Assessing the Use of Molecular Barcoding and qPCR for Investigating the Ecology of *Prorocentrum minimum* (Dinophyceae), a Harmful Algal Species

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Abstract: *Prorocentrum minimum* is a species of marine dinoflagellate that occurs worldwide and can be responsible for harmful algal blooms (HABs). Some studies have reported it to produce tetradotoxin; however, results have been inconsistent. qPCR and molecular barcoding (amplicon sequencing) using high-throughput sequencing have been increasingly applied to quantify HAB species for ecological analyses and monitoring. Here, we isolated a strain of *P. minimum* from eastern Australian waters, where it commonly occurs, and developed and validated a qPCR assay for this species based on a region of ITS rRNA in relation to abundance estimates from the cultured strain as determined using light microscopy. We used this tool to quantify and examine ecological drivers of *P. minimum* in Botany Bay, an estuary in southeast Australia, for over ~14 months in 2016–2017. We compared abundance estimates using qPCR with those obtained using molecular barcoding based on an 18S rRNA amplicon. There was a significant correlation between the abundance estimates from amplicon sequencing and qPCR, but the estimates from light microscopy were not significantly correlated, likely due to the counting method applied. Using amplicon sequencing, ~600 unique actual sequence variants (ASVs) were found, much larger than the known phytoplankton diversity from this region. *P. minimum* abundance in Botany Bay was found to be significantly associated with lower salinities and higher dissolved CO2 levels.

Keywords: *Prorocentrum minimum*; harmful algae; next-generation sequencing

1. Introduction

In recent decades, there has been an apparent global increase in the range, intensity, and frequency of harmful algal blooms (HABs) linked to a variety of factors, including range expansions, increases in anthropogenic nutrients into coastal water bodies, and increased aquaculture [1–6]. *Prorocentrum minimum* is a planktonic marine dinoflagellate that forms HABs and is found commonly in temperate estuarine and coastal waters [7]. *P. minimum* blooms are most common in eutrophic coastal waters of the northern hemisphere; however, they have also been reported in tropical and subtropical regions globally [1,7–10]. Although few studies have been conducted on *P. minimum* in Australia, it is known to occur in high abundances in some regions, with frequent blooms in the Hawkesbury River in New South Wales (NSW) [11]. In line with the global increase of HABs, *P. minimum* appears to have expanded its geographical range over the last 40 years [1,10,12]. *P. minimum* usually blooms in warm brackish waters that are heavily impacted by excess nutrients, which has led to its presence being used as an indicator of eutrophication in water bodies in the northern hemisphere [1,11,13].

The abundance and even dominance of *P. minimum* in dynamic estuarine and coastal systems may be due to its broad salinity tolerance range of 5–17 PSU [9,14] and broad

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temperature tolerance range of 3–30 °C [1,15,16]. *P. minimum* typically blooms in low-turbulence environments during periods of high irradiance levels [1]; however, it has been demonstrated that the species can survive complete darkness for extended periods [17], which may allow it to survive in ship ballast waters. *P. minimum* is considered to be a mixotroph, able to supplement its nutrient intake due to feeding on smaller microbes, such as *Cryptomonas* spp., in response to depleted nutrients in the water [7,11,18]. Despite the ability to survive with low nutrients, *P. minimum* preferentially grows in water bodies with high nutrient loadings, typical of eutrophic water bodies [1,9]. *P. minimum* growth has been found to be associated with high inorganic nitrogen (N) and phosphorus (P), strongly linked to anthropogenic sources, such as fertilisers [7].

*P. minimum* blooms have been associated with several different marine biotoxins [19–22]; however, the identities of the compounds and their modes of toxicity are debated. *P. minimum* blooms have shown toxic effects on shellfish, including mortality, poor development, and altered behaviours [1,23–25]. Recently, a *P. minimum* bloom has been associated with the neurotoxin tetrodotoxin (TTX) [19,26,27], possibly due to bacterial species associated with *P. minimum* [26,28]. It has been suggested that *P. minimum* toxicity is variable depending on the strain of the species and the environmental conditions under which it is grown [1,26]. Due to incidences of toxin accumulation in shellfish and the impacts on shellfish growth of *P. minimum* toxins, it is an important HAB species to monitor in shellfish-harvesting regions [1,11,23,24,26].

Until recently, light microscopy has been the only routine method available to identify and manually count HAB species [29–32]. However, this method is relatively time-consuming, requires a very high level of taxonomic expertise, and is not able to identify cryptic species, which may appear morphologically indistinguishable from one another despite toxicological differences. For these reasons, alternative methods of monitoring HABs have been developed. Molecular genetic techniques can provide rapid and sensitive HAB monitoring [29,30,33,34]. Two molecular genetic methods used are quantitative polymerase chain reaction (qPCR) and molecular barcoding. Molecular barcoding, also referred to as amplicon sequencing, is becoming invaluable in studying marine ecological assemblages, as it allows for uncultured cells in samples to be identified [29,35,36]. However, due to the existence of variability in the copy numbers of genes among microalgal species, particularly in dinoflagellates [37–39], the number of gene copies amplified may not reflect the relative abundance of species in the sample. There is also a bias introduced with the use of broad-range primers, which can lead to certain sequences being preferentially amplified, giving a skewed proportional abundance of target species [29,40]. For this reason, the quantification of dinoflagellate species using amplicon sequencing is uncertain and not accurate when compared with other methods, including qPCR and light microscopy [29,41]. The use of molecular barcoding, which provides an overview of the genetic composition of microbial communities, in conjunction with qPCR, may improve the quantitative assessment of the impact of HAB species in the context of, for example, seasonal changes in the wider microbial community.

The aim of the study was to develop and assess new molecular genetic approaches to investigate the dynamics, community, and environmental drivers of *P. minimum* in an Australian estuary. To do this, a local isolate of *P. minimum* from Australia was established, and qPCR approaches were designed and tested for the detection and quantification of *P. minimum*. In addition, 18S rRNA amplicon sequences from estuarine water samples were examined to compare the specificity, detection limits, and quantification accuracy of the methods. Environmental samples, including physico-chemical data, were collected monthly for 14 months from 2016 to 2017 from two sites in Botany Bay, an estuary in southeast Australia. Data of the entire microbial community, the abundance of *P. minimum*, and the corresponding physico-chemical variables were examined to assess the factors impacting the presence and abundance of *P. minimum* in an Australian estuary.
2. Materials and Methods

2.1. Sampling Sites

Fortnightly, phytoplankton samples were collected from two sites, Towra Point (−34.007, 151.19) and Bare Island (−33.992, 151.23), which are both located in Botany Bay, a heavily modified estuary in southeast Australia (Figure 1), as part of the coastal and benthic sampling for the Marine Microbes project, conducted by Bioplatforms Australia (BPA)\[42\]. Water samples were also collected from Towra Point as part of the NSW Food Authority’s Shellfish Safety program for the purpose of identification and enumeration of phytoplankton. A phytoplankton net was towed to collect a dense sample to verify species identity by microscopy. Lugol’s iodine (elemental iodine (5%) and potassium iodide (KI, 10%) and distilled water used at 1 mL/50 mL sample) was added immediately after collection to preserve cells [43]. In the laboratory, gravity-assisted membrane filtration was used to concentrate samples, and cell counts were completed using a Sedgewick Rafter counting chamber following a previously published protocol [43–45]. Highly toxic species were counted to a minimum level of detection of 50 cells\(^{-}\), while others, including \(P.\) \(\text{minimum}\), were counted to a minimum level of detection of 500 cells\(^{-}\). All counts were completed using Zeiss Axiolab or Zeiss Standard microscopes with a maximum magnification of 1000×. All cells were identified to the nearest taxon able to be accurately identified, and if separation of species was not possible, the cells were assigned to a species group.

![Figure 1. Map of Botany Bay in southeast Australia, highlighting the two sampling points used for the Marine Microbes project, Bare Island and Towra Point.](image)

2.2. Sampling, DNA Extraction for Amplicon Sequencing, Metabarcoding, and Physico-chemical Data

All samples followed standard operating procedures (SOPs) outlined in Sections 1.5.1 and 1.7 of the Australian Microbiome Scientific Manual (version 2020) [46]. Briefly, 2 L samples were filtered in triplicate through 0.22 µm dia. pore-size polyethersulfone filters (Sterivex\(^{TM}\), Merck KGaA, Darmstadt, Germany) to concentrate algal and bacterial cells. DNA was extracted using the QIAGEN DNeasy PowerSoil Kit according to the man-
manufacturer’s instructions (QIAGEN, Hilden, Germany). Amplicon sequencing was completed using the HTS Illumina MiSeq technology at the Ramaciotti Centre for Genomics, University of New South Wales (UNSW). Samples were collected fortnightly at Towra Point and monthly at Bare Island. A series of 4× samples were collected during and after a rainfall event at both sites from 3 to 14 June 2016.

2.3. Cell Culture and Culturing Conditions

Several 600 mL water samples were collected from Berowra Creek (~33.5342, 151.1459), a tributary of the Hawkesbury River in NSW, about 50 km north of Botany Bay, on 12 April 2019 following reports of a bloom of this species on 8 April 2019. The bloom was reported by Ana Rubio from Hornsby Shire Council, who regularly monitors and samples Berowra Creek. Clonal cultures of cells with the morphological features typical of P. minimum were isolated under a light microscope using a micropipette and cultured in a 96-well plate format. The Berowra Creek sample was successful for the isolation of P. minimum, and one isolate survived and was successively inoculated into 50 mL of media. The clonal isolate was grown in K media [47] in an incubator at 18 °C at a salinity of 35 PSU under a 12/12 h light cycle (±100 µmol photon m⁻² s⁻¹). A salinity of 35 PSU closely matches the salinity found at Berowra Creek where the strain was isolated from. A cultured strain of a closely related species, Prorocentrum cf. balticum (obtained from the Cawthron Institute Culture Collection, Nelson, New Zealand, CICCM, CAWD38), was used as a negative control in the qPCR assay. P. cf. balticum was maintained in culture in identical conditions to P. minimum in K media [47]. Prorocentrum lima (SM43), Prorocentrum concavum (SM46), and Prorocentrum cassubicum (CS881, from the Australian National Algal Culture Collection) cultures were grown in F/10 media [48] under a 12/12 h light cycle. P. concavum and P. lima were incubated at 28 °C, and P. cassubicum at 25 °C.

2.4. Toxin Analysis

To prepare samples for toxin analysis using liquid chromatography–mass spectrometry (LC–MS, ThermoFisher Scientific Q Exactive, Waltham, MA, USA), 20 mL of a dense (28,000 cells mL⁻¹) culture was centrifuged at 4000 g for 10 min to form a pellet, and the supernatant was discarded. The sample was then freeze-dried and stored at −20 °C. Analysis of TTX presence in the culture was completed by Dr. Chowdhury Sarowar at the Sydney Institute of Marine Science (SIMS) following an adapted method from [49].

Briefly, 5 mL of 1 mM acetic acid was added to the sample and vortexed for 90 s, after which the samples were placed in a boiling water bath for 5 min and then cooled to room temperature. The cooled sample was placed in an ultrasonic bath for 1 min and then centrifuged to pellet the cellular debris, and the supernatant was used with or without dilution for LC–MS analysis. A Thermo Scientific™ Q EXACTIVE™ MS (Waltham, MA, USA) was used for the detection of TTX. The source parameters for detection were as follows: sheath gas and auxiliary gas flow rates of 50 and 13, respectively (arbitrary units); a spray voltage of 3.5 kV; a capillary temperature of 263° C; and an auxiliary gas heater temperature of 425° C.

Chromatographic separation was performed on a Thermo Scientific™ ACCELATM UPLC system (Waltham, MA, USA). Analysis was performed using an Acquity UPLC BEH Phenyl 1.7 µm 100 × 2.1 mm column with an injection volume of 5 µL. The mobile phases used were A (water/formic acid/NH4OH at 500:0.075:0.3 v/v/v) and B (acetonitrile/water/formic acid at 700:300:0.1 v/v/v). Initial condition starts at A/B 2:98 at a flow rate of 400 µl/min and held for 5 min. The condition was then linearly changed for over 3.5 min from A/B (2:98) to A/B (50:50). The flow rate was then changed from 400 µl/min to 500 µl/min for over 2 min. The chromatographic condition was then rapidly changed to initial buffer conditions A/B (2:98) for over 0.5 min, while the flow rate was kept at 500 µl/min. The flow rate was then increased to 800 µl/min for over 0.5 min and held for 0.6 min. The flow rate was then decreased back to the initial flow rate of 400 µl/min, and
the condition changed to A/B 100:0. A certified standard solution of TTX was sourced from Enzo Life Sciences (Exeter, UK).

2.5. DNA Extraction and PCR for Strain Identification

Cell density was determined by enumeration with Lugol’s iodine-stained cells using a Sedgewick Rafter counting chamber [44,45]. Following microscopic counts, samples of the *Prorocentrum* spp. cultures were harvested by centrifugation at 4,000 × g for 10 min to be used for DNA extraction. DNA was extracted from the *P. minimum* (and other *Prorocentrum* spp. to be used for negative controls) cell pellets using the QIAGEN DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. The samples were eluted in 100 µL of buffer and stored at −20 °C until analysis. The quantity and quality of the extracted DNA were measured with a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Following DNA extraction, PCR amplification was completed using appropriate primers for the LSU rRNA and ITS rRNA regions. PCR amplification was conducted using the Bio-Rad T100 Thermal Cycler [50] (Bio-Rad Laboratories Inc., Hercules, CA, USA), targeting two rRNA regions. The LSU rRNA region was run using d1F (F) and d3B (R) primers, and the ITS rRNA region was run with ITSfwd (F) and ITStrv (R) primers (Table 1). PCR was run in 25 µL reactions with 12.5 µL of ImmoMix (Bioline, Sydney, NSW, Australia), 1 µL of BSA (bovine serum albumin), 7.5 µL of sterile water, 1.5 µL of forward primer (10 µM), and 1.5 µL of reverse primer (10 µM). The protocol used for PCR was 10 min at 95 °C, followed by 35 cycles of 95 °C for 20 s, 57 °C for 30 s, and 72 °C for 1 min, then held at 72 °C for 7 min. DNA fragments were cleaned using the DNA Clean and Concentrator (Zymo Research, Irvine, CA, USA) according to the manufacturer’s protocol and sequenced at Macrogen (Seoul, Korea). Contigs were formed using the sequences in Geneious Prime (v2019.2.1, Biomatters, Ltd., Auckland, NZ). Following the assembly of the contigs for each gene region, each sequence was uploaded to NCBI BLAST nucleotide sequence search to identify the strain and confirm that it was *P. minimum* (Tables 2 and 3).

**Table 1.** Names and sequences of all the primers used in this project. Primers 200F and 525R are taken from [51]. The other primers were designed using the online Primer-BLAST software (NCBI). All reverse primers are in reverse complement.

| Primer Sequences for qPCR | Name (Forward) | Name (Reverse) |
|---------------------------|----------------|----------------|
| Pm 200F                   | TGTGTTTATTAGTTCAGACAGAC | Pm 525R       |
| 1F Pmin                   | CGGAGGCAAGGTGATAGGGAC | 1R Pmin TCTGGGAAGGCCAGAAGGCTG |
| 2F Pmin                   | TCGGCTCGAACAGATGAAG | 2R Pmin AAGGGTCTGGAAAGGCGCAG |
| 3F Pmin                   | TTCTGGCCGTTTCCAGAAGGC | 3R Pmin CATGCCCAACAAACAGGCA |
| 4F Pmin                   | CGTATACTGCGTCTTCCGGGA | 4R Pmin CACACAGAACAACACAAGCAGT |
| 5F Pmin                   | CTTTCCAGAAGCTGTGTTG | 5R Pmin CTGGGACTAGACGGAAC |
| 6F Pmin                   | CAGGCTCGAAGCTGTGCTTGG | 6R Pmin AGCGTTGCTGAAAGGCGCAG |
| 7F Pmin                   | CAACAGTGGTGTGGCCTCTTGG | 7R Pmin ATTCAAAAACAGAGAAGCAG |
| 8F Pmin                   | AAAAAACAGAGAGAAGTCAGGAAC | 8R Pmin CAAACAGAAGAGAQAGAC |
| 9F Pmin                   | GTGAGGCTGCTGGCTGG | 9R Pmin CAAACAGAAGAGATCATGAGAC |
| 10F Pmin                  | TCACTGGACCGCATTCATTCC | 10R Pmin AAGGACAGAGCAGAAGGAC |
| 11F Pmin                  | TCTGATGCAAGGAGGTCC | 11R Pmin GTTCTGGTGAAGTGTCGCTG |
| 12F Pmin                  | CGCTTCTCCAAGCGTCTGTTGG | 12R Pmin GCTGACCTAAGCCTGACG |
| 13F Pmin                  | CGCTTGTGTTTCTCAGTGT | 13R Pmin CATGCCCAACAAACAGGCA |
| 14F Pmin                  | TCTTCAGGCAAGGCAACTC | 14R Pmin CGGTTTCTGACCTAACTT |


Table 2. Top 5 BLAST nucleotide sequence hits of the LSU rRNA sequence of Prorocentrum minimum from Berowra Creek, CAWD359, as compared with sequences of P. minimum strains on the NCBI database, including linked accession numbers.

| Strain Description | Percent Identity | Accession       |
|--------------------|-----------------|-----------------|
| Prorocentrum minimum strain DAB02 28S | 99.66% | KU999985.1 |
| Prorocentrum minimum strain D-127 | 99.66% | JX402086.1 |
| Prorocentrum minimum isolate PIPV-1 | 99.54% | JQ616823.1 |
| Prorocentrum minimum isolate SERC | 99.54% | EU780639.1 |
| Prorocentrum minimum strain Pmin1 | 99.54% | AY863004.1 |

Table 3. Top 5 BLAST nucleotide sequence hits of the ITS rRNA sequence of P. minimum from Berowra Creek, CAWD359, as compared with sequences of P. minimum strains on the NCBI database.

| Strain Description | Percent Identity | Accession       |
|--------------------|-----------------|-----------------|
| Prorocentrum minimum strain D-127 | 99.67% | JX402086.1 |
| Prorocentrum minimum strain AND3V | 100.00% | EU244473.1 |
| Prorocentrum minimum isolate PIPV-1 | 99.35% | JQ616823.1 |
| Prorocentrum minimum strain PMDH01 | 99.35% | DO054538.1 |
| Prorocentrum minimum strain NMBjah049 | 99.67% | KY290717.1 |

2.6. qPCR Assay Development

2.6.1. Primer Design

A published set of primers designed for a P. minimum-specific qPCR assay was tested [51]. Forward (F) and reverse (R) primers were designed to amplify a 325 bp region from the partial 18S rDNA sequence of P. minimum accessed from GenBank (AY421791.1) (Table 1). Twenty-two new sets of primers were designed after the above primer did not pass testing using the NCBI Primer-BLAST tool, targeting ITS regions 1 and 2 of partial sequence of the P. minimum strain CCMP698 (EU927537.1). The sizes of the qPCR products were from 70 to 130 bp in length, with the primers 20 to 25 bp in length, with the optimal Tm (melting temperature) set at 60 °C (Table 1). All sets were expected to be specific to the target sequence of P. minimum, as they were compared with all available sequences in the NCBI database and were unique to P. minimum. All 22 primer sets were run using an identical protocol (see qPCR Assays) with P. minimum DNA to determine the most sensitive and efficient assay. All valid primer sets were then subjected to specificity testing with other Prorocentrum spp. DNA. Primer sets that amplified other Prorocentrum spp. were disregarded, and then standard curve testing followed.
2.6.2. qPCR Assays

qPCR was conducted using a 20 µL mix with 1 µL of DNA, 10 µL of Bio-Rad iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 1 µL (at 10 µM concentration) of each of the forward and reverse primers, and 7 µL of sterile water. qPCR was performed with the following thermal cycling program of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve was performed on all runs, from 55 °C to 95 °C in 5 °C increments for 0.05 s each. All qPCR analyses and testing were run on the Bio-Rad CFX96 Touch Real-Time PCR Detection System in 96-well plates with a clear seal or clear plastic strips [52] (Bio-Rad Laboratories Inc., Hercules, CA, USA). The previously published primers [51] and primer sets 1–12 followed the original qPCR protocol. Primers 13–22 were run with a modified protocol of 95 °C for 2 min, followed by 35–40 cycles of 95 °C for 15 s and 60 °C for 30 s. All assays were run using a generic thermal profile, as per above. The assays were tested for sensitivity using DNA extracted from the *P. minimum* cultures in duplicate and two negative controls containing no DNA (no template control (NTC)). Cross-reactivity of the primers was tested by running each assay on four other *Prorocentrum* spp. as negative controls. This step is crucial to ensure that the primers would only bind to *P. minimum* specifically. For other species, see Table 4. Unique primer sets developed using the NCBI Primer-BLAST tool were subjected to identical testing used for the published primer set [51]. Primer sets that were unable to amplify *P. minimum* were not sensitive (amplified past 35 cycles); those that amplified other *Prorocentrum* spp. at similar *Cq* values to *P. minimum* and those that had efficiencies outside 90–110% were disregarded. Those primer sets that passed specificity tests were then tested for their efficiency using a dilution series of gBlocks® gene fragments (IDT, USA) of the ITS region and *P. minimum* DNA, both with known concentrations. Standard curves were created using a 10-fold dilution series over five different concentrations and plotted with the threshold cycle (*Cq*) (*x*-axis) and natural log of concentration (cells/µL). The curves were used to calculate the efficiency (E) of the assay using 

\[
E = -\frac{1}{\Delta q_{\text{opt}}} \ln(2)\]

Efficiency of qPCR assays should fall between 90% and 110% [53]. This standard curve will also be used to quantify the amount of *P. minimum* cells in unknown concentrations from environmental samples [54,55]. The assay that had acceptable efficiency and specificity was used for analysing BPA samples. After the development of the assay, qPCR was run on all extracted DNA samples from Towra Point and Bare Island collected during the BPA project from 2016 to 2017 using only one of the primer sets that passed efficiency and specificity testing. If amplification was found in the no template control (NTC), a conservative cutoff value of 3.3 *Cq* points or more below the *Cq* of the NTC was set to accept the amplification of *P. minimum* in the samples. These data on the distribution and abundance of *P. minimum* were then compared with results obtained using light microscope identification and amplicon sequencing.

**Table 4.** All species used as negative controls for specificity testing of the *P. minimum* qPCR assay. Species names and which culture collection they can be found are listed, as well as the strain ID.

| Species Name         | Culture Collection | Strain ID |
|----------------------|--------------------|-----------|
| *Prorocentrum* cf. balticum | Cawthron Culture Collection (CICCM) | CAWD38 |
| *Prorocentrum* cassubicum | Australian National Culture Collection (ANAAC) | CS-881 |
| *Prorocentrum* concavum | Seafood Safety Team, University of Technology Sydney (UTS) | Pmona (SM46) |
| *Prorocentrum* lima | Seafood Safety Team, University of Technology, Sydney (UTS) | SM43 |
2.7. Bioinformatic Analysis

Samples collected as part of the Marine Microbes project were subjected to amplicon sequencing using primer sets to target different organisms: eukaryotes, bacteria, archaea, and fungi (Table 1). The primer sets used targeted the V4 region of 18S rRNA (Table 1) found in all eukaryotes [56]. After sequencing, the reads were trimmed, merged, concatenated, and taxonomically classified using the Earth Microbiome Project (EMP) protocol [57,58]. Following this, the resulting actual sequence variants (ASUs) were assigned to taxonomic lineages and species using the PR2 taxonomic database (version 4.12) [59] with the DADA2 (version 1.16.0) [60] assignTaxonomy and assignSpecies functions in R. The resulting data were used to extract the occurrence and relative abundance of *P. minimum* in the sequence samples; these data were used to compare with the qPCR results. The classified data were also used to discover phytoplankton species that co-occur with *P. minimum*. The relative abundance of *P. minimum* was multiplied by 100 (1 µL was used for amplicon sequencing) to give the approximate abundance in the 2 L original sample and then divided by 2 to give cells L\(^{-1}\) to be able to compare with microscope count and qPCR abundance data. Data are submitted as supplementary material, S2: Towra Point ASVs and S3: Bare Island ASVs.

2.8. Environmental Parameters

Physico-chemical data were collected during each sampling point according to the SOPs laid out in Australian Microbiome Scientific Manual Section 1.5. [46]. The physical parameters collected were temperature (°C), salinity (PSU), dissolved oxygen (% and mg/L), conductivity (s/m), total alkalinity (µmol/kg), and pH. The nutrients measured in the samples were nitrite, nitrate, oxidised nitrogen, phosphate, ammonium (all in µg/L), and total carbon dioxide (in µmol/kg). These data were statistically analysed with the qPCR *P. minimum* abundance data.

2.9. Statistical Analysis

To test for relationships between *P. minimum* and environmental variables, the data were first checked for normality using the Shapiro–Wilks test due to the small dataset. After failing normality (p < 0.05), all variables were log-transformed and tested again for normality. Several variables remained non-normally distributed (p < 0.05), so a nonparametric testing approach was applied. The two sites were not found to show any significant differences (p > 0.05), so data were pooled for both sites for further analysis. Due to the disparate nature of the dataset, multiple regression was deemed inappropriate. Instead, Kendall’s tau-b correlation was used as it is more suitable for nonparametric small datasets and is more robust to error [61]. It was found to be a suitable analysis to determine preliminary relationships that could be investigated with further higher temporal data. A two-tailed correlation was run between all variables to assess the correlation between *P. minimum* abundance and environmental variables. Analyses were run in SPSS (v.26, IBM Corp, Armonk, NY, USA).

To determine whether there were any significant correlations between *P. minimum* and other phytoplankton species, co-occurrence analysis was run using the probabilistic model developed by Veech (2013), which is included in the R package “cooccur” [62,63]. This method tests all possible pairwise associations between species across samples/sites, and the output is the probability that two species co-occur at a level that is more or less frequent than the observed frequency of co-occurrence [64]. The output provides information specific to *P. minimum* and its associations, as well as the number of random and significant associations between all species in the dataset. The amplicon sequencing output was used to create a presence/absence matrix with all phytoplankton species across all the BPA sampling dates to use in the analysis.
3. Results

3.1. Strain Isolation, Identification, and Toxin Testing

A strain of *P. minimum* was successfully isolated from a water sample from Berowra Creek, NSW, in April 2019. It was initially identified as *P. minimum* based on light microscopy. The strain was also identified as *P. minimum* based on sequencing of rRNA barcoding regions, as sequencing of the LSU (GenBank accession number MT856373) and ITS rRNA regions (GenBank accession number MT895109) matched eight different *P. minimum* strains (>99.5%) when queried against the NCBI nonredundant database using blastn (Tables 3 and 4). The strain has been submitted to the Cawthron Institute Culture Collection (http://cultures.cawthron.org.nz/) as strain number CAWD359. The strain was tested for the presence of tetrodotoxin using a TTX standard. No TTX was detected, while TTX was detected in the spiked positive control.

3.2. qPCR Assay Development and Testing

A previously published assay with specific primers for *P. minimum* [51] was tested. This assay did not pass initial testing due to the amplification of other *Prorocentrum* species tested: *P. cf. balticum*, *P. lima*, *P. cassubicum*, and *P. concavum*. The assay was also not able to distinguish between products in the melt curve analysis (Table 5). The assay had a low efficiency of 70%. Following this, 22 new primer sets were tested (Table 5) to find one that was specific to *P. minimum*, sensitive, and efficient (90% ≤ E ≤ 110%). Only one primer set (20, Pmin 20F and Pmin 20R) displayed acceptable specificity and efficiency: E = 101% for *P. minimum* standard curve using DNA extracted from our strain (from 1.91 × 10⁴ to 1.91 × 10⁰ cells, Figure 2) and E = 99.3% for the standard curve using the gBlock synthetic gene fragment of the ITS region of *P. minimum* (from 1.64 × 10⁷ to 1.64 × 10⁰ copies, Figure 2) at an annealing temperature of 60 °C. Although this primer set was found to amplify other *Prorocentrum* species, this amplification occurred at similar or higher Cq values than that of the lowest *P. minimum* dilution point, which was the DNA equivalent of ~1.9 cells of *P. minimum*. This was even though all samples contained the DNA equivalent of >10⁴ cells of that *Prorocentrum* species. We considered this to be an acceptable level of cross-reactivity. The assay had a reliable detection limit of ~13 cells L⁻¹ when only values of at least 3.3 Cq points or less than the NTC were taken into consideration [65,66]. Primer set 20 was then used to analyse the abundance of *P. minimum* in environmental samples collected from Botany Bay.

| Primer Set | *P. minimum* | *P. cf. balticum* | *P. cassubicum* | *P. concavum* | *P. lima* | gBlock (%) | *P. min DNA (%) |
|------------|--------------|------------------|----------------|--------------|-----------|------------|----------------|
| Pm 200F/525R | +            | +                | −              | /            | /         | 70         | −              |
| 3          | +            | /                | −              | −            | −         | 65         | −              |
| 4          | +            | +                | −              | −            | −         | 64         | −              |
| 5          | +            | +                | +              | +            | −         | 62         | −              |
| 6          | +            | +                | −              | +            | +         | 65         | −              |
| 7          | +            | /                | −              | −            | −         | 57         | −              |
| 8          | +            | +                | −              | −            | −         | 56         | −              |
| 9          | +            | +                | −              | −            | −         | 58         | −              |
| 10         | +            | +                | N/A            | /            | /         | 60         | −              |
| 11         | +            | −                | N/A            | −            | −         | 76         | −              |
| 12         | +            | /                | N/A            | −            | −         | 85         | −              |
| 13         | +            | −                | +              | N/A          | +         | 43         | −              |
| 14         | +            | +                | N/A            | +            | +         | 328        | 335            |
3.3. Comparison of qPCR, Light Microscope Count, and Amplicon Sequencing Abundance Results

During 2016–2017, *P. minimum* was recorded using the qPCR assay on 21 out of 27 sampling dates for Towra Point and 7 out of 17 dates for Bare Island, the two sites in Botany Bay (Figures 3 and 4). Abundances varied from 0 cells L\(^{-1}\) to 8100 cells L\(^{-1}\) at Towra Point and from 0 cells L\(^{-1}\) to 14,800 L\(^{-1}\) at Bare Island. The highest peaks were recorded in early June for Towra Point and Bare Island (Figures 3 and 4). No *P. minimum* was found on the 9 sampling dates between July 2016 and October 2016 at Bare Island (Figure 4).

The estimates of the cell abundances of *P. minimum* using all three methods showed results that were of the same order of magnitude and often relatively similar (Figures 3 and 4). The estimates of the abundance of *P. minimum* based on amplicon sequencing appeared to be consistently higher when compared with the qPCR and microscope count data (Figure 3). The highest abundances of *P. minimum* were found in June 2016; however, no microscope counts were completed for this month, so these data points were excluded in the following comparisons (Figures 3 and 4).

A highly significant relationship \((p = 1.61 \times 10^{-14}/p = 0.00, \text{ Table 6})\) was found between the *P. minimum* abundance estimates derived from amplicon sequencing data and the *P. minimum*-specific qPCR, while the relationships between microscope counts and qPCR and microscope counts and amplicon sequencing were not significant \((p > 0.05, \text{ Table 6})\).

It is likely that the higher standard deviation in the method used for the light microscope cell count (Figure 3) may have led to the apparent differences in the cell counts of *P. minimum* using this method compared with that of the two molecular genetic methods.
Figure 3. Towra Point *P. minimum* cell counts L$^{-1}$ with amplicon sequencing, qPCR, and microscope counts across the BPA sampling period. Standard error bars are included for qPCR (too small to detect) and count data.

Figure 4. Bare Island *P. minimum* cell counts L$^{-1}$ with amplicon sequencing and qPCR across the BPA sampling period. Standard error bars are included for qPCR (too small to detect).

Table 6. Linear regression between qPCR, metabarcoding, and light microscopy.

|                      | qPCR vs. Amplicon Sequencing | qPCR vs. Count | Amplicon Sequencing vs. Count |
|----------------------|------------------------------|----------------|-------------------------------|
| Multiple R          | 0.90                         | 0.18           | 0.23                          |
| R$^2$                | 0.82                         | 0.03           | 0.05                          |
| Adjusted R$^2$       | 0.81                         | -0.02          | 0.00                          |
| Standard Error       | 1172.31                      | 670.05         | 662.94                        |
| df                   | 35                           | 20             | 20                            |
| $p$ (Significance)   | 0.00                         | 0.43           | 0.31                          |

3.4. Amplicon Sequencing Results

Using the amplicon sequencing method, 644 different phytoplankton ASVs in 428 genera were identified from the 27 samples from Towra Point, and 623 ASVs in 419 genera were identified from the 17 samples from Bare Island (Figure 5).
3.5. Factors Influencing the Growth of *P. minimum* in Botany Bay

The abundance of *P. minimum* in Botany Bay was found to be significantly correlated to the environmental variables, total dissolved CO$_2$ (R = 0.34, p = 0.008), and salinity (R = −0.28, p = 0.035). All other variables were not found to be significantly correlated to *P. minimum* abundance. Data are submitted as supplementary material, S1: Physicochemical Data.

Co-occurrence Analysis

At Bare Island, *P. minimum* was found to have positive significant co-occurrence (p < 0.05) with four other phytoplankton species, *Pyramimonas gelidicola*, *Alexandrium pacificum*, *Euglyphida sp.*, and *Goniomondales sp.* At Towra Point, *P. minimum* was found to have significant negative co-occurrence (p < 0.05) with two other phytoplankton species, *Prymnesiophyceae Clade F* sp. and *Bildingia dawsonii*, and positive significant associations (p < 0.05) with *Psammodictyon sp.*, *Surirella sp.*, *Tryblionella apiculata*, *Vampyrellida sp.*, *Actinocyclus curvatulus*, *Cryothecomonas sp.*, *Dino-Group-II-Clade-26* sp., *Massiteriidae sp.*, *Navicula cryptoechola*, and *Navicula gregaria*.

4. Discussion

*Prorocentrum minimum* is a marine dinoflagellate that commonly occurs throughout the world and can form HABs [1,7,10]. HABs due to this species often occur in estuarine and coastal waters where aquaculture takes place, and in relation to that, death of shellfish has been reported [1,10]. *P. minimum* has been reported to show physiological flexibility with a global distribution across a range of conditions from temperate to subtropical [1,7,67]. It has been reported to produce TTX, a harmful neurotoxin [19,68–70]. Due to the potential harmful impacts of *P. minimum* on shellfish aquaculture, in this study, we aimed to develop new methods of investigating this species and apply them to environmental samples. In this study, a new culture of *P. minimum* was successfully isolated from Berowra Creek, Hawkesbury River, Australia. *P. minimum* has been linked to the production of TTX after a bloom in Vistonikos Bay, Greece, was positively correlated with TTX.
TTX was not detected in our strain. Genetic variability among strains may influence the toxicity of *P. minimum*, as well as the environmental conditions under which it is grown [1,24,26]. Due to the reported variability in toxicity in this species, future studies will be required to evaluate the toxicity of strains of *P. minimum*. As the alga is now successfully in culture with the Cawthron Institute Culture Collection, it allows future studies to look at more in depth toxin profiles, including how different environmental stressors and relationships with other known toxic algae or bacteria influence its toxicity.

qPCR assays have been developed over the past ~15 years for the detection and monitoring of HAB species [51,71,72]. qPCR has advantages over traditional light microscopy methods in that it is sensitive and rapid and allows for possible future automation. In the development of qPCR assays for the detection and quantification of specific taxa in environmental DNA, the most important considerations are the specificity of the assay in that it amplifies only the species of interest, and the amplification efficiency of assays with an efficiency of less than 90% will not give quantitative results across its full detection range [53,73]. Assays with an efficiency greater than 110% are considered to show significant inhibition to PCR amplification [74]. Amplification greater than 100% can be due to contamination in the sample, pipetting errors, inaccurate dilution series, and primer dimers [29]. For this study, a previously published qPCR assay developed for *P. minimum* was originally tested [51], which targeted a fragment of the small subunit ribosomal (SSU/18S) RNA gene. However, it was found to amplify several other nontarget *Prorocentrum* spp. and have a low efficiency (Table 5, 70%). Therefore, new primer sets were designed to develop a new qPCR assay for *P. minimum* with the aim of being specific, sensitive, and efficient. Twenty-two unique primer sets were designed and tested with variable results (Table 5). Only one of the primer sets was found to be sufficiently specific and efficient and was used to examine environmental samples for the presence of *P. minimum* (primer set 20, Table 5). The newly designed assay was based on the ITS rDNA gene region, which is more variable and faster evolving than the SSU rDNA gene among dinoflagellate species [75,76]. The assay did have a low level of cross-reactivity with the most genetically similar species, *P. cf. balticum*. However, *P. cf. balticum* could be distinguished from *P. minimum* due to a higher temperature on the melt curve profile. Several studies have used melt curve differences to discriminate similar species [77,78]. When analysed for efficiency, the new primer set showed E = 101% (*P. minimum* DNA) and E = 99% (gBlock synthetic DNA) (Table 5). The new assay amplifies a much shorter fragment than the previously published assay (71 bp compared with 325 bp), and this may account for its greater amplification efficiency [79]. The qPCR assay developed for *P. minimum* is more sensitive than most light microscopy counting methods, with a reliable detection limit of 13 cells L⁻¹ [65,66].

Molecular barcoding, or amplicon sequencing, which involves PCR amplification of environmental DNA (eDNA) and then sequencing of short (~600) [80] “barcoding” gene regions using high-throughput sequencing (HTS), is another molecular genetic method that has begun to be used in phytoplankton research [81–83]. Amplicon sequencing uses broad-range primers designed to amplify conserved regions across whole domains of life—in this case, eukaryotes [56,84]. A major problem with the use of amplicon sequencing as a tool for quantifying microbial eukaryotes is that the “barcoding” genes may be present in multiple copies that can be variable among microalgal populations and species, meaning that sequenced gene amplicons may not reflect the true abundance of a species in the sample. qPCR is not immune to this problem; however, the impact is minimised by designing primers that amplify gene regions only present in a specific species and using a standard curve with known amounts of target.

However, in this study, the sequencing of amplicons of eukaryotic V4 regions of SSU rDNA from samples from Botany Bay did not show a significantly different quantity of *P. minimum* compared with the quantification based on qPCR (Figures 3 and 4 and Table 6). In addition, the results of this method have shown a previously unknown level of phytoplankton diversity in Botany Bay, detecting over 600 eukaryotic microbial ASVs between
the two sites in Botany Bay. Previously, phytoplankton identification using light microscopy had led to the detection of only ~100 species or fewer in 10 years of phytoplankton monitoring at Botany Bay [85,86]. In this study, only 43% of all phytoplankton ASVs were able to be classified to species level using the 18S V4 primer set and the PR2 database [59]. Further development of reference databases of 18S V4 sequences from reference “voucher” specimen taxa curated by taxonomists is an important factor in the future of HTS to enable a more complete and accurate picture of microbiome species composition [87,88]. Another possible approach that may lead to a more specific identification of taxa is the use of other primer pairs that amplify other amplicon regions, such as the LSU rDNA region in dinoflagellates, the SSU (16S) plastid genes, CO1, cytochrome b, or other mitochondrial gene regions [82,83,89,90]. In previous studies, it was found that some groups of taxa, such as dinoflagellates, can be identified more readily using LSU rDNA regions, rather than SSU rDNA [83].

The collection and preservation of water samples for the identification and manual counting of cells with light microscopy has been the “gold standard” method used to study phytoplankton abundances [91–93]. The accuracy of light microscope-based microalgal enumeration is highly variable depending on the particular technique chosen, the counting effort, and the taxonomic expertise of the technician [81]. Compared with light microscopy enumeration, amplicon sequencing has been shown to be extremely sensitive and has the capacity to identify all phytoplankton species in a sample without requiring any taxonomic expertise. qPCR is an optimal technique for the enumeration of a particular target species, as the limit of detection is low, and the accuracy of the method is independent of the effort or taxonomic skills of the operator. It is relatively inexpensive, rapid, sensitive, and specific and, therefore, is highly suited to adoption for ongoing monitoring programs. qPCR can also be completed in situ at the time of sampling to get rapid results and can be used by trained shellfish producers to get results of HABs on-site.

The light microscopy counting method utilised in this study had a larger-than-average error rate, a high detection threshold of *P. minimum* (500 cells L$^{-1}$ compared with 13 cells L$^{-1}$ with qPCR), and comparatively fewer data points when compared with the molecular methods. Adoption of other light microscope counting methods, like the Utermöhl counting chamber [94], and the use of a DNA-based stain (i.e., a fluorescence in situ hybridisation (FISH) probe [95]) may have led to more accurate assessments of the abundance of *P. minimum* and the detection of cryptic species. For research into HAB ecology, a combination of the use of amplicon sequencing, to first determine the diversity of phytoplankton, particularly cryptic and small species, and then qPCR, to quantify the exact cell abundance, would appear to give optimal information for ecological inference and understanding of co-occurrence patterns. This two-step molecular pathway appears to be the most appropriate method for future development [29,35,71,92,96].

Botany Bay is an estuary in southeast Australia that is extensively modified [43], containing an international shipping port (Port Botany), an oil fuelling station, recreational beaches, industrial estates, and urban developments [97]. The bay is a highly populated area and is impacted by freshwater flows from the Georges and Cooks Rivers, both also extensively modified and surrounded with urban developments [97]. Despite the modification, Botany Bay is also home to a Ramsar wetland and one remaining oyster farm, both at Towra Point [98]. Thus, it is an important site for ongoing monitoring of HAB species, as they can impact not only the shellfish production but also the quality of the water for recreational and industrial purposes. Botany Bay and the Georges River have both previously experienced HABs, including *Noctiluca scintillans*, *Alexandrium pacificum*, other *Alexandrium* spp., and *Heterocapsa* spp. [99].

Two sites in Botany Bay were sampled from April 2016 to June 2017: Bare Island and Towra Point (Figure 1). Due to the extensively modified nature of the bay and its surroundings, and the nutrient input that can occur in relation to this land runoff, it was expected that *P. minimum* may be abundant at these sites. It was also expected that *P. minimum* may be particularly high in abundance at Towra Point, which is impacted by
freshwater flows, as this species has been shown to flourish in low salinities with high nutrient freshwater inputs [1,70]. *P. minimum* was found to be in low abundance for most of the sampling period at both sites, detected at ~30 cells L\(^{-1}\) at both sites for most of the year of sampling (Figures 3 and 4). There was one peak in the abundance of *P. minimum* (8000–14,000 cells L\(^{-1}\)) at both sites, on 7 June 2016 (Figures 3 and 4). This could still be considered a low value for *P. minimum*, which has been detected at “bloom” levels upwards of 10 million cells\(^{-1}\) [11]. The low presence of *P. minimum* is an important current baseline for monitoring the health of Botany Bay and other southeast Australian estuaries.

The abundance of *P. minimum* was found to have a significant positive relationship with total CO\(_2\), contrary to a previous finding that found that increased CO\(_2\) had no relationship with the abundance of *P. minimum* [100]. *P. minimum* was also found to have a weak but significant negative relationship with salinity, which supports previous findings that *P. minimum* grows preferentially in decreasing salinities [1,70,101]. *P. minimum* was not found to have a significant relationship with any other environmental variables; however, it is likely that there may be a time lag between an environmental change and increase or decrease in *P. minimum* [102,103]. Incorporating a measure of exposure of *P. minimum* to environmental variables would require a higher temporal sampling frequency than what was undertaken in the present study. However, the correlations we found (+ve CO\(_2\) and –ve salinity) between *P. minimum* and the environmental variables measured are hypothesis forming and should be further investigated in Australian waters.

An analysis of phytoplankton species that significantly co-occurred with *P. minimum* in Botany Bay is useful, as in the past, toxicity attributed to blooms of *P. minimum* may have been also associated with the presence of *Dinophysis* spp., which are the main causative agents of diarrhetic shellfish poisoning (DSP), even when present in low abundances, such as ~100 cells L\(^{-1}\) [1,21,22,104]. Due to the potential uncertainties of amplicon sequencing-based estimates of the absolute abundance of cells in a sample, the data were analysed as a presence/absence matrix across all sampling dates [29,37]. *P. minimum* at Towra Point was found to significantly co-occur with 12 other phytoplankton species and at Bare Island with 4 other phytoplankton species. Of all the co-occurring species, only 1 is a toxin-producing species, *Alexandrium pacificum*. *A. pacificum* is an important HAB species due to the severity of bloom impacts in Australia, New Zealand, Korea, Japan, and other countries [72,105–107]. *A. pacificum* produces paralytic shellfish toxins (PSTs). *P. minimum* blooms have previously been associated with symptoms characteristic of PSTs [68,69]; however, there is still a possibility that it can produce other toxins not yet classified [19,20].

5. Conclusions

A sensitive, specific, and efficient *P. minimum* qPCR assay was successfully developed and will allow for high-throughput information to be collected on the distribution and abundance of this species. A new strain of *P. minimum* was also isolated from Berowra Creek, NSW, and shown to not produce tetrodotoxin. *P. minimum* was found to generally be in a low abundance in Botany Bay across all seasons during the BPA Marine Microbes sampling period (April 2016–June 2017), with one peak in its abundance at Towra Point and Bare Island in June. *P. minimum* was found to be significantly correlated to total CO\(_2\) and to a decrease in salinity at the sites in Botany Bay. Further field and laboratory studies may be useful to determine more detailed information on the environmental variables associated with blooms of *P. minimum* in southeast Australia. *P. minimum* was found to positively co-occur with *A. pacificum*, which produces PSTs. This association may be relevant to the management of harmful algal blooms in Botany Bay and other oyster-producing estuaries in southeast Australia. qPCR is a useful method for the monitoring of particular HAB species as it is rapid, specific, sensitive, and efficient, while the use of amplicon sequencing based on the V4 region of the 18S rDNA found a level of microbial eukaryotic species diversity that was approximately six times greater than that previously known...
from this site. In the future, these two methods may be combined as a valuable tool for HAB research in Australian waters.

**Supplementary Materials:** The following are available online at www.mdpi.com/2076-2607/9/3/510/s1, Table S1: Physicochemical data, Table S2: Towra Point ASV data, Table S3: Bare Island ASV data.

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**References**

1. Heil, C.A.; Glibert, P.M.; Fan, C. Prorocentrum minimum (Pavillard) Schiller: A review of a harmful algal bloom species of growing worldwide importance. *Harmful Algae* 2005, 4, 449–470, doi:10.1016/J.HAL.2004.08.003.
2. Gobler, C.J.; Doherty, O.M.; Hattemrath-Lehmann, T.K.; Griffith, A.W.; Kang, Y.; Litaker, R.W. Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. *Proc. Natl. Acad. Sci. USA* 2017, 114, 4975–4980, doi:10.1073/pnas.1619575114.
3. Nixon, S.W. Coastal marine eutrophication: A definition, social causes, and future concerns. *Ophelia* 1995, 41, 199–219, doi:10.1080/00785236.1995.10422044.
4. Cloern, J.E. Our evolving conceptual model of the coastal eutrophication problem. *Mar. Ecol. Prog. Ser.* 2001, 210, 223–253.
5. Méneguex, A.; Lacroix, G. Modelling the marine eutrophication: A review. *Sci. Total Environ.* 2018, 636, 339–354, doi:10.1016/J.SCITOTENV.2018.04.183.
6. FAO The State of World Fisheries and Aquaculture: Meeting the Sustainable Development Goals; FAO: Rome, Italy, 2018.
7. Glibert, P.M.; Mayorga, E.; Seitzinger, S. Prorocentrum minimum tracks anthropogenic nitrogen and phosphorus inputs on a global basis: Application of spatially explicit nutrient export models. *Harmful Algae* 2008, 8, 33–38, doi:10.1016/J.HAL.2008.05.023.
8. Hajdu, S.; Pertola, S.; Kuosa, H. Prorocentrum minimum (Dinophyceae) in the Baltic Sea: Morphology, occurrence—a review. *Harmful Algae* 2005, 4, 471–480, doi:10.1016/J.HAL.2004.08.004.
9. Pertola, S.; Kuosa, H.; Olsen, R. Is the invasion of Prorocentrum minimum (Dinophyceae) related to the nitrogen enrichment of the Baltic Sea? *Harmful Algae* 2005, 4, 481–492, doi:10.1016/J.HAL.2004.08.005.
10. Skarlato, S.; Tellesh, I.; Mantnaseva, O.; Pozdnjakov, I.; Berdleva, M.; Schubert, H.; Filatova, N.; Knyazev, N.; Pechkoukskaia, S. Studies of bloom-forming dinoflagellates Prorocentrum minimum in fluctuating environment: Contribution to aquatic ecology, cell biology and invasion theory. *Protistology* 2018, 12, 113–157.
11. Ajani, P.A.; Larsson, M.E.; Woodcock, S.; Rubio, A.; Farrell, H.; Brett, S.; Murray, S.A. Bloom drivers of the potentially harmful dinoflagellate Prorocentrum minimum (Pavillard) Schiller in a south eastern temperate Australian estuary. *Eurmar. Coast. Shelf Sci.* 2018, 215, 161–171, doi:10.1016/j.ecss.2018.09.029.

12. Heisler, J.; Gilbert, P.; Burkholder, J.; Anderson, D.; Cochlan, W.; Dennison, W.; Goble, C.; Dortch, Q.; Heil, C.; Humphries, E.; et al. Eutrophication and Harmful Algal Blooms: A Scientific Consensus. *Harmful Algae* 2008, 8, 3–13, doi:10.1016/j.hal.2008.08.006.

13. Jeong, B.; Jeong, E.-S.; Malazarte, J.M.; Sin, Y. Physiological and Molecular Response of Prorocentrum minimum to Tannic Acid: An Experimental Study to Evaluate the Feasibility of Using Tannic Acid in Controlling the Red Tide in a Eutrophic Coastal Water. *Int. J. Environ. Res. Public Health* 2016, 13, 503, doi:10.3390/ijerph13050503.

14. Hajdu, S.; Edler, L.; Olenina, I.; Witek, B. Spreading and Establishment of the Potentially Toxic Dinoflagellate Prorocentrum minimum in the Baltic Sea. *Int. Rev. Hydrobiol.* 2000, 85, 561–575, doi:10.1002/(SICI)1098-1060(200011)85:5<561::AID-IRHY561>3.0.CO;2-3.

15. Telesh, L.; Schubert, H.; Skarlato, S.O. Ecological niche partitioning of the invasive dinoflagellate Prorocentrum minimum and its native congeners in the Baltic Sea. *Harmful Algae* 2016, 59, 100–111, doi:10.1016/J.HAL.2016.09.006.

16. Tango, P.J.; Magnien, R.; Butler, W.; Luckett, C.; Luckenbach, M.; Lacouture, R.; Poukish, C. Impacts and potential effects due to Prorocentrum minimum blooms in Chesapeake Bay. *Harmful Algae* 2005, 4, 525–531, doi:10.1016/J.HAL.2004.08.014.

17. Tyler, M.A.; Seliger, H.H. Selection for a red tide organism: Physiological responses to the physical environment. *Limnol. Oceanogr.* 1981, 26, 310–324, doi:10.4319/lo.1981.26.2.0310.

18. Stoecker, D.; Li, A.; Coats, D.; Gustafson, D.; Nannen, M. Mixotrophy in the dinoflagellate Prorocentrum minimum. *Mar. Ecol. Prog. Ser.* 1997, 152, 1–12, doi:10.3354/meps152001.

19. Denardou-Queneherve, A.; Grzebyk, D.; Pouchus, Y.; Sauvait, M.; Alliot, E.; Biard, J.; Berland, B.; Verbiest, J. Toxicity of French strains of the dinoflagellate Prorocentrum minimum experimental and natural contaminations of mussels. *Toxicicon* 1999, 37, 1711–1719, doi:10.1016/S0041-0101(99)00113-0.

20. Grzebyk, D.; Denardou, A.; Berland, B.; Pouchus, Y.F. Evidence of a new toxin in the red-tide dinoflagellate Prorocentrum minimum. *J. Plankton Res.* 1997, 19, 1111–1124, doi:10.1093/plankt/19.8.1111.

21. Langeland, G.; Hasselgård, T.; Tangen, K.; Skulberg, O.M.; Hjelle, A. An outbreak of paralytic shellfish poisoning in western Norway. *Sarