosynthesis [24-26] such as *sxtA4* [27], may be more reliable as the variation in genomic copy numbers may be less than that of ribosomal RNA genes. While copy numbers of *sxtA4* genes show variation, it is of a lesser degree, up to one order of magnitude [21, 22]. Moreover, a positive correlation has been found between *sxtA4* copies per cell and the quantity of PSTs synthesised per cell, showing a dosage effect such that the quantification of *sxt4A* may be particularly pertinent to quantifying the likelihood of certain PST concentrations [22, 28]. For the purposes of seafood safety risk assessment, the identity of the species is less important than the indication of the presence of target genes linked to PST biosynthesis. For this reason, a commercial qPCR-based assay Phytoxigene™ DinoDtec has been developed, based on the detection of a gene region, *sxtA4*, that is only found in dinoflagellate species that produce PSTs. While development of the kit has been carried in the laboratory, this study represents the first field and on-farm applications of this kit.

Phytoplankton monitoring for shellfish safety risk management using eDNA [19, 29-31] is currently carried out in dedicated molecular biology laboratory facilities. With the emergence of portable qPCR equipment, the quantification of target genes *in situ* has been made possible. To conduct qPCR on-farm, a simplified and standardised workflow is necessary. Such an early detection tool should be able to be conducted with minimal training by non-specialists, be rapid, relatively inexpensive, use no toxic or harmful chemicals, use relatively inexpensive equipment, have minimal electricity requirements, and be able to be conducted outside of a controlled laboratory setting.

Here, we developed a simplified and standardised workflow that allows for the detection of PST producing microalgae on site with results available within ~2 hours. The workflow included three stages: 1) water collection and filtration using the Phytoxigene™ Portable Water Sampler; 2) cell lysis for DNA extraction and buffer selection for sample preservation; and 3) the Phytoxigene™ DinoDtec lypholised qPCR assay and data interpretation. We then evaluated this early detection tool both temporally and spatially during two HAB events of PST-producing *A. catenella*.

2. Materials and Methods

2.1 Cell Recovery using the Phytoxigene™ Portable Water Sampler

The gravity operated Phytoxigene™ Portable Water Sampler (Diagnostic Technology) (Fig. 1) was designed to standardise the water filtration of phytoplankton of the size class relevant for HAB analysis from 3 L seawater. The first stage of this filtration consists of a 100 µm nylon mesh, as a pre-filter and to remove zooplankton, debris and larger phytoplankton, and the second stage is a 11 µm nylon fabric designed to capture and retain HAB cells. To operate, seawater is poured from the top until the chamber is full. The valve is then released to allow the seawater to flow through the 11 µm mesh. The flow-through seawater is collected with a squeeze bottle. The second stage filter is then removed from the sampler and the cells which have collected on the 11 µm filter are back-flushed with the filtered seawater into a collection chamber such as a 50 mL falcon tube. Finally, the water sampler should be rinsed with tap water after each sampling event. If either filter becomes clogged, then the mesh should be sprayed with diluted bleach (10 %) under pressure, followed by a thorough rinse with tap water (2 mins).

To determine the cell recovery efficiency of the water sampler, 3 L of 5 µm filtered seawater was spiked in triplicate with 100, 1,000, or 5,000 cells of *Alexandrium catenella* (Group 1 genotype, strain ATTR/F, Triabunna Tasmania, Australia). All samples were then passed through the sampler, and the sample recovered as described above. One mL from each concentrated sample was counted using a Sedgewick-Rafter counting chamber (ProSciTech, Australia) and cell recovery estimated from counts before and after the filtration.

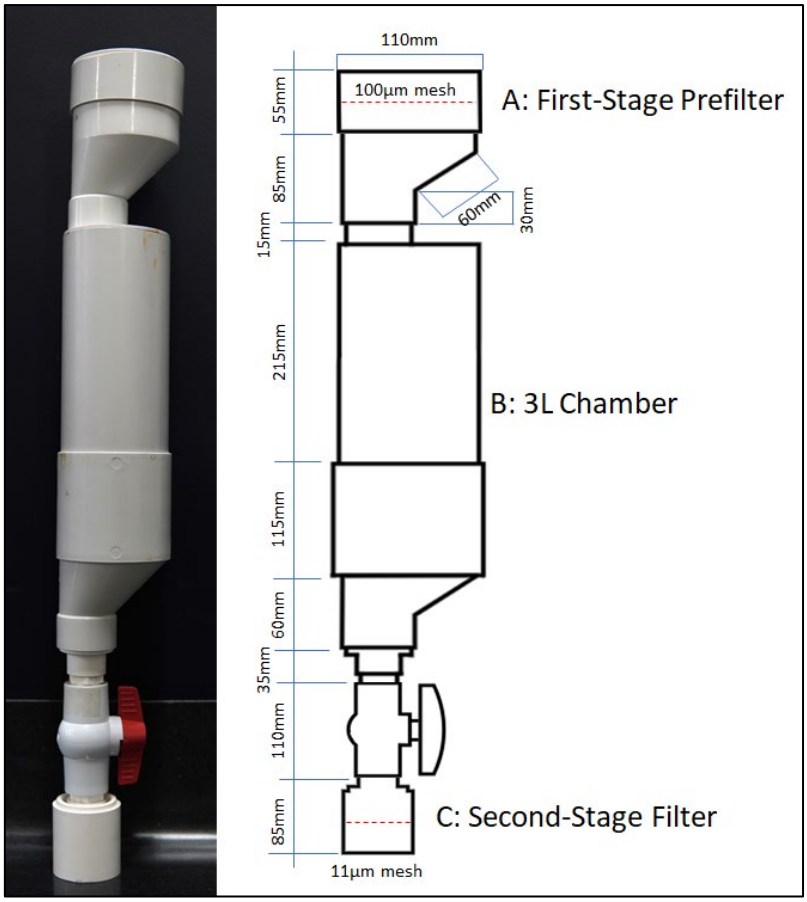

Figure 1. Photo and technical diagram of the gravity operated Phytoxigene™ Portable Water Sampler (Diagnostic Technology) showing: A. First stage prefilter with 100 µm mesh; B. 3 L water chamber; and C. Second-stage filter with 11 µm mesh designed to capture and retain HAB cells such as dinoflagellates; and all measurements. The sampler is made from PVC piping with a midline ball valve/tap.

2.2 Performance of cell lysis method for DNA extraction and preservation

To develop a simple cell lysis protocol for DNA extraction from seawater samples and examine its performance, a culture of *Alexandrium pacificum* (strain MMWA 83) was serially diluted from 170,798 ten times at a 1:2 dilution rate. Diluted samples were filtered through a 25 mm Swinnex filter holding an 8 µm nitrocellulose filter (Merck Millipore, Massachusetts, US). Each filter was removed and inserted into a BioGX lysis tube (BioGX, Birmingham, USA) containing ~300 mg of sterilised glass beads and 500 µL of sterilized bead lysis buffer. Tubes were then vortexed at maximum speed for 10 mins using a Vortex-Genie 2 ® (Scientific Industries, New York, USA) equipped with a 24-place adapter for 1.5 – 2.0 mL tubes (Qiagen, Venlo, Netherlands). Tubes were subsequently centrifuged at 1000 ×g for 1 min to pellet the debris. Five µL of the supernatant from each tube was transferred into the qPCR reaction plate.

The DinoDtec qPCR reaction mix was rehydrated with 80 µL of nuclease-free water, and 20 µL added to each well resulting in a 25 µL qPCR reaction volume. The cycling conditions for qPCR were 95°C for 2 mins, followed by 45 cycles of 95°C for 10 sec and 64°C for 45 sec on a CFX96 Touch Real-Time PCR Detection System (Biorad). The fluorescence signal from the probe was quantified in the FAM channel, and the copy numbers of the gene calculated by establishing a standard curve using the sample quantification cycle (Cq) (y-axis) and the natural log of concentration (x-axis). The percentage efficiency of each reaction was then calculated by the equation $E = -1 + 10^{(-1/slope)}$. A satisfactory amplification efficiency was accepted if between 90 and 110% [32]. The internal amplification control (IAC), which showed whether or not the reaction amplified, was measured on the HEX channel. In samples where the IAC Cq is 1.5 cycles higher than the Blank or non-template control IAC CT, the result is considered invalid. All samples were below this threshold.

The inhibitory effect of three common DNA and tissue preservative solutions, Longmire's buffer [33], RNA Later (Sigma Aldrich) and Lugol's Iodine (Sigma Aldrich) on the DNA extraction and qPCR processes, was tested using two DNA extraction methods. The inhibitory effect was visualised by examining the Cq of qPCR reactions using DNA extracted using each method. A total of twenty-four, 8 µm nitrocellulose filters (25 mm diameter) with 31,000 cells of *Alexandrium catenella* strain ATTR/F on each filter were prepared by filtering a culture using syringe filtration. The samples were then split into two sets of twelve. The first set (1) underwent an extraction using a FastDNA for Soil kit (MP Biomedicals) and the second underwent DNA extraction using BioGX Lysis tubes (2). Membrane filters containing *A. catenella* ATTR/F were inserted into either the FastDNA bead beating tubes for set 1, or in the BioGX Lysis tube for set 2, and 1mL of each of the 3 preservative buffers was added (n=3 per buffer, and n=3 for control with no preservative buffer). Tubes with fixed cells were then vortexed for 5 min and centrifuged at 1000g for 1 min. The qPCR reactions were performed with the same conditions as the test of lysis tube efficiency described previously, with the primers for *sxtA4,* and 1 µL sample DNA.

2.3 Phytoxigene™ DinoDtec qPCR specificity and efficiency test

The DinoDtec kit (Diagnostic Technology, Australia) is a commercialised qPCR assay developed based on primers targeting the *sxtA4* gene (Murray et al 2011). To test the specificity and efficiency of the kit, qPCR reactions were compared between six non-PST producing *Alexandrium* species (*A. affine, A. concavum, A. leii, A. margalefi, A. fraterculus, A.*

pseudogonyaulax) and three PST producing species (*A. pacificum, A. catenella* and *A. minutum)* (Table S1). DNA was extracted from 50,000-75,000 cells of each strain using the FastDNA Spin kit for soil and eluted in 80 µL of elution buffer. The DinoDtec mix was rehydrated with 80 µL of nuclease-free water, and 20 µL aliquot was mixed with 2 µL DNA and 3 µL PCR grade water to a total of 25 µL for each qPCR reaction. Cycling conditions and signal quantification were same as above except this time using a MyGo Mini portable thermocycler (IT-IS LifeScience, Cork, Republic of Ireland). Phytoxigene™ DinoNAS standards were used to develop the standard curve for *sxtA4* copy number quantification. Phytoxigene™ DinoNAS is a DNA standard for the DinoDtec kit developed by the National Measurement Institute (NMI).

Efficiency tests of the DinoDtec *sxtA4* qPCR assay were performed by developing standard curves from strains of *A. pacificum*, *A. catenella*, and *A. minutum*. Serial dilutions were carried out (1:2) on three extracts for each strain with DNA amount equivalent to 3000 cells. qPCR reactions were carried out in triplicate, with temperature settings of 95°C for 2 mins, followed by 45 cycles of 95°C for 10 sec and 64°C for 45 sec. Standard curves were established as above and the percentage efficiency of each reaction calculated.

2.4 On-farm early detection tool testing

For *in situ* pipeline testing ~10 L of seawater was collected from a depth of 5 m using a peristaltic pump from a lease offshore from Spring Bay Seafoods, Tasmania (42.59 S, 147.97 E) every Sunday between 9:30-11am from 22nd May 2016 to 30th October 2017. The seawater was then transported to the hatchery in Triabunna (Tasmania) and subsequently filtered using the Phytoxigene™ Portable Water Sampler, followed by syringe filtration and drying as described earlier. Filters were removed and inserted into a BioGX cell lysis tube, vortexed at maximum speed for 10 min, and centrifuged at 1000 g for 1 min to precipitate cell debris. Five µL of cell lysate and 20 µL of DinoDtec was transferred and mixed in a qPCR tube and qPCR carried out as previously described. A negative control (5 µL of PCR grade water) and Phytoxigene™ DinoNAS standard representing 5,000 copies of *sxtA4* was included in each qPCR run.

To infer the temporal relationship between *sxtA4* copies L^{-1} in seawater and PSTs in mussel tissue (*Mytilus galloprovincialis*), the concentration of PSTs was measured in mussels weekly across the sampling period. Approximately 10 - 12 individual mussels were sampled, the flesh

was pooled, frozen and couriered on ice to Symbio Laboratories Sydney, a National Association of Testing Authorities (NATA) accredited commercial laboratory for initial screening using HPLC (Lawrence 2005). Shellfish flesh was homogenized, and 5 g was added to 3 mL of 1% acetic acid and boiled (100 ºC) for 20 min in a water bath. The sample was allowed to cool and centrifugation subsequently performed for 10 min at 1000× g. The supernatant was then collected and the remaining pellet was resuspended with 3 mL of 1% acetic acid. This prepared sample was centrifuged again at 1000 g to separate the supernatant which was then mixed with water to get a final volume of 10 mL. A SPE C18 cartridge was used to perform the clean-up of this mixture. Standards, PST positive reference matrices, and sample were oxidised with a matrix modifier. After periodate oxidation, screening of the PST analogues including STX, GTX2, 3, C1, 2, GTX5, NEO, dcNEO, and GTX1, 4 was performed. If a positive result was reported, precolumn oxidation was used to confirm concentrations of STX, GTX2, 3, C1, 2, GTX5, dcSTX, dcGTX2, 3, NEO, dcNEO, GTX1,4, C3,4. AST (domoic acid (DA)), and DSTs (OA, dinophysistoxin 1 (DTX-1), dinophysistoxin 2 (DTX-2)), and pectenotoxin 2 (PTX-2) were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (AB ScieX Triple Quad 6500) [34, 35]. Positive toxin results were reported by the Tasmanian Shellfish Quality Assurance Program as equivalent to ≥1.00 mg/kg DA (AST), ≥0.25 mg/kg OA equivalents (DSTs) and ≥0.10 mg STX eq/kg (PSTs).

Pearson correlations of *sxtA4* in seawater and PST concentrations in mussel tissue with no lag-time, a lag time of 1, 2, and 3 weeks for the period from May 2016 to Oct 2017 using GraphPad Prism 7.04 (Graphpad Software Inc).

2.5 Workflow case-study: Spatial patterns of an Alexandrium catenella *bloom in southeast Tasmania*

2.5.1 Sample collection

Seawater samples were collected during 26-28 August 2016, on board the RV *Southern Cross*, along inshore-offshore transects on the east coast of Tasmania (-42 S, 148 E) (Table S1). At each of the 18 stations a Seabird SBE 19 PlusV2 CTD (Sea-Bird Scientific, Washington, USA) was used to measure temperature (°C), salinity (ppt), and pH, and a 10 L Niskin bottle was used to collect water samples from each depth. From each of these 10 L samples, 3 L of seawater was subsequently collected and filtered using the Phytoxigene™ Portable Water Sampler as previously described. Concentrated samples were then stored in 50 mL tubes ~4 ºC for several hours, before being syringe-filtered onto triplicate filters. The filters were then

transferred to 2 mL cryogenic tubes and 1 mL of Longmire's buffer was added to each. Samples were stored at room temperature until further downstream processing.

2.5.2 Light Microscopy cell identification and counting

From each 10 L Niskin water sample collected, 1 L subsample was collected and preserved with Lugol's iodine (final concentration of 1%). Each of these 1 L samples were then concentrated using a sedimentation technique. For this, each sample was allowed to stand for 48 hours before the supernatant was siphoned off, leaving a final volume of 10 mL. The remaining 10 ml was then well mixed, and settled again in a 5cm diameter petri dish and the entire contents enumerated for *A. catenella* cells with an inverted light stereomicroscope at magnification of x200 (Leica, Wetzlar, Germany).

2.5.3 DNA Extraction and gene quantification

Samples concentrated using the sampler were extracted using a FastDNA Spin kit for Soil (MP Biomedicals, Solon, OH, United States) and processed using the sampling workflow previously described before running the Phytoxigene™ DinoDtec qPCR assay.

3. Results and Discussion

3.1 Cell recovery using the Phytoxigene™ Portable Water Sampler

The gravity sampler was designed to be used in the field and without electricity, as it does not require the use of a pump. The sampler collects and concentrates 3 L of water, which is much more than the volume of current phytoplankton monitoring protocols used in Tasmanian monitoring marine biotoxin programs [36]. This high sample volume reduces the potential for random sampling error at early bloom development concentrations of *A. catenella*, which can lead to PST accumulation in mussels. The effectiveness of the sampler was demonstrated by the >90% recovery of *A. catenella* at all concentrations tested (100, 1,000, and 5,000 cells per 3 L) (Table S2). Cell recovery was lowest when the sampler filtered ~100 cells (~92%) and highest when recovering ~1000 cells (~95%). The slightly higher cell loss at low concentrations may be due to attachment of cells to the pre-filter membrane, or increasingly low precision of Sedgewick-Rafter chambers at low cell concentration [20, 37, 38].

With a mesh size of 11 µm, the sampler also concentrates phytoplankton species responsible for Diarrhetic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP), in addition

to the target PSP associated microalgae. With the availability of assays specifically designed for the detection the toxic genera *Pseudo-nitzschia* spp. [39-42] and *Dinophysis* spp. [43, 44], the sampler device would be an effective tool for sampling other HAB taxa. However, additional work to validate species recovery would be necessary before on-farm use.

3.2 *Performance of Phytoxigene™ cell lysis for DNA extraction and preservation*

To test the efficiency of the cell lysis in our workflow, a qPCR standard curve was developed using triplicate, ten-fold 50% serial dilutions of *A. pacificum*. The efficiency of the assay was 99.07% which was deemed acceptable (Fig. 2).

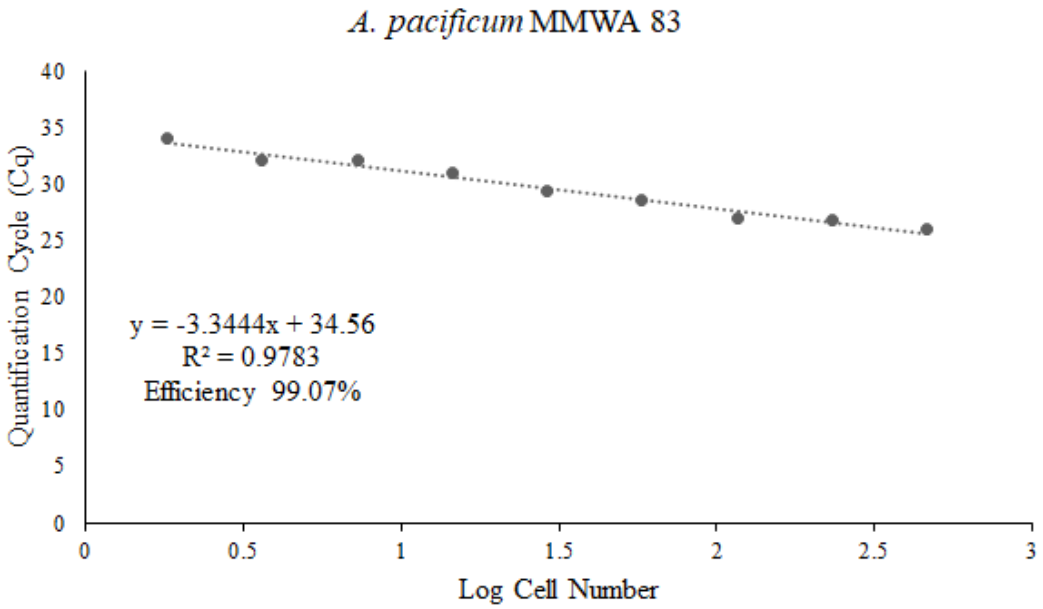

Figure 2. Standard curve of DinoDtec assay using lysed cells of *A. pacificum* strain MMWA 83 as the template DNA, showing the quantification cycle (y-axis) versus the known cell number in log-scale (x-axis).

The Cq of the sample preserved with Longmire's buffer was not significantly different (one tailed t-test p = 0.037, d.f =3) to the non-preserved sample (Table S3). The Cqs from the samples preserved with RNALater and Lugol's were 4 - 6.5 units higher than the non-preserved sample. Samples preserved in all three buffers did not amplify when processed using BioGX lysis tubes.

One of the main challenges analysing environmental samples are sample matrix effects that interfere with the quantification of the analyte of interest [45]. Environmental samples contain a variable range and concentration of substances that co-extract with DNA and inhibit the efficiency of PCR amplification process. To address this issue of inhibition, a well-equipped molecular laboratory has DNA extraction protocols that can be applied to remove the inhibitory compounds from field samples [46, 47]. However, this is not the case with the DinoDtec assay when used *in situ*. The use of a simple bead lysis to break open cells, instead of conducting a full DNA extraction, combined with centrifugation to precipitate and remove the cell debris before the qPCR amplification, can be an alternative method. Additionally, we have shown that Longmire's Buffer does not inhibit the qPCR reaction (see Fig. 2), however it should not be added directly to the lysis tube, but instead, the membrane filter should be removed from the buffer and transferred to lysis tube immediately prior to qPCR. Longmire's buffer has been shown to be an effective preservative for environmental DNA, and our study supports this as an alternative to refrigeration or freezing of environmental samples.

3.3 *Phytoxigene™ DinoDtec qPCR specificity and efficiency test*

The DinoDtec assay was found to be specific to PST-producing species, as it did not amplify DNA from any of the non-producing species tested (Table S4). The standard curves of DNA extracted from multiple strains of three different species of PST-producing *Alexandrium* spp. were within an acceptable range of 90-110%, with a regression coefficient of 0.99 or higher (Table 1).

Table 1. Standard Curve Efficiencies of Phytoxigene™ DinoDtec qPCR assay with different species and strains of *Alexandrium*.

Species	Strain	% Efficiency	R^2
A. pacificum	CS300	93.72	1.000
	CAWD44	95.75	0.999
	ACTRA02	90.74	0.999
	CS798	95.54	0.999
	CS313	106.89	0.997
	CS315	100.69	0.999
A. minutum	CS324	95.45	0.991
A. catenella	TRIA-E	95.31	0.999

Previous studies have shown the specificity and utility of assays targeting *sxt4A* for detecting PST-producing dinoflagellate species in the marine environment [21, 24, 26, 48-50]. The

Phytoxigene™ DinoDtec assay is in a lyophilised form enabling long term storage at room temperature and transport into the field. It contains enzyme, probe, primers, dNTP and Internal Amplification Control (IAC) target. In terms of phytoplankton monitoring, the impact of false positives could be significant, as it could trigger unnecessary closures and delays in the harvest of shellfish products. Our data show that the DinoDtec assay is efficient for three species of PST producing *Alexandrium* and while some variation was observed between strains of *A. pacificum*, the efficiency remained within acceptable limits (90-110%).

3.4 *On-farm workflow testing*

During the 2016 *A. catenella* bloom, the number of *sxtA4* copies L^{-1} began increasing at Spring Bay on 3rd July 2016 (Fig. 3). The *sxtA4* copies L^{-1} then decreased to almost zero on 17 July 2016, and increased again in the following week. The PST levels in mussels started to increase during the week starting on 31st July 2016, which is approximately four weeks after the first *sxtA4* copies L^{-1} were observed. The PST concentration then increased until the week starting on 25th Sept 2016, after which it continuously decreased (Fig. 3).

In 2017, the rapid increase *sxtA4* copies L^{-1} number started on the week of the 21st Aug 2017, and the increase continued until 2nd Oct 2017 (Fig. 4). PST concentrations began to elevate during the week commencing on the 5th Sept 2017. The PST concentration reached its peak on the 10th Oct 2017 (140 mg kg^{-1}), after which it gradually declined. This coincided with decreasing numbers of *sxtA4* copies L^{-1} during this time. As cell numbers were extremely high on the week starting on 10th Oct 2017 and 17th Oct 2017, Spring Bay Seafoods was not operational and sampling was suspended. Hence, no qPCR data were obtained.

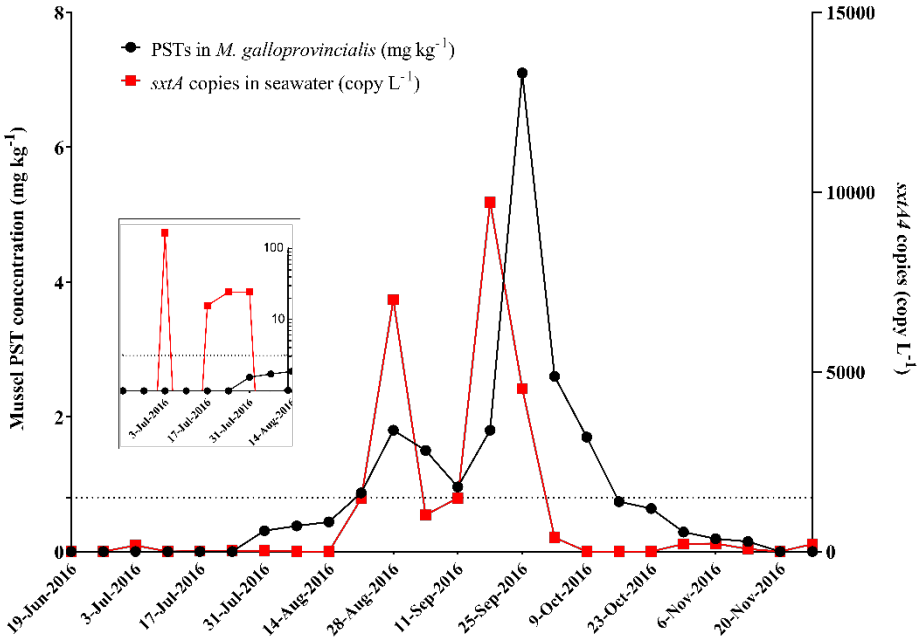

Figure 3. Copy number of *sxtA4* L^{-1} in seawater and the total concentration of PST in mussel tissue (mg kg^{-1}) during the 2016 *A. catenella* bloom from Spring Bay Seafoods. The inset shows a portion of the same data from June-July 2016, with *sxtA4* L^{-1} in seawater on a log scale, highlighting that the qPCR assay detected significant *sxtA4* copies prior to the detection of toxins in mussel tissue.

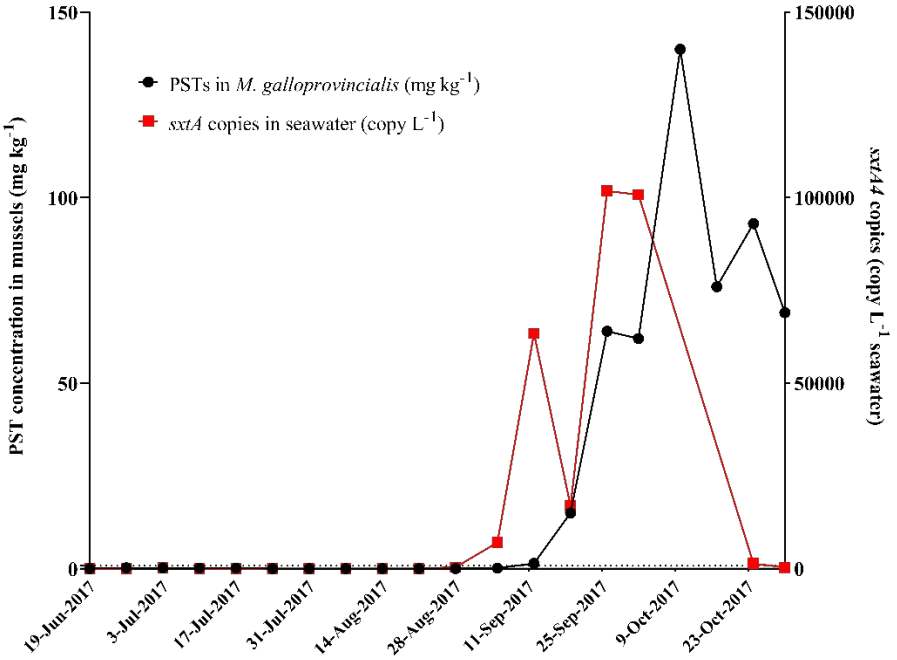

Figure 4. Dynamics of *sxtA4* L^{-1} copies in seawater and the total concentration of PST in mussel tissue during the 2017 *A. catenella* bloom from Spring Bay Seafoods.

Our correlation analyses of *sxtA4* L^{-1} and PST mg kg^{-1} in mussels demonstrates the use of the DinoDtec qPCR assay as an early warning indicator of PST in Tasmanian commercial mussels, *M. galloprovincialis* (Table S5). Over the course of two *A. catenella* blooms indicates that the DinoDtec workflow provides a two-week early warning of PST (r = 0.95, Table S5). Additionally, a Pearson correlation coefficient with a lower r value was also found when modelling the *sxtA4* copies L^{-1} and PST mg kg^{-1} in mussels, suggesting that up to 3 weeks' early warning may be possible (r = 0.7, Table S5). Our results with *M. galloprovinvcialis* are consistent with previous observations showing an approximately two-week lag between the addition of PST-producing dinoflagellates to the water, and accumulation of toxin in *Mytilus* spp. [51].

The rate of PST accumulation and depuration may also differ depending on factors such as the ploidy level of the shellfish and the ambient water temperature [15]. Depuration in *M. galloprovincialis* is additionally influenced by size, age, soft tissue weight, and reproductive stage [52]. For example, increased acidification and increased temperature could potentially cause lower maximum PST accumulation, but slower depuration in *Mytilus galloprovincialis* [53]. Farmed mussels are grown from the same batch, which means that they have a similar size, age, and weight, potentially minimising differences among individuals. Given the differences among species and ambient environmental factors, it is likely that the optimal lag time established between *sxtA4* copies L^{-1} and PST mg kg^{-1} established in this study will be specific to mussels impacted by *A. catenella* during the austral winter, which on the east coast of Tasmania, is generally characterised by water temperatures of 10 - 15 ºC.

Toxin accumulation and detoxification dynamics of shellfish are complex and depend on many factors to modify uptake processes (e.g. filtration and feeding rate/efficiency, particle size, toxin distribution in the planktonic food web). As a result, PST uptake, PST biotransformation and depuration rates can differ greatly between shellfish species [15, 54-57]. In green-lipped mussel (*Perna viridis*), accumulation of PST can exceed the regulatory level within 2 days of exposure, with significant amount of toxins removed within three weeks [56]. Blue mussels (*Mytlis edulis*) accumulate toxins quickly and show limited toxin metabolism (and thus are useful indicators of the toxigenic source), and can take weeks to detoxify [54]. Australian abalone uptake PST at about 10 times slower rate than mussels, but with comparable depuration rates [58]. In scallops, the accumulation and depuration of toxins is much slower than mussels, and can take up to

several months [54]. In Pacific oysters (*Crassostrea gigas*), a PST level higher than the regulatory limit can be reached in three weeks when the concentration of *A. minutum* is between 9 and 140 cell L^{-1} in the surrounding water) [59].

3.5 *Spatial patterns of an* Alexandrium catenella *bloom*

During the August 2016 bloom, the surface abundance *sxtA4* copies was highly variable across sites, with no consistent pattern with distance from shore in any sample zone (Fig. 5). The highest *sxtA4* copy abundance (in surface waters) was observed in Spring Bay (maximum abundance of 2,764 *sxtA4* copies L^{-1} at SB3), Coles Bay (max. abundance 2,017 *sxtA4* copies L^{-1} at CB4), and Little Swanport (max. abundance 1,939 *sxtA4* copies L^{-1} at OB4).

At the deeper (>30 m) stations of SB3 and OB1, *A. catenella* cell abundance was noticeably stratified, with maximum cell density observed between 5-10 m and decreasing below 10 m (Fig. S1A-B). Salinity was uniform with depth at these sites, however temperature stratification was evident (0.2 - 0.4 ºC) (oceanographic data presented in Condie et al. 2019). For stations that were shallower (<10 m), the highest number of cells were found at a depth of ~5 m (Fig. S1C-D). In estuaries (<5 m), the *A. catenella* cell numbers did not vary greatly between the surface and ~ 3 m (Fig. S1E-G). These patterns were consistent with both qPCR and light microscopy results, with the exception of two sites (CB1 and CB3), which showed variability between methods (Fig. S1D).

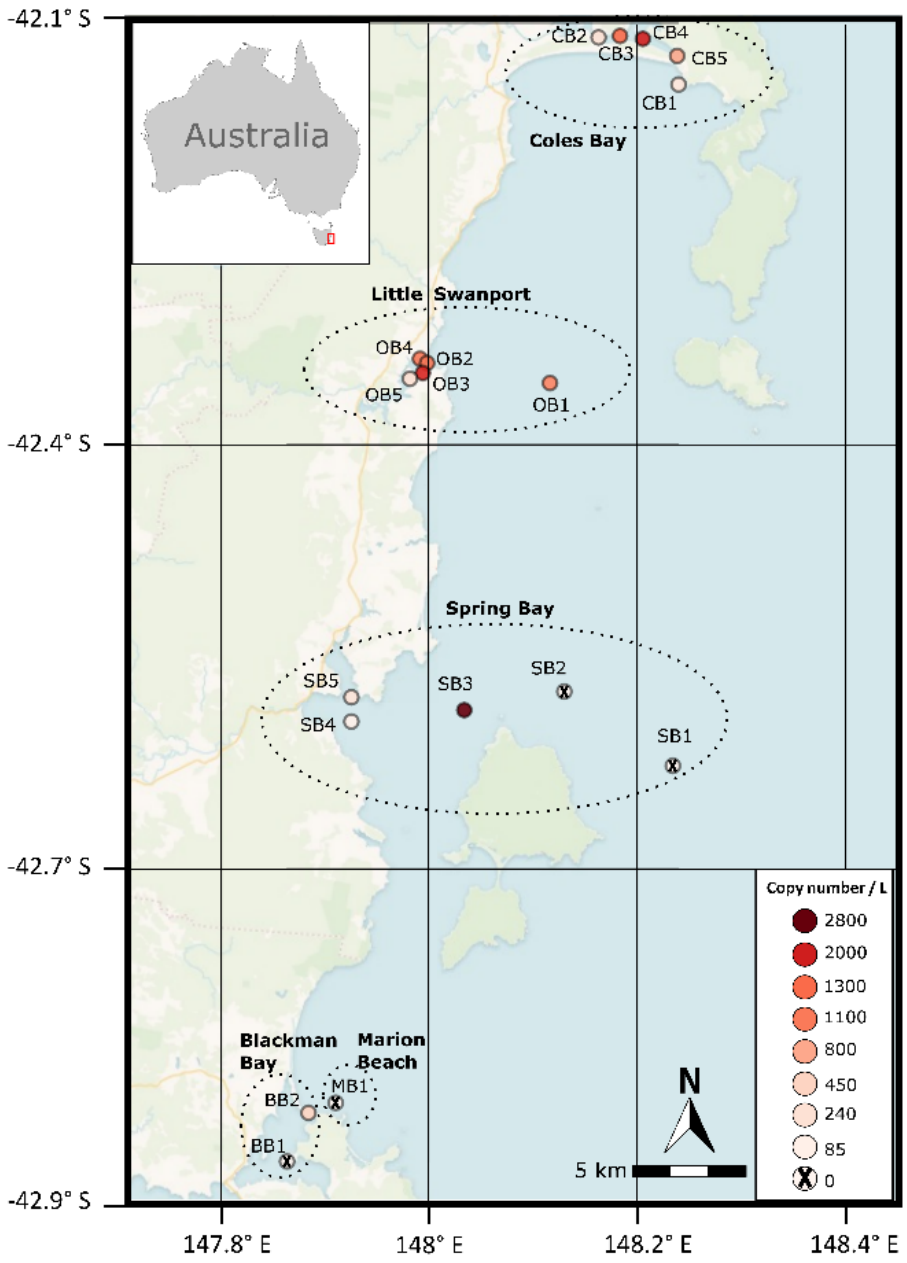

Figure 5. The sampling stations of the RV Southern Cross survey along the east coast of Tasmania, Australia, with the concentration of surface-*sxtA4* copy L^{-1} detected at each site during the peak (26-28 August) of the *A. catenella* bloom in 2016.

3.6 Implications for Monitoring Strategies

During the 2016 and 2017 *A. catenella* blooms in Tasmania, the detection of *sxtA4* copies L^{-1} was used to make decisions regarding mussel harvest management of Spring Bay Seafoods, prompting voluntary harvest closures prior to official PST detection and harvest closure notice from the TSQAP. Advantages of this early detection included capacity to switch harvest/production to alternative sites not impacted by PSTs, and continued production throughout primary site closure periods. Declining *sxtA4* copy number also provided an early indication of the bloom decline allowing farmers to continue to source production from the alternative site until the PST-level decreased to below the regulatory limit (0.8 mg kg^{-1}).

During 2016, the decrease of *sxtA4* copies L^{-1} occurred from the week starting on 25th September 2016 (Fig.S1), while PST mg kg^{-1} in mussel tissue was still above the regulatory limit. While weekly samples were adequate to inform management decisions and modelling, more frequent sampling may provide information on finer scale dynamic shifts in *A. catenella* and improve the resolution of the results.

The vertical profile of *A. catenella* cell abundance across depths during the August 2016 bloom event indicated that cell abundance was well mixed throughout the water column in estuaries, but became stratified in oceanic conditions (Fig S1). At these later stations, the highest *A. catenella* cell abundance and *sxtA4* copies were observed at a depth of 5-10 metres below the sea level, decreasing in deeper samples. Such a vertical trend was also observed in bloom in the Gulf of Maine in 2001 [60], where the highest concentration of cells was found in a thin layer depth of ~10-11 metres. The maximum cell abundance in the present study was linked to stratification in temperature and chlorophyll fluorescence, significant for the vertical migration of *A. catenella* [61]. The vertical distribution of *Alexandrium* implies therefore, that sample depth, stratification and time of day are important considerations for cell quantification in oceanic systems.

The spatial distribution of *sxtA4* copies L^{-1} appeared to be patchy in August 2017 (Fig. 5), and no discernible pattern was observed between oceanic and estuarine stations. It is likely that factors such as upwelling and downwelling, rainfall, and current flow affected the spatial distribution of the bloom [61]. As qPCR is relatively fast to implement, additional sampling stations and replicates can be used by monitoring agencies, to increase accuracy and prediction in such circumstances.

To conclude, we have established a three-step tool for the detection and quantification of PST-producing dinoflagellates that is applicable for use on site. This protocol is standardised, relatively simple and inexpensive to operate, and not in need of specialised laboratory facilities. The cell recovery of the sampler was found to be high, and the lysis method for DNA extraction and the DinoDtec assay were shown to be sensitive, specific and efficient. We successfully used this protocol to identify and describe two significant blooms of *A. catenella* in Tasmania, Australia, with our results suggesting that these blooms are well mixed in estuaries, but appear to be sub-surface in deeper, more oceanic sites, suggesting important implications for future monitoring strategies.

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Supporting Information for Publication: Tables S1-S5: sampling sites/depths; results of cell recovery using sampler; preservative agent; qPCR specificity and relationship between *sxtA4* copies L^{-1} and PSTs (mg kg^{-1}) in *M. galloprovincialis.* Figure S1 field quantification of *A. catenella*.

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