# Mapping the development of a *Dinophysis* bloom in a shellfish aquaculture area using a novel molecular qPCR assay

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Short Title: Using qPCR to detect *Dinophysis* in SE Australia.

**Abstract**

Diarrhetic shellfish toxins produced by certain species of the marine dinoflagellate *Dinophysis* can accumulate in shellfish in high concentrations, representing a significant food safety issue worldwide. This risk is routinely managed by monitoring programs in shellfish producing areas, however the methods used to detect these harmful marine microbes are not usually automated nor conducted onsite, and are often expensive and require specialized expertise. Here we designed a quantitative real-time polymerase chain reaction (qPCR) assay based on the ITS-5.8S ribosomal region of *Dinophysis* spp. and evaluated its specificity, efficiency, and sensitivity to detect species belonging to this genus. We designed and tested twenty sets of primers pairs using three species of *Dinophysis* - *D. caudata*, *D. fortii* and *D. acuminata.* We optimized a qPCR assay using the primer pair that sufficiently amplified each of the target species (*Dacu_11F/Dacu_11R*), and tested this assay for cross-reactivity with other dinoflagellates and diatoms in the laboratory (11 species) and *in silico* 8 species (15 strains) of *Dinophysis*, 3 species of *Ornithocercus* and 2 species of *Phalacroma*. The qPCR assay returned efficiencies of 92.4% for *D. caudata*, 91.3 % for *D fortii*, and 91.5 % for *D. acuminata*, while showing no cross-reactivity with other phytoplankton taxa. Finally, we applied this assay to a *D. acuminata* bloom which occurred in an oyster producing estuary in south eastern Australia, and compared cell numbers inferred by qPCR to those determined by microscopy counts (max abund. ~$6.3 \times 10^3$ and $5.3 \times 10^3$ cells $L^{-1}$ respectively). Novel molecular tools such as qPCR have the potential to be used on-farm, be automated, and provide an early warning for the management of harmful algal blooms.

**Keywords:** Diarrhetic shellfish Toxins, *Dinophysis*, Diarrhetic Shellfish Poisoning, qPCR, shellfish

## 1. Introduction

Global bivalve production has significantly grown (~1 - 18 million t) over the past 70 years (FOA, 2020), with approximately 90% of the produce coming from aquaculture (Wijsman et al., 2019). In this context, the occurrence of harmful algal blooms (HABs) represents a considerable and ongoing issue for the shellfish industry, as some species of microalgae can produce marine biotoxins which can bioaccumulate in bivalves (after consuming algae from the water column), enter the food web, and cause sicknesses and/or death of higher trophic organisms, including humans.

While multiple studies have quantified the economic impacts of HABs on fish aquaculture (Anderson et al., 2016; Skjoldal and Dundas, 1991), there have been fewer studies focused on the economic impact of HABs on the shellfish industry (Anderson et al., 2000; Mardones et al., 2020; Sanseverino et al., 2016). A recent study to assess the economic impact of HABs on Scottish shellfish aquaculture over the last 10 years, found that biotoxins from species belonging to the dinoflagellate genus *Dinophysis* Ehrenberg, generated annual losses of approximately 15% of total production, which is equivalent to a loss of 1080 ton of shellfish per year and estimated at £ 1.37 m (Martino et al., 2020). Conversely, some economic benefits can result from extra food safety measures. A survey focused on shellfish consumer's confidence revealed that some consumers would be willing to pay extra if doing so would provide extra food safety guarantees (Garza-Gil et al., 2016) .

Worldwide, the distribution and frequency of HABs appear to be changing as a result of human impacts such as climate change, eutrophication, increase in global aquaculture ventures, and the introduction of new species to new areas (Ajani et al., 2016; Ajani et al., 2020; Gobler, 2020). Such is the case of the distribution and persistence of Diarrhetic Shellfish Toxins (DSTs) produced by species of the dinoflagellate genus *Dinophysis*. Diarrhetic Shellfish Toxins are a group of polyketide toxins that comprise okadaic acid (OA) and dinophysistoxins (DTXs), both which inhibit protein phosphatase and can cause diarrheagenic effects in mammals; and pectenotoxins (PTXs), polyether lactones which have been found to cause liver damage in mice (Reguera and Blanco, 2019). These toxins can bioaccumulate in the food chain and cause Diarrhetic Shellfish Poisoning (DSP) in humans. Symptoms include diarrhea, nausea, vomiting, abdominal pain and potentially stomach tumours, but the effects of chronic

exposure to DSTs are not well known (Cordier et al., 2000; Fujiki and Suganuma, 1993; Fujiki et al., 1988; Suganuma et al., 1988).

Since the identification of *Dinophysis fortii* as the causative organism of a severe DSP outbreak in Japan, in 1976 (Yasumoto et al., 1980; Yasumoto et al., 1978), nine other species of *Dinophysis* have been reported globally to produce DSTs, even at very low cell densities ($<10^3$ cells $L^{-1}$) (Reguera et al., 2014; Reguera et al., 2012; Simoes et al., 2015). These include *D. acuminata*, *D. acuta*, *D. caudata*, *D. infundibulum*, *D. miles*, *D. norvegica*, *D. ovum*, *D. sacculus* and *D. tripos* (MacKenzie et al., 2005; Reguera et al., 2014; Reguera et al., 2012). The geographical distribution of DSP episodes reported has also gradually increased over the last forty years, and now includes Asia, Australia, Europe, New Zealand, and South America (Lembeye et al., 1993; Taylor et al., 2013; Whyte et al., 2014; Yasumoto et al., 1980).

In Australia, three DSP outbreaks have been caused by the consumption of 'pipis' (*Plebidonax deltoides*), which are a common bivalve found on beaches in south eastern Australia (Dakin, 1976), where they have been commercially harvested over the past ~20 years (O'Connor and O'Connor, 2011). The first outbreak was in 1997, when 47 cases of gastroenteritis were reported (Quaine et al., 1997), the second in March 1998 with 20 cases reported (Madigan et al., 2006), and a third in 2000 when only one individual was affected (Burgess and Shaw, 2001). Since this time, routine monitoring for the presence of *Dinophysis* and their biotoxins has commenced in all major harvest areas in Australia, and as a result of this monitoring, DST was responsible for a recall of mussels in Tasmania in 2016, reported at a maximum of 0.56 mg $kg^{-1}$ OA , almost x 3 the Australian regulatory limit of 0.2 mg $kg^{-1}$ OA (Ajani et al., 2021a; Hallegraeff et al., 2021).

Since the first report of biotoxins in shellfish in 1937, the mouse bioassay (MBA) became the most common analytical monitoring tool for toxic events (Schantz et al., 1957). From the late 1980's onwards, liquid chromatography with tandem mass spectrometry began to be implemented in certain countries throughout the world (LC-MS/MS and LC-MS) (McNabb et al., 2005; Quilliam et al., 1995). This method, along with morphotype-based identification of plankton species which is currently done with light microscopy, are now the standard monitoring tools to assess toxin concentrations in shellfish and enumerate HAB species. Molecular genetic methods to detect and quantify harmful algae such as quantitative

Polymerase Chain Reaction (qPCR) assays however, are growing in popularity (Dyhrman et al., 2006; Erdner et al., 2010; Galluzzi et al., 2010; Galluzzi et al., 2004; Godhe et al., 2008; Hosoi-Tanabe and Sako, 2005; Kamikawa and Sako, 2007; McLennan et al., 2021). The qPCR method shows high sensitivity and delivers rapid results for the monitoring and detection of HAB species compared to other methods. It is also comparatively low cost and requires less sophisticated laboratory equipment compared to other molecular methods. Quantitative PCR assays amplify and track the targeted genetic material in real-time, can be set up to measure multiple HAB species at once (multiplex assays), and their development and use for detecting HABs is increasing worldwide (Andree et al., 2011; Antonella and Luca, 2013; Engesmo et al., 2018; Kretzschmar et al., 2019; McLennan et al., 2021; Murray et al., 2019; Ruvindy et al., 2018; Smith et al., 2014; Zamor et al., 2012). There have been few examples however, of the use of molecular genetic methods to detect *Dinophysis in situ* (Edvardsen et al., 2013; Takahashi et al., 2005), one of which developed *Dinophysis* clade specific primers for amplification of the LSU rDNA (Hart et al., 2007), and only one which has used qPCR for the detection of *Dinophysis* (Kavanagh et al., 2010). This latter study used the highly conserved, large ribosomal subunit (LSU) D1-D2 region, and delineated *D. acuta* and *D. acuminata* based on melt-peak temperature. To date, there has been no further progress in the use of qPCR for the detection of *Dinophysis*.

The aim of this study was to develop and assess a qPCR assay for the detection and quantification of this harmful algal genus. We first developed and tested the assay on cultured cells, and then using environmental samples from a bloom of *Dinophysis* which occurred in a major oyster growing estuary in southeastern Australia, we tested our assay on natural samples with the potential aim of applying this powerful tool into on-farm shellfish safety management strategies.

## 2. Materials and Methods

### 2.1 Isolation of clonal strains and maintenance of cultures

*Mesodinium rubrum* and *Teleaulax amphioxeia* were isolated from Inokushi Bay (32.7998 N, 131.8923 E) in Oita Prefecture, Japan, at the end of February 2007 (Nagai et al., 2008). The *M. rubrum* culture was maintained by mixing 50 ml of the culture (7.0–9.0 × $10^3$ cells $mL^{-1}$) with 100 ml of a modified f/2 medium (Guillard 1975, Nagai et al. 2004), with the addition of 25-100 µL of *T. amphioxeia* culture (containing 0.5–2.0 × $10^4$ cells) as food source. The

culture medium was prepared with 1/3 nitrate, phosphate, and metals and 1/10 vitamins, plus any enrichment from autoclaved natural seawater collected from Tokyo Bay (35.3460 N, 139.6570 E). Seawater was filtrated through three cartridge filters with 5, 1, and 0.5 µm (STG-10-5, STG-10-1, STG-10-0.5, Kankyotechnos, Japan) at the sampling site and brought back to the laboratory. Salinity was adjusted to 30 practical salinity units [psu] before autoclaving. A part of the ciliate culture was transferred into fresh culture medium containing the food source once a week, and they were maintained at a temperature of 18°C under a photon irradiance of 100 µmol $m^{-2}$ $s^{-1}$, provided by cool-white fluorescent lamps, with a 12:12 h light: dark cycle.

*Dinophysis caudata* cells were isolated by micropipetting from a seawater sample collected from Nagasaki, Japan (32.8088 N, 129.7708 E) in 2013 and incubated in individual wells of a 48-well microplate (Iwaki, Japan). Similarly, *D. acuminata* cells were isolated from Mombetsu, Hokkaido, Japan (44.3368 N, 143.3808 E) in 2017, and *D. fortii* cells were isolated from the Saroma Lake, Hokkaido, Japan (44.1405 N, 143.8009 E) in 2015 and incubated in individual wells of a 48-well microplate (Iwaki), respectively. Each cell was grown in 1.0 mL of the culture medium containing ca. $1.0 \times 10^3$ cells of the marine ciliate *M. rubrum* as the prey species. *Dinophysis* cells were incubated under the same conditions as those for the *M. rubrum* culture, except for *D. caudata* set at 25°C. After one month of incubation, several strains were established for each species, and clonal strains of DA_MOM02 (*D. acuminata*), DC_NAG01 (*D. caudata*), and DF_SAL90 (*D. fortii*) were used for further experiments. For ongoing culture maintenance, 0.1 mL of each culture was inoculated into 2.9 mL of fresh *M. rubrum* culture (ca. $2 \times 10^3$ cells $mL^{-1}$, without *Teleaulax*) every three weeks, and maintained under the same conditions as stated above.

For scale-up of the cultivation, 3 mL of *Dinophysis* cells (ca. $3 \times 10^3$ cells $mL^{-1}$,) were inoculated into 150 mL of fresh *M. rubrum* culture (ca. $2 \times 10^3$ cells $mL^{-1}$, without adding *Teleaulax* culture) of 250 mL capacity polycarbonate Erlenmeyer flasks (Corning). Five flasks were prepared for each strain, and they were incubated for one month under the same conditions as those used for the maintenance culture. Flasks of each strain were then combined and 1 mL of each culture was sampled in triplicate for cell counts and toxin analyses. *Dinophysis* cells were harvested using a nylon sieve (mesh size 10 µm, to remove *M. rubrum*), washed with 50 mL of fresh culture medium, and inoculated into 2 mL flat bottom plastic

tubes. The tubes were centrifuged at 14,000 rpm for 2 min, and the supernatant was removed by pipetting. Samples were kept at -80°C until use.

2.2 Sequences of 5.8S rDNA with the ITS region

Genomic DNA was extracted from ~10 cells of each species by 5% Chelex buffer (Nagai et al., 2012). PCR amplification was carried out on a thermal cycler (PC-808, ASTEC, Fukuoka, Japan) with a reaction mixture consisted of 1 µL template DNA, 1 µM each of ITS (5.8S rDNA with the ITS region) primer sets (Adachi et al. 1994), 0.2 mM of each dNTP, 1× PCR buffer, 1.5 mM $Mg^{2+}$, 1U KOD-Plus-Ver.2 (TOYOBO, Osaka, Japan), and RNA free dH2O to bring up to a final volume of 25 µL. The PCR cycling conditions were as follows: 2 min at 94 °C, 30 cycles at 94°C for 15 sec, 56°C for 30 sec, and 68°C for 40 sec. Sequences of the target regions were obtained by the direct Sanger sequencing method using the Dynamic ET terminator cycle sequencing kit (GE Healthcare, Little Chalfont, UK) and a DNA sequencer (ABI3730, Applied Biosystems). The sequences were aligned using MEGA version 10 (Kumar et al., 2018) and the consensus sequences were obtained for each species. The BLAST search was performed to confirm the availability of sequences of the same species on the GenBank. All newly obtained sequences were then deposited into the DDBJ databank.

2.3 DNA extraction for qPCR assay development

DNA was extracted from pellets corresponding to ~$1.1 \times 10^5$, $1.2 \times 10^5$ and $4.3 \times 10^5$ cells of *D. acuminata, D. fortii* and *D. caudata* respectively, using the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen, Hilden Germany). Minor modifications were made to the manufacturer's protocol during the extraction process e.g. centrifugation instead of a vacuum pump. Cells pellets were preserved at 4°C in Longmire buffer (Williams et al. 2016) prior to the extraction process. The buffer was then heated to 65°C for 10 mins and cells were lysed using 0.7 mm garnet beads (Capella Science Pty Ltd) on a vortex adapter (Qiagen/Scientific Industries Inc.) at top speed for 10 mins. Six hundred and fifty microliters of buffer CD3 (provided by manufacturer, Qiagen) was then added to the lysate and the mixture was added onto silica-based spin columns (provided by manufacturer, Qiagen). The liquid was removed through centrifugation and purified using ethanol-based buffers (as per manufacturer's protocol) and finally eluted in 80 µL of buffer C6 (provided by manufacturer). DNA from these samples were stored in -20°C until further analysis.

2.4 Toxin Determination

Samples were frozen at –30°C until toxin analysis using a modified solid-phase extraction (SPE) method (Suzuki et al., 1996; Suzuki et al., 1998; Suzuki et al., 2009). Each 1 mL sample was thawed to room temperature and applied to a MonoSpin C18 centrifuge cartridge column (GL Science Inc., Tokyo, Japan). The SPE column was then washed with 0.5 mL distilled water and the toxins were eluted with 0.1 mL methanol. The methanol elutes were directly analyzed by LC-MS/MS. LC-MS/MS analysis of the toxins was carried out according to a previous method (Suzuki and Quilliam, 2011). A Nexera-20XR series liquid chromatograph (Shimadzu, Kyoto, Japan) was coupled to a QTRAP 4500 mass spectrometer (SCIEX, MA, USA) of hybrid triple quadrupole/linear ion trap. Separations were performed on LC columns (internal diameter [i.d.], 100 mm × 2.1 mm) packed with 1.9 µm Hypersil GOLD C8 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and maintained at 30°C. Eluent A was water, and eluent B was acetonitrile water (95:5), containing two mM ammonium formate and 50 mM formic acid. Toxins were eluted from the column with 50% B at a flow rate of 0.3 mL $min^{-1}$. Multiple reaction monitoring (MRM) LC-MS/MS analysis with negative-mode ionization was carried out using the target parent ions and the fragment ions in Q1 and Q3 for each toxin as follows: OA, m/z 803.5 > 255.1; DTX-1, m/z 817.5 > 255.1; PTX-2, m/z 857.5 > 137.0; PTX-1 and PTX-11, m/z 873.5 > 137.0; PTX-2 Seco acid (PTX-2 SA), m/z 875.5 > 137.0. The lowest detection limits of OA/DTX-1 and PTX-2 were 0.1 and 1.2 ng $mL^{-1}$. These levels are equivalent to 0.2 pg $cell^{-1}$ of OA (and DTX-1) and 2.4 pg $cell^{-1}$ of PTX-2, when 100 cells of the toxic plankton were analyzed using our LC-MS/MS method.

2.5 qPCR assay development

*2.5.1 Primer design and specificity*

In order to design a specific and efficient qPCR assay for *Dinophysis,* eighteen ITS1/5.8S/ITS2 rRNA sequences from nine *Dinophysis* species, were initially downloaded from GenBank (http://www.ncbi.nlm.nih.gov), aligned using ClustalW v1.6 (Thompson et al. 1994), and examined by eye for regions of similarity and differences. Due to the largely conserved ITS region across all sequences, primers were designed based on *Dinophysis acuminata in silico* tool NCBI Primer-BLAST. This resulted in twenty sets of primers pairs ranging from 106 to 150 bp in length. To determine which primer set would sufficiently

amplify the DNA extracted from each of the *Dinophysis* cell pellets described above, qPCR assays were subsequently undertaken.

Each qPCR assay was conducted using triplicate 20 µL reactions containing 1 µL of DNA from template from each of the three *Dinophysis* species, 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA), 0.5 µL of each of the forward and reverse primers, and 8 µL of DNA nuclease-free water (Ambion®). The qPCR assay was performed on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System™ platform with the following thermal cycling program: 95°C for 3 mins, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and finally a temperature gradient for melt curve construction at a resolution of 0.5 °C. A negative control using nuclease-free water instead of the template DNA to detect for contamination was also included in the test run. This, and all subsequent assays, were run in 96 and/or 384 well plates with a clear seal (Bio-Rad, CA, USA).

An evaluation of the cross-reactivity of the most appropriate primer set followed. This was first assessed *in silico*, by downloading and aligning ITS1/5.8S/ITS2 rRNA sequences from the closely related genera *Phalacroma* and *Ornithocercus* from GenBank (http://www.ncbi.nlm.nih.gov). The number of Single Nucleotide Polymorphisms (SNPs) were then identified in the binding sites of the *Dacu_11F/Dacu_11R* qPCR primers (deemed the most appropriate primer set - see Results). Specificity was also tested in the laboratory using DNA from other available phytoplankton genera/species that commonly occur with *Dinophysis* in Australian waters (Ajani et al., 2013). This included 12 dinoflagellates and 1 diatom (Table 1). The qPCR assay protocols for this specificity testing remained identical to those outlined above.

*2.5.2 qPCR assay efficiency*

To evaluate the mean qPCR efficiency (or performance) of the novel *Dinophysis* specific assay, standard curves were established using both a cell-based calibration and a gene-based approach (Bustin et al., 2009). In order to do this, the DNA from all three *Dinophysis* species was five-fold serially diluted. Dilutions ranged from 700 to 0.07 cells $\mu L^{-1}$ for *D. acuminata,* 1500 to 0.15 cells $\mu L^{-1}$ for *D. fortii* and 5,350 to 0.54 cells $\mu L^{-1}$ for *D. caudata*. For the gene-based calibration curve, a ten-fold dilution series was established using a synthetic gene

fragment (gBlock® IDT, USA) which was 257 base pairs in length and based on the ITS region of *D. acuminata*. The molecular weight and the amount of gBlock was supplied by IDT, from which the exact copy number of the gene fragment per microliter was calculated as described in Conte et al. (Conte et al., 2018). Copy numbers used in the qPCR assay ranged from $3.8 \times 10^7$ to $3.8 \times 10^1$ gene copies $\mu L^{-1}$ (see Supplementary Table 1). All samples were amplified in triplicates as per the qPCR protocol outlined above. Standard curves were then established for all three species and the gene fragment assay 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 ranged between 90 – 110% (Bustin et al., 2009). Finally, to determine the relationship between cell number of each species and gene copy number, the slope of the log-linear standard curve was used to solve for x (concentration) for both species and gBlock equations, and the resulting 'factor' antilogged to return a number of gene copies per cell for each of the three species. The quantification of this relationship was then used in the interpretation of qPCR assay results from environmental samples downstream (see Supplementary Table 1).

2.6 Comparison of cell counts and qPCR assay for *Dinophysis* bloom dynamics

*2.6.1 Water sampling for* Dinophysis *cell enumeration and eDNA analysis*

Water samples (500 ml) were collected at approximately 2-weekly intervals from a depth of 0.5 m from the Manning River (-31.89S, 152.64E) for microscopic phytoplankton identification and enumeration in accordance with the NSW Marine Biotoxin Management Plan (NSW MBMP) and the Australian Shellfish Quality Assurance Program (ASQAP) (Fig. 1). Once collected, samples were immediately preserved with 1% Lugol's iodine solution, and returned to the laboratory for concentration using gravity-assisted membrane filtration. Detailed cell examination and counts were then performed using a Sedgewick Rafter counting chamber and a Zeiss Axiolab or Standard microscope equipped with phase contrast. Cells were identified to the closest possible taxon using light microscopy (maximum magnification

× 1000), and cell counts to determine the abundance of individual *Dinophysis* species carried out with a minimum detection threshold of 50 cells $L^{-1}$.

As part of a large scale research project (www.foodagility.com/research/food-safety-in-the-oyster-industry), water samples were collected at a second sampling site for environmental DNA (hereafter known as eDNA) (Fig. 1) at approximately weekly intervals. This sampling program provided us with a unique opportunity to test our *Dinophysis* specific qPCR assay on environmental samples both before, during and after a *Dinophysis* bloom event, which was reported on 17 February 2019 at a maximum cell concentration of 5,300 cells $L^{-1}$ of *D. acuminata*. Triplicate 3 L surface water samples (0.5 m) were collected weekly from this site using the water sampler described in Ruvindy et al. (Ruvindy, 2019). In brief, water samples were passed firstly through a 100 µm (pore size) nylon mesh, then a second 11 µm mesh, and then backwashed with filtered seawater to retain the phytoplankton. Finally, the sample was filtered one last time using a syringe filter with a 8 µM filter (Merck), and the filter placed into a 5mL tube (Eppendorf) containing 2 mL Longmire buffer. Samples for eDNA were then stored at 4 °C until further downstream processing.

*2.6.2 qPCR assay using eDNA for bloom dynamics*

Prior to DNA extraction, filtered samples in 2 mL Longmire buffer were incubated at 65 °C for 10 mins and vortexed for 10 mins using Vortex Genie 2 (at top speed). The eDNA was then extracted using the QIAcube HT automated nucleic acid isolation system and the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen), with all buffers provided by the manufacturer. In brief, 1 mL of buffer was loaded onto the S-block and the addition of 650 µL of buffer CD3 then followed. The resulting mixture was then added onto the QIAamp 96 plate and liquid removed using a vacuum pump. The eDNA was then purified on a column using ethanol-based buffers (as per manufacturer's protocol) and eluted into 80 µL of buffer C6. Finally, the eDNA samples were stored in -20°C until further analysis.

Triplicate eDNA samples and gene fragment serial dilution samples were prepared for qPCR analysis. For this final assay, the reaction volumes were 5 µL, comprising of 2.5 µL SYBR Green Mix (Bio-Rad), 1.1 µL nuclease free water, 0.2 µL of forward and reverse primer (0.5 µM final concentration) and 1 µL of eDNA template (1:8 dilution with milliQ water). Two negative controls were also run to detect for contamination. The plate was prepared with an

epMotion®5075l Automated Liquid Handling System. The qPCR assay was performed using the BIORAD CFX384 Touch™ Real-Time PCR Detection System™ using the cycling conditions as described above.

## 3. Results

### 3.1 *Dinophysis* species identification.

The three monoculture strains were unequivocally identified as *D. acuminata* (strain DA_MOM_02), *D. fortii* (DF_SAL_90) and *D. caudata* (DC_NAG_01) (accession numbers: LC634028- LC634030).

### 3.2 Toxin Determination

Three toxin analogues (OA, DTX-1, PTX-2) were detected in all three strains tested, with the exception of DTX-1 in *D. caudata* (Table 2). Mean (±SE) OA pg cell$^{-1}$ ranged from 0.01 (± 0.00) in *D. caudata,* to 1.3 (± 0.10) in *D. acuminata*, and to 13.21 (±1.54) in *D. fortii* respectively. Mean DTX-1 was detected at 17.38 (±3.04) pg cell$^{-1}$ in *D. fortii* and 23.90 (±3.31) pg cell$^{-1}$ in *D. acuminata*. Mean PTX-2 ranged from 52.77 (±9.96) pg cell$^{-1}$ in *D. caudata*, 63.19 (±1.42) pg cell$^{-1}$ in *D. acuminata,* and to 185.93 (±27.66) pg cell$^{-1}$ in *D. fortii* respectively (Table 2).

### 3.3 qPCR assay development

#### *3.3.1 Primer design and specificity*

The *Dacu_11F/Dacu_11R* primer pair, which comprised 133 bp from the ITS region of *Dinophysis*, was the only primer set which showed sufficient specificity to amplify all three *Dinophysis* species (Table 3). This primer pair amplified a single peak at approximately the same temperature (*D. acuminata* 80.5°C, *D. fortii* 80.5°C and *D. caudata* 80.5-81°C), with an average Cq value of 15.29 for *D. acuminata*, 14.17 for *D. fortii* and 16.17 for *D. caudata.* This specificity was subsequently examined *in silico* against three species of *Ornithocerus* and two species of *Phalacroma*, which resulted in 8-10 SNPs in forward primer binding region and 4 (no sequence data available in this region for *Phalacroma* spp.) in the reverse primer-binding region of the *Dacu_11F/Dacu_11R* primer pair respectively (Supplementary Table 1) in comparison to the non-target species. In addition, no cross-reactivity was observed in the laboratory against any other phytoplankton species tested (Table 1).

*3.3.2 qPCR assay efficiency*

To test for primer efficiency, five-fold serially diluted cell-based curves were established for each species (see Supplementary Table 1 for the detection limit in cells of the assay). The percentage efficiency of each reaction was determined to be 91.5% for *D. acuminata*, 91.3% for *D fortii*, and 92.4% for *D. caudata*, all which were deemed acceptable (Fig. 3A-C, Supplementary Table 1). The eight-fold serially diluted gene fragment-based curve also reported a suitable efficiency of 99.38% (ie. slope for Cq vs. gene copy number = -3.33) (Fig. 4, Supplementary Table 1).

To determine the relationship between cell number of each species and the copy number of the ITS1/5.8S/ITS2 gene, the slope of the log-linear standard curve was solved for x (concentration) for all species and gblock equations. The resulting factors were × 538 for *D. acuminata*, × 1253 for *D. fortii* and × 80 for *D. caudata* (Supplementary Table 1).

3.5 Evaluation of qPCR for *Dinophysis* bloom dynamics

To evaluate the effectiveness of the *Dinophysis* qPCR assay for the detection of *Dinophysis* in environmental samples, we compared microscopy-based *D. acuminata* and *D. caudata* cell counts with eDNA samples collected from the Manning River across the same time period. Sixteen water samples collected from 10/9/2018 to 31/3/2019 showed *D. acuminata* peaked on 17/2/2019 at a cell concentration of 5,300 cells $L^{-1}$, while *D. caudata* reached a maximum cell concentration of 300 cells $L^{-1}$ on 3/12/2018 (Fig. 5, Supplementary Table 1). Using the *Dinophysis* assay developed in this study, we then screened twenty-four eDNA samples (in triplicate) across this similar time period (11/9/2018 to 26/3/2019) and successfully detected gene copies of *Dinophysis* in 62 out of 72 replicate samples. Mean gene copy number peaked on 9/2/2019 and corresponded to 3,682,268 gene copies $L^{-1}$ (Fig. 5, Supplementary Table 1). Assuming the bloom was dominated by *D. acuminata* (as reported by microscopy) at this time, we then used the x factor for *D. acuminata* (× 538) to determine the peak cell concentration of *D. acuminata* to be ~6,316 cells $L^{-1}$ (Fig. 5). A Lomb-Scargle Periodogram (Lomb 1976; Scargle 1982) was applied to the microscopy and qPCR data to compare the periodicity in the unevenly sampled time-series. The correlation coefficient generated from this analysis was 0.31 which was significant with a student's t-test ($p$<0.1) (Fig. 6A-B).

## 4. Discussion

*Dinophysis* is a cosmopolitan genus of dinoflagellates producing toxins harmful to human health, which is of growing concern to the shellfish industry globally. In response to this, we successfully designed, tested and substantiated the first application of a specific, efficient and rapid qPCR assay for the detection and quantification of species of this genus. We found a detection limit for the three *Dinophysis* species tested as <0.1 – 1 cells per assay (Fig. 2A-C), which is easily sufficient to detect *Dinophysis* cells prior to the accumulation of ~500-1000 cells $L^{-1}$, which is the detection threshold of *Dinophysis* species of some harmful algal monitoring programs. We then examined environmental samples collected from a bloom of *D. acuminata* which occurred in a large oyster-growing estuary in southeastern Australia in February 2019, to confirm this assay's ability to identify and replicate the simultaneous quantification of *Dinophysis* cells using light microscopy, the currently used routine method for regulatory monitoring in NSW (NSW Food Authority, 2015)

With ten *Dinophysis* species unambiguously found to be toxic and monitoring efforts increasing worldwide, global records over the past thirty years have seen an increase in *Dinophysis* species observations, toxic events, and the distribution of DSP (Hallegraeff et al. 2021). *Dinophysis* is also widespread in Australian waters, with 36 species reported (Ajani et al., 2011; Hallegraeff and Lucas, 1988; McCarthy, 2013), and the toxic representatives *D. acuminata*, *D. caudata*, and to a lesser extent *D. fortii*, *D. tripos* and *D. acuta* all widely distributed in south eastern Australia (Ajani et al., 2013; Ajani et al., 2016; Hallegraeff and Reid, 1986; Richardson et al., 2020). Data spanning from July 1928 to March 2021 and sourced from the Australia Phytoplankton Database (Davies et al., 2016), shows the two most common toxic species enumerated in Australian waters are *D. acuminata* and *D. caudata*. Their abundance showed distinct seasonal patterns – *D. acuminata* highest in austral spring and *D. caudata* highest in summer (Ajani et al., 2016; Richardson et al., 2020) (Table 4). Species with cell densities that triggered shellfish testing over this time have been *D. acuminata*, *D. caudata, D. acuta* and *D. fortii* (see Table 4 for regulatory action limits), and while long-term biotoxin monitoring has shown a low incidence of DSTs in shellfish aquaculture areas in Australia overall, reports of DSTs from *Dinophysis* spp. in wild harvest pipis collected from open beaches along the southeastern Australian coastline are common (Ajani et al., 2021a; Ajani et al., 2016; Farrell et al., 2015; NSW Food Authority, 2017; Richardson et al., 2020) (Table 4).

In studies aimed at harmful algal detection, it is often important that qPCR or microarray assays be species-specific, as only a small subset of species produce toxins, while closely related species in the same genus often do not produce those toxins. However, in the case of *Dinophysis*, the majority of the most abundant species, and the most commonly found species, all produce the same class of toxins, the DSTs. Therefore, we have developed an assay that will detect all *Dinophysis* species. While there is a small risk that this assay may detect non-DST producing *Dinophysis* species, data from the Australian Phytoplankton Database (Davies et al., 2016) reveals these species are relatively rare in Australian coastal waters and will likely not lead to false positives. In the event that low frequency false positives are reported the impact is likely to be limited provided that any positive detections can be verified via light microscopy and/or biotoxin analysis of shellfish.

Although toxic species belonging to the genus *Dinophysis* pose a significant threat to aquaculture worldwide, there has been surprisingly little research on their genetic and morphological variability. Where limited studies have been carried out, it has been commonly reported that traditional morphological criteria and/or universally used genetic markers, such as the nuclear SSU/LSU and/or ITS rDNA regions, or the mitochondrial *cox1* and/or *cob* genes, provide ambiguous species diagnosis (Edvardsen et al., 2003; Guillou et al., 2002; Hart et al., 2007; Jensen and Daugbjerg, 2009; Park et al., 2019; Raho et al., 2008; Sechet et al., 2021; Wolny et al., 2020). In particular, there is ongoing difficulty in discriminating between *D. acuminata, D. ovum* and *D. sacculus* (together known as the "*D. acuminata* complex"), with the former two species hypothesized to be ecotypes of the same taxa, with their phenotypic differences postulated to be linked to contrasting ecological niches (Park et al., 2019). Niche differentiation has been demonstrated between *D. acuminata* and *D. acuta*, whereby vertical distribution, daily vertical migration, and division rates vary between these species depending on environmental conditions and prey availability (Baldrich et al., 2021).

Similarly, when we examined the ITS region *in silico* between all *Dinophysis* species sequences available on Genbank, there was minimal sequence diversity observed between them (Supplementary Fig. 1). This was also reflected in the qPCR melt peak temperature for each target species tested in our study (ie. the temperature at which 50% of the oligonucleotide is hybridized in the assay). Each strain/species examined - *D. caudata*, *D fortii* and *D. acuminata* - showed a single, and similar, melt curve peak at ~80 °C. As we lacked live cell

cultures available to test in the laboratory, we conducted *in-silico* analyses of sequences from closely related Dinophysiales, such as *Ornithocercus* and *Phalacroma,* and found sufficient sequence divergence to support the specificity of our assay to *Dinophysi*s (Supplementary Fig. 1). A clear genetic delineation between *Dinophysis* and other closely related genera is also supported by a study which combined data from 26 genetically identified individuals (SSU, 5.8S, LSU and ITS1 and ITS2 regions), all Dinophysiacean Genbank submissions (22), and environmental clone sequences from bulk environmental DNA samples (86), and remains the largest molecular phylogeny carried out across four dinophysoid genera to date (Handy et al., 2008).

Quantitative PCR assays have frequently been developed to target a molecular barcoding region such as the ITS, LSU or the small subunit (SSU) rRNA, and as such can often be species specific. Examples include assays for *Prorocentrum minimum* (McLennan et al., 2021), various *Alexandrium* species (Galluzzi et al., 2004; Hosoi-Tanabe and Sako, 2005; Kamikawa and Sako, 2007; Ruvindy et al., 2018) and *Gambierdiscus lapillus* (Kretzschmar et al., 2019). Alternatively, qPCR assays can target a clade of cryptic species, such as *Pseudo-nitzschia pseudodelicatissima* complex Clade I (Ajani et al., 2021c), or be genus specific such as *Alexandrium* (Godhe et al., 2008), *Gambierdiscus* or *Fukuyoa* (Smith et al., 2017). Finally, qPCR has been used to target functional gene(s) that encode for toxin biosynthesis such as *sxtA* (Murray et al., 2019; Murray et al., 2011).

The quest to find a more rapid and cost-effective testing method for the presence of DSTs has also included the development of rapid toxin testing (Mcleod et al., 2015). These include an antibody-based enzyme-linked immunosorbent assay (ELISA) test, a functional protein phosphatase inhibition activity (PPIA) assay, and a lateral flow analysis (LFA) rapid test. In a recent study to compare these kits in both naturally contaminated and spiked shellfish (oysters, mussels and pipis), however, all kits delivered an unacceptably high level (25–100%) of falsely compliant results for spiked samples, while the LFA and the PP2A kits performed satisfactorily for naturally contaminated pipis (0%, 5% falsely compliant, respectively) (Ajani et al., 2021b). While qPCR detection of *Dinophysis* cannot directly measure levels of toxins, it does have several advantages when compared to rapid toxin detection methods. It is rapid (1-2 h), requires only basic molecular biology experience, has the potential to be used on-farm utilising cell lysis kits for DNA extraction and portable qPCR machines (Ruvindy,

2019), and/or be automated as part of a larger environmental sampler such as a large-scale qPCR instrument or an onsite Environmental Sample Processor (ESP) (Cox and Goodwin, 2013; Doucette et al., 2009). Finally, qPCR can be used for the detection of harmful raphidophyte species which, without any evidence of known toxin production, can cause fish kills.

Aquaculture industries are keen to adopt efficient, fast and cost-effective management tools for biotoxins and the phytoplankton producing them. Farmers, have a regulatory requirement to have marine biotoxin testing conducted on their produce, and as such, qPCR is a relatively simple screening method that provides a rapid result. Quantitative PCR is cheaper than more traditional toxin detection methods such as liquid chromatography with tandem mass spectrometry (LC-MS/MS), which require expert analysts in dedicated laboratories. Finally, a rapid onsite test for the presence of DST producing microalgae such as qPCR, will allow harvest management to become simpler, faster and with fewer closures.

For the application of our assay, we detected and quantified *Dinophysis* in environmental samples collected before, during and after a *D. acuminata* bloom that occurred in the Manning River in February 2019. As part of our assay development, we determined the relationship between cell number of each species and copy number of the ITS1/5.8S/ITS2 gene for each species. We determined that there were 538 copies cell$^{-1}$ in *D. acuminata*, 1,253 copies cell$^{-1}$ in *D. fortii* and 80 copies cell$^{-1}$ in *D. caudata* (Supplementary Table 1), representing a >15-fold difference in copy number between the three species of *Dinophysis* tested. Using a universal primer set based on the V7-V9 SSU region and accompanied by a labeled probe, Yarimizu et al. (Yarimizu et al., 2021) examined single cells from 16 phytoplankton strains including *Dinophysis fortii* (also isolated from Japan), the first time any species from this genus has been examined in this context. Average copy number per cell of this rRNA gene across all taxa examined was from 44 in *Pseudochatonella verruculosa* to 1,800,000 in *Gambierdiscus sp.,* with *Dinophysis fortii* having on average ~16,000-29,000 copies cell$^{-1}$ (2 cells from each of two strains of *D. fortii* were examined).

It is well understood that most ribosomal genes are in multiple copies within a dinoflagellate genome (Murray et al., 2019), yet dinoflagellates such as *Alexandrium* can show up to a >10-fold variation even within a single species ((Brosnahan et al., 2010; Erdner et al., 2010;

Galluzzi et al., 2004; Hou et al., 2019; Ruvindy et al., 2018; Savela et al., 2016; Yarimizu et al., 2021). For example, genomic copies per cell$^{-1}$ for *Alexandrium australiense* strains have been found to vary between 70,000-150,000, and between 500,000-100,000 for *A. catenella* (Murray et al., 2011). Similarly, rRNA copies range between 860-1,020 for *A. minutum* (Galluzzi et al., 2004) and 190,000-2,489,000 for *A. pacificum* (reported as *A. catenella*) (Galluzzi et al., 2010). Other studies have shown similar levels of variation between closely related dinoflagellates species. Gong and Marchetti (Gong and Marchetti, 2019), used computational methods to estimate the average 18S V4-region copy numbers between two species of *Symbiodinium (S. kawaguitii* and *S. minutum),* with variation between 161-116 respectively reported. Similarly, large gene copy variation has also been reported both within and among populations of other eukaryotic protists (Andree et al., 2011; Gong and Marchetti, 2019; Kim et al., 2017).

The *Dinophysis* bloom reported in the Manning River was shown to be dominated by *D. acuminata* by routine microscopy, and using qPCR we found a similar abundance level (Fig. 4). Our mean gene copy number, as determined from field samples, is an average of the variability in gene copies present in the environment, and therefore takes into consideration all the factors that may affect copy number within *Dinophysis acuminata* as we outlined above. With the phytoplankton action limits in New South Wales (Australia) set as 1000 cell L$^{-1}$ for *D. acuminata* and 500 cells L$^{-1}$ for both *D. caudata* and *D. fortii*, this equates to 538,000, 40,000 and 626,500 gene copy numbers respectively. A multi-species bloom of *Dinophysis* in contrast would be more challenging to quantify. Fortunately, however, blooms of *Dinophysis* are often monospecific with many species having restricted geographical ranges or temporal windows (Reguera et al., 2012), and even closely related species display different ecological preferences. This was clearly observed in a twelve-year time series of abundance and environmental data to model *Dinophysis* bloom formation in a southeastern Australia oyster growing estuary (Ajani et al., 2016). The highest abundance of *D. acuminata* occurred during the austral spring, while the highest abundance for *D. caudata* occurred in the summer to autumn. Modelling also revealed that *D. acuminata* in this estuary was significantly driven by season, thermal stratification and nutrients, while *D. caudata* was linked with nutrients, salinity and dissolved oxygen. A similar temporal pattern was also reported from the offshore coastal waters of eastern Australia, with peak abundance of *D. acuminata* observed between August and December, and *D. caudata* between December and

March (Farrell et al., 2018). Species-specific temporal patterns and environmental drivers have also been observed for other *Dinophysis* species across the globe, suggesting that our qPCR assay would be a valuable early warning tool in many locations (Campbell et al., 2010; Hallfors et al., 2011; Koukaras and Nikolaidis, 2004; Peperzak et al., 1996; Reguera et al., 2014).

Another consideration for the rapid detection and assessment of *Dinophysis* blooms is the varying toxin content and profiles between and within species. Due to the difficulty in cultivating mixotrophic dinoflagellates such as *Dinophysis*, and the first successful establishment of cultures in recent years (Park et al., 2006), there is only limited information on the inter- and intra-strain variability in toxin content of *Dinophysis* species, and minimal or none on the change (none) in toxin profile (Nagai et al., 2011), or change in toxin concentration (unknown) over the life cycle of these taxa. Uchida et al. 2018 picked and pooled individual *Dinophysis* cells from locations around Japan, and using LC-MS, determined their toxin content and relative toxin profiles. The dominant toxin found in *D. acuminata* was PTX2, with only minor concentrations of DTX1, although this varied across locations, with the detection of OA in samples from one location only. *Dinophysis fortii* on the other hand, showed a toxin profile dominated by PTX2, and to a lesser degree DTX1 and OA, although DTX1 was higher than reported for *D. acuminata*. Other *Dinophysis* species including *D. caudata*, *D. norvegica* and *D. tripos* were dominated by PTX2 (Uchida et al., 2018).

Similarly, our strains/species showed considerable differences in their toxin profiles, with *D. fortii* having the greatest concentration of OA and PTX2 per cell compared to *D. acuminata*, while *D. caudata* had very low OA and no DTX1 detected (Table 2). Moreover, our *D. acuminata* strain showed higher DTX1 (max. 27 pg cell$^{-1}$) than is reported elsewhere in the literature, *D. fortii* having higher PTX2 (222 pg cell$^{-1}$), and *D. caudata* having lower OA (<0.01 pg cell$^{-1}$) (Uchida et al., 2018). It has also been found that some strains of the same species produce okadaates while other strains produce pectenotoxins, and yet again, some produce both (Reguera et al., 2014). A combination of cultures grown under constant conditions, and/or across well documented environmental conditions, combined with genetic analysis is needed to deliver a more rigorous assessment of the link between cell growth, synthesis pathways and toxic profiles (Reguera et al., 2014). Furthermore, molecular

networking (MN), a method using the fragmentation data obtained by untargeted high-resolution mass spectrometry (HRMS) has been recently used to visualize and tentatively identify unknown analogues which are closely related to the DST family. Using this novel method to examine toxin profiles between strains of *D. acuta*, *D. caudata* and the "*D. acuminata* complex", amongst significant uncharacterized diversity within this toxin group, five new putative PTX analogues were discovered (Sibat et al., 2021).

## 5. Conclusions

Our study reports on at quantitative, real-time polymerase chain reaction (qPCR) assay to detect species belonging to the HAB genus *Dinophysis.* We evaluated its performance, demonstrating it was specific to this genus, and showed no cross-reactivity to other phytoplankton taxa tested. Finally, we used this assay to accurately map the development of a *Dinophysis acuminata* bloom from an oyster-producing estuary in south eastern Australia. Novel molecular tools such as qPCR have the potential to be used on-farm, be automated, and provide an early warning of toxic algal blooms. Future work should include the development and validation of a simplified and commercialised qPCR pipeline for the detection of *Dinophysis*, and further still, the development of a multiplex protocol for the detection and quantification of a suite of species/toxin genes for the early detection and management of HABs in marine waters (Eckford-Soper and Daugbjerg, 2015).

This technology offers shellfish producers the potential for more rapid identification of food safety risks associated with HABs. Rapid detection of food safety risks benefits producers through improved food safety outcomes, as well as reduced magnitude and frequency of product recalls. The ability to analyse environmental samples on farm negates the cost and time delay of sample transport, one of the main barriers to rapid detection of food safety risks in shellfish harvest areas. Future development work should also consider the needs of industry to develop methods that can be implemented without specialist technical knowledge or complex laboratory equipment.

## 6. Acknowledgements

This project was partially supported by funding from Food Agility CRC Ltd, funded under the Commonwealth Government CRC Program. The CRC Program supports industry-led collaborations between industry, researchers and the community. The authors would like to

thank the Department of Primary Industries and the University of Technology Sydney for also funding this work. We thank all the oyster farmers in the Manning River for collecting weekly samples. Routine phytoplankton monitoring sample data for the Manning River were funded by the NSW Food Authority and the shellfish industry. This project was also partly supported by the Fisheries Research and Development Corporation Project 2017:203. Finally, we would also like to thank Prof. Manfred Lenzen (USyd) for statistical analysis and Phil Baker (DPI) for the map.

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Table 1. Cross-reactivity of the selected qPCR *Dacu_11F/Dacu_11R* primer pair on *Dinophysis* spp. and other available phytoplankton species including strain code and location of strain isolation. NSW=New South Wales; SA=South Australia; QLD=Queensland

| Template | Strain code | Location of Isolation | ITS PCR amplification |
|---|---|---|---|
| *Dinophysis acuminata* | DA_MOM_02 | Mombetsu, Hokkaido, Japan | + |
| *Dinophysis fortii* | DF_SAL_90 | Saroma Lake, Hokkaido, Japan | + |
| *Dinophysis caudata* | DC_NAG_01 | Nagasaki, Japan | + |
| *Alexandrium pacificum* | HRP4-2 | Hawkesbury River, NSW, Australia | - |
| *Pseudo-nitzschia* cf. *cuspidata* | P_WAG170419_1 | Wagonga Inlet NSW, Australia | - |
| *Coolia malayensis* | MAB | Malabar, NSW, Australia | - |
| *Heterocapsa ovata* | SA20 | Port Lincoln, SA, Australia | - |
| *Gambierdiscus polynesiensis* | CG14 | Rarotonga, Cook Islands | - |
| *Fukuyoa yasumotoi* | OIRS230 | Orpheus Island, QLD, Australia | - |
| *Prorocentrum lima* | SM43 | Raine Island, QLD, Australia | - |
| *Amphidinium massartii* | CS259 | Kirrimine Beach, Qld, Australia | - |
| *Ostreopsis* cf. *siamensis/O.* sp. 9 | HER24 | Heron Island, QLD, Australia | - |
| *Thecadinium kofoidii* | THE | Gordons Bay, NSW, Australia | - |

Table 2. Toxin analogues and their concentrations as determine for the three strains *Dinophysis acuminata*, *D. fortii* and *D. caudata* used for cell-based qPCR assay development.

| Strain | Rep | Cells/mL | OA pg cell$^{-1}$ | DTX1 pg cell$^{-1}$ | PTX2 pg cell$^{-1}$ |
|---|---|---|---|---|---|
| *D. acuminata* | 1 | 2417 | 1.12 | 27.43 | 64.96 |
| | 2 | 2367 | 1.31 | 26.99 | 64.22 |
| | 3 | 2633 | 1.48 | 17.28 | 60.39 |
| | Mean | | 1.30 | 23.90 | 63.19 |
| | SD | | 0.10 | 3.31 | 1.42 |
| *D. fortii* | 1 | 800 | 15.63 | 17.63 | 203.75 |
| | 2 | 733 | 13.64 | 22.51 | 222.37 |
| | 3 | 1033 | 10.36 | 12.00 | 131.66 |
| | Mean | | 13.21 | 17.38 | 185.93 |
| | SD | | 1.54 | 3.04 | 27.66 |
| *D. caudata* | 1 | 3000 | 0.007 | - | - |
| | 2 | 3200 | 0.009 | - | 42.81 |
| | 3 | 2200 | 0.009 | - | 62.73 |
| | Mean | | 0.01 | - | 52.77 |
| | SD | | 0.00 | - | 9.96 |

Table 3. List of Primers tested based on *Dinophysis acuminata* ITS consensus file ~50 bp sequences; those bolded are those that were used in the final *Dinophysis* assay.

| Primer Pair | Primer Name | Sequence (5'->3') | Template Strand | Product Length |
|---|---|---|---|---|
| 1 | Dacu_1F | Forward primer | AAGCGGGAGCAAGTTTACGA | |
| | Dacu_1R | Reverse primer | GCAACCACAGCAAAGCTTGA | 110 |
| 2 | Dacu_2F | Forward primer | GCAAGCGGGAGCAAGTTTAC | |
| | Dacu_2R | Reverse primer | ACCACAGCAAAGCTTGAGGA | 109 |
| 3 | Dacu_3F | Forward primer | CAAGCGGGAGCAAGTTTACG | |
| | Dacu_3R | Reverse primer | TCATCGCAACCACAGCAAAG | 116 |
| 4 | Dacu_4F | Forward primer | AAATCAAAGCAAGCGGGAGC | |
| | Dacu_4R | Reverse primer | CTCATCGCAACCACAGCAAA | 126 |
| 5 | Dacu_5F | Forward primer | AGCGGGAGCAAGTTTACGAG | |
| | Dacu_5R | Reverse primer | CATCGCAACCACAGCAAAGC | 113 |
| 6 | Dacu_6F | Forward primer | GCGGGAGCAAGTTTACGAGT | |
| | Dacu_6R | Reverse primer | AACCACAGCAAAGCTTGAGG | 106 |
| 7 | Dacu_7F | Forward primer | ATCAAAGCAAGCGGGAGCAA | |
| | Dacu_7R | Reverse primer | GGTTATGCTCATCGCAACCAC | 131 |
| 8 | Dacu_8F | Forward primer | AATCAAAGCAAGCGGGAGCA | |
| | Dacu_8R | Reverse primer | AGGTTATGCTCATCGCAACCA | 133 |
| 9 | Dacu_9F | Forward primer | CCTAGTGGGTCATTGTGGGT | |
| | Dacu_9R | Reverse primer | CCACAGCAAAGCTTGAGGATG | 150 |
| 10 | Dacu_10F | Forward primer | CAAAGCAAGCGGGAGCAAG | |
| | Dacu_10R | Reverse primer | ACCACAGCAAAGCTTGAGGAT | 113 |
| ***11*** | ***Dacu_11F*** | ***Forward primer*** | ***AAGCAAGCGGGAGCAAGTTT*** | |
| | ***Dacu_11R*** | ***Reverse primer*** | ***GCAGAAGGTTATGCTCATCGC*** | ***133*** |
| 12 | Dacu_12F | Forward primer | AAAGCAAGCGGGAGCAAGTT | |
| | Dacu_12R | Reverse primer | CAACCACAGCAAAGCTTGAGG | 114 |
| 13 | Dacu_13F | Forward primer | ATCAAAGCAAGCGGGAGCA | |
| | Dacu_13R | Reverse primer | ATCGCAACCACAGCAAAGC | 121 |
| 14 | Dacu_14F | Forward primer | TAAATCAAAGCAAGCGGGAGC | |
| | Dacu_14R | Reverse primer | GGTTATGCTCATCGCAACCA | 134 |
| 15 | Dacu_15F | Forward primer | CCTAGTGGGTCATTGTGGGTT | |
| | Dacu_15R | Reverse primer | CCACAGCAAAGCTTGAGGAT | 150 |
| 16 | Dacu_16F | Forward primer | TCAAAGCAAGCGGGAGCAAG | |
| | Dacu_16R | Reverse primer | CTCATCGCAACCACAGCAAAG | 123 |
| 17 | Dacu_17F | Forward primer | TCAAAGCAAGCGGGAGCAA | |
| | Dacu_17R | Reverse primer | AACCACAGCAAAGCTTGAGGA | 115 |
| 18 | Dacu_18F | Forward primer | AGCAAGCGGGAGCAAGTTT | |
| | Dacu_18R | Reverse primer | ATGCTCATCGCAACCACAGC | 122 |
| 19 | Dacu_19F | Forward primer | AAGCAAGCGGGAGCAAGTT | |
| | Dacu_19R | Reverse primer | TATGCTCATCGCAACCACAG | 124 |
| 20 | Dacu_20F | Forward primer | AAAGCAAGCGGGAGCAAGT | |
| | Dacu_20R | Reverse primer | GTTATGCTCATCGCAACCACA | 127 |

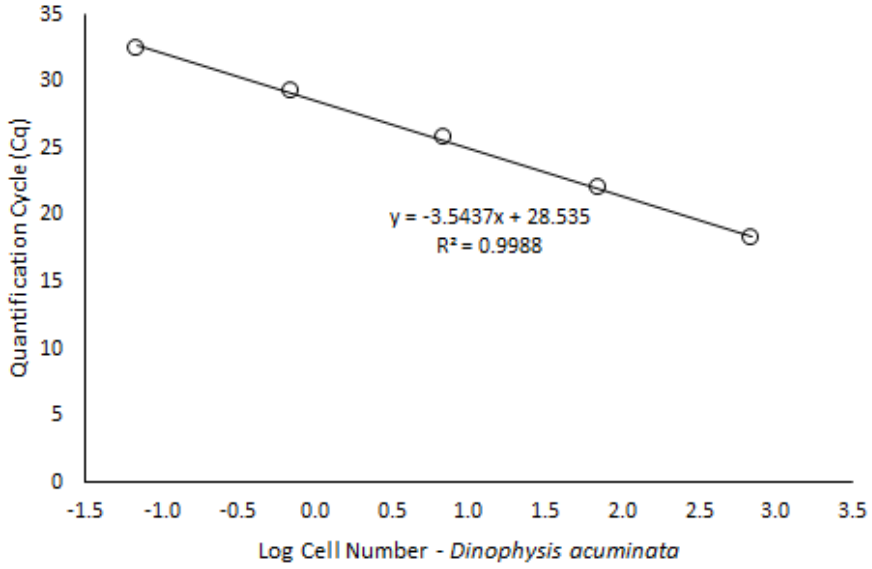
Table 4. Toxin producing *Dinophysis* species (their maximum abundance, location and date of maximum abundance) as sourced from the Australian Phytoplankton Database (https://portal.aodn.org.au, Davies et al., 2016). ND=Not detected; NS=Not specified; #NSWFA (2015); NSW=New South Wales; NT=Northern Territory; WA=Western Australia.

| Toxin producing *Dinophysis* species | Maximum Abundance Cells $L^{-1}$ | Location of Max Abundance | Date of Max Abundance | Total No. of Counts in Database | Trigger Level for Shellfish Testing# Cells $L^{-1}$ | No. of Samples over Trigger Level | % Samples Trigger Sampling of Shellfish Flesh |
|---|---|---|---|---|---|---|---|
| *Dinophysis acuminata* | 13,900 | Ballina, NSW | 20/10/2008 | 2365 | 1000 | 89 | 4 |
| *Dinophysis acuta* | 3,500,000 | Manly Lagoon, NSW | 24/04/2013 | 95 | 500 | 52 | 55 |
| *Dinophysis caudata* | 12,000 | Berowra Creek, NSW | 10/4/2013 | 1110 | 500 | 105 | 9 |
| *Dinophysis fortii* | 4,000 | Berowra Creek, NSW | 12/01/2004 | 77 | 500 | 2 | 3 |
| *Dinophysis infundibulum* | ND | ND | ND | ND | NS | - | - |
| *Dinophysis miles* | 100 | Darwin, NT | 9/02/2018 | 4 | NS | - | - |
| *Dinophysis norvegica* | 147 | North West Shelf, WA | 24/01/2015 | 2 | NS | - | - |
| *Dinophysis ovum* | ND | ND | ND | ND | NS | - | - |
| *Dinophysis sacculus* | ND | ND | ND | ND | NS | - | - |
| *Dinophysis tripos* | 10,000 | Berowra Creek, NSW | 11/12/2007 | 234 | NS | - | - |

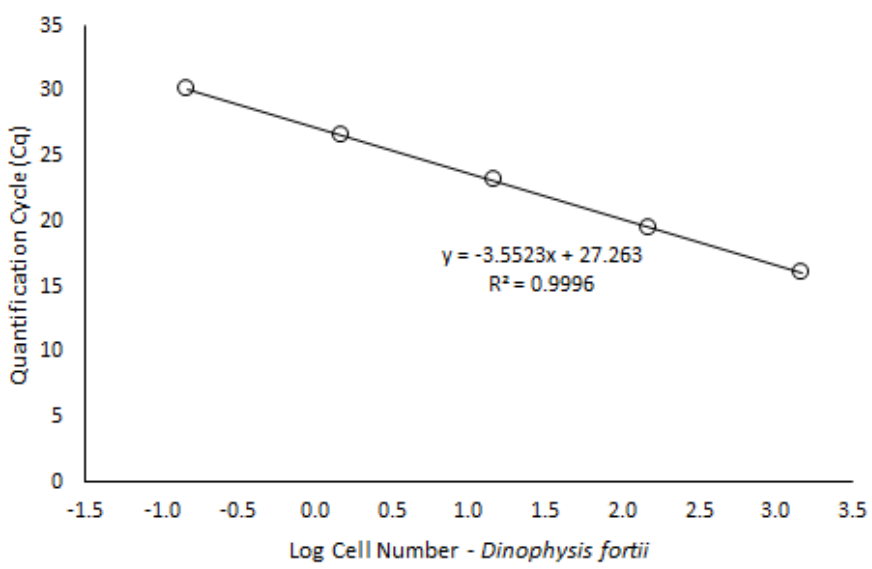

Figure 1. Map of Manning River (-31.89S, 152.64E), southeastern Australia (see insert), showing phytoplankton sampling site (black circle) and eDNA (sensor) sampling site (black square).

Figure 2A-C. Light microscopy images of A. *Dinophysis acuminata*, B. *D. caudata*, and C. *D. fortii*. Scale bar is 30 µm.

A.


Quantification Cycle (Cq)
y = -3.5437x + 28.535
R² = 0.9988
Log Cell Number - Dinophysis acuminata

B.


Quantification Cycle (Cq)
y = -3.5523x + 27.263
R² = 0.9996
Log Cell Number - Dinophysis fortii

C.

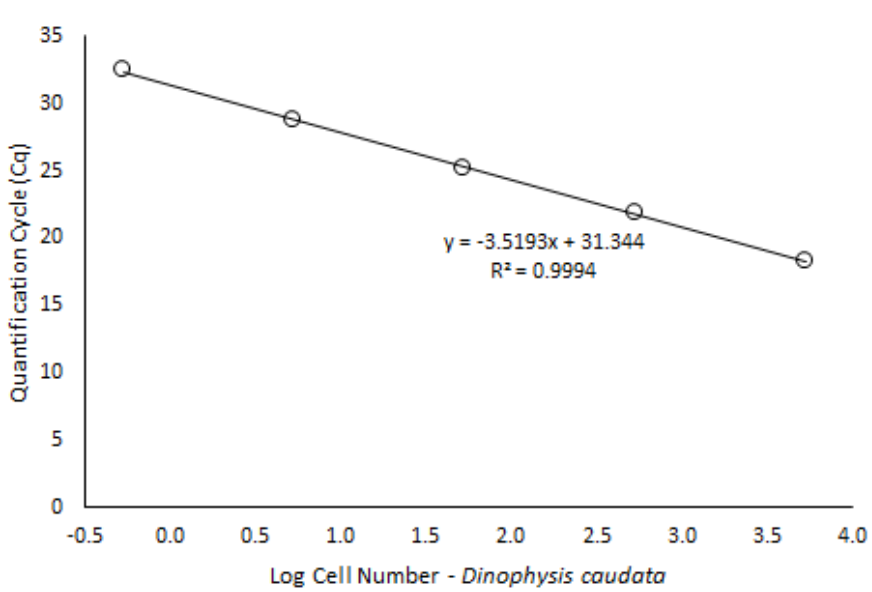

Quantification Cycle (Cq)
y = -3.5193x + 31.344
R² = 0.9994
Log Cell Number - Dinophysis caudata

Figure 3A-C. Standard curves for *Dacu_11F/Dacu_11R* primer pair using cell-based serial dilutions of A. *Dinophysis acuminata*; B. *Dinophysis fortii*; and C. *Dinophysis caudata*. All error bars were <5% for each data point so for clarity are not shown.

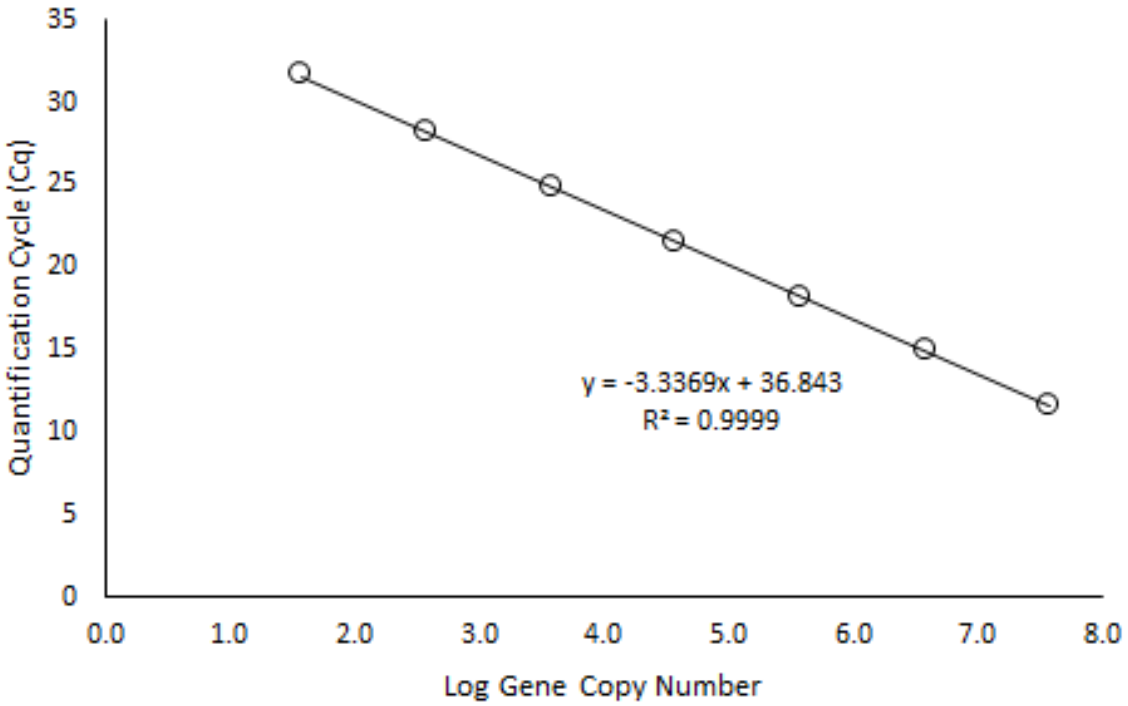



Figure 4. Standard curve for *Dacu_11F/Dacu_11R* primer pair using ITS gene fragment-based serial dilutions. All error bars were <5% for each data point so for clarity are not shown.

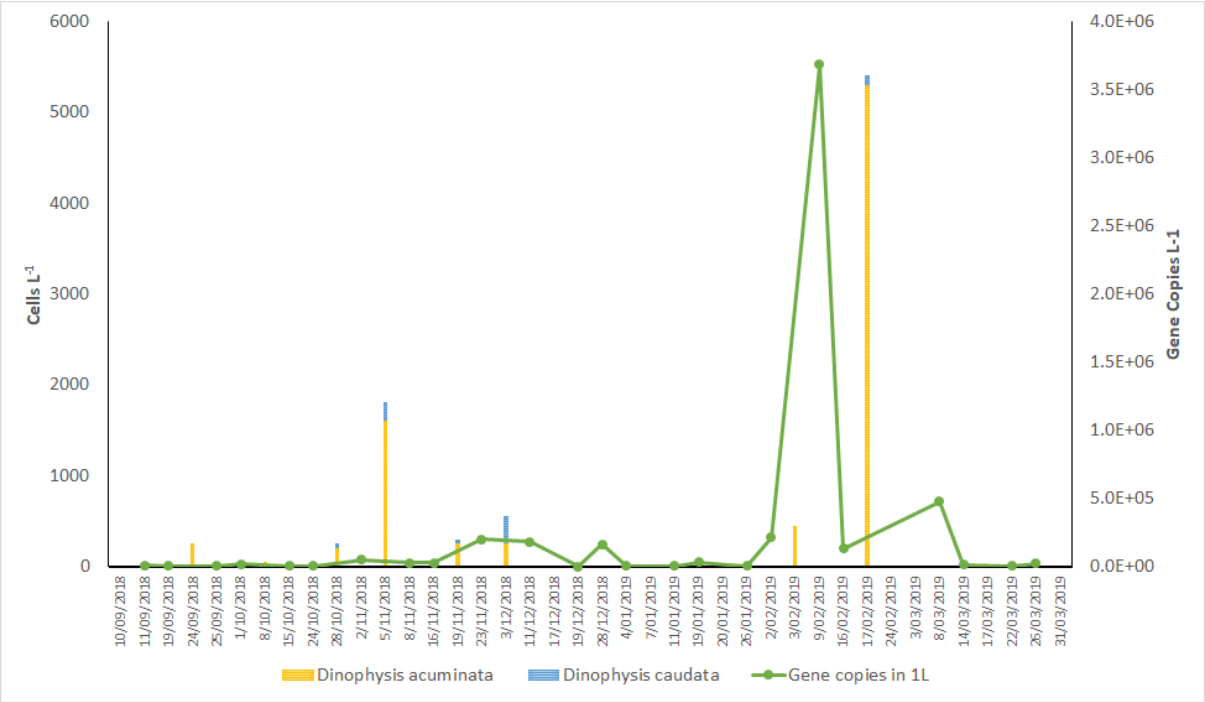



Figure 5. Comparative quantification of *Dinophysis acuminata* (yellow bar) and *D. caudata* (blue bar) using A. light microscopy (cells $L^{-1}$); and B. using qPCR (gene copies $L^{-1}$, green line) for *Dinophysis* spp. in the oyster-growing Manning River estuary.

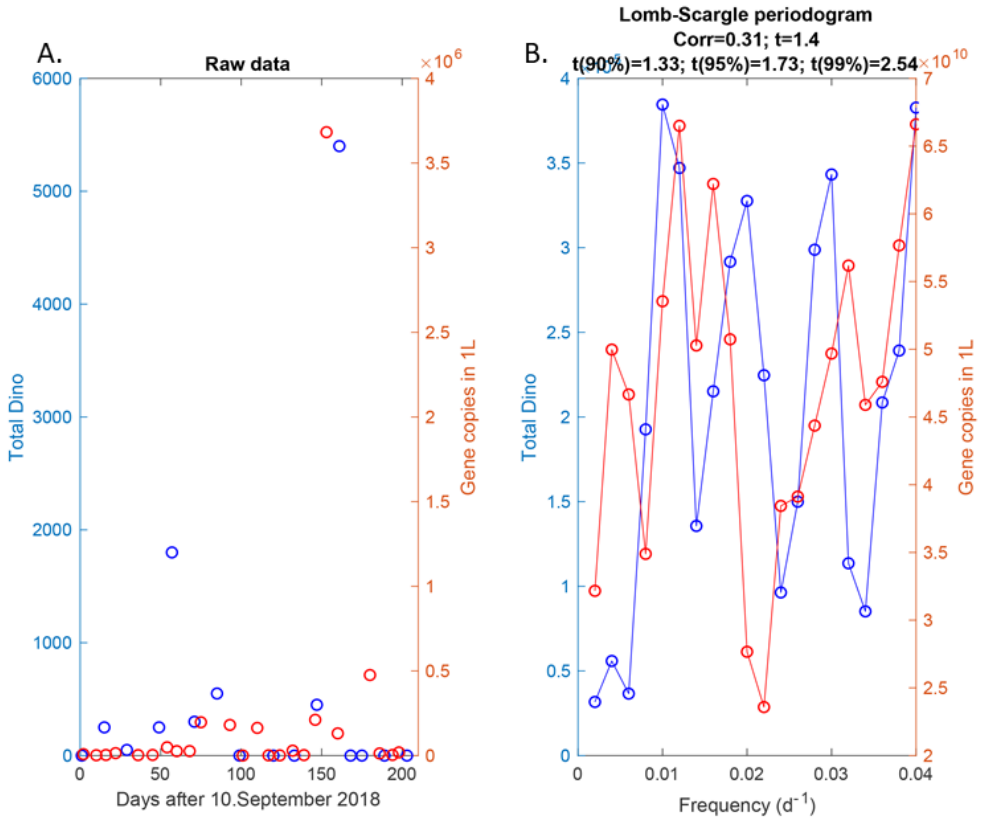
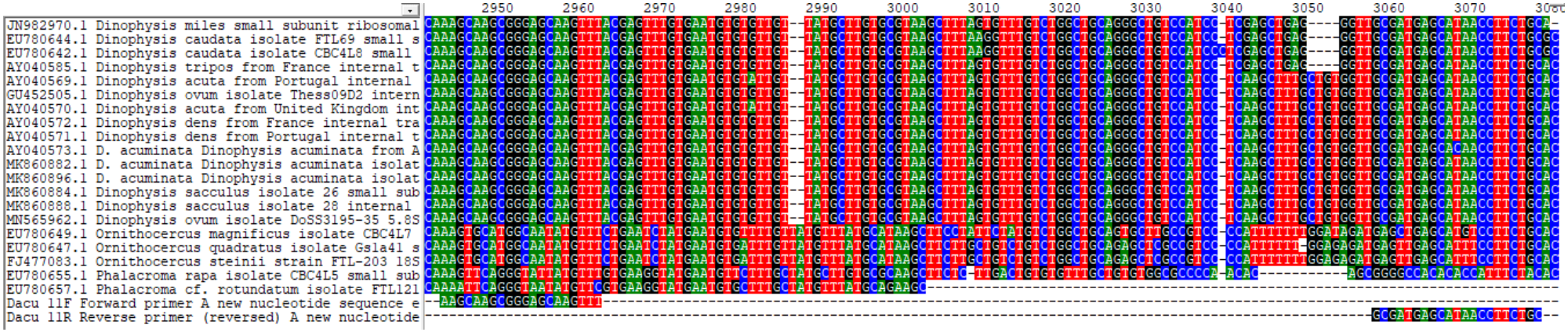



Figure 6. A. Raw data of both total *Dinophysis* enumeration using microscopy (cells $L^{-1}$, blue) and qPCR (gene copies $L^{-1}$, red); used for B. Lomb–Scargle periodogram to compare the periodicity in the unevenly sampled time-series

Supplementary Figure 1. Fasta file showing dissimilarity between ITS1/5.8S/ITS2 rRNA sequences for *Dinophysis* spp., *Phalacroma* spp. and *Ornithocercus* spp. with *Dacu_11F/Dacu_11R* primer pair; and B. List of the number of SNPs identified in the binding sites of the qPCR primers.




| Species | SNPs in Forward primer binding region | SNPs in reverse primer binding region |
|---|---|---|
| *O. magnificus* | 9 | 4 |
| *O. quadratus* | 9 | 4 |
| *O. steinii* | 9 | 4 |
| *P. rapa* | 8 | - |
| *P. cf. rotundatum* | 10 | - |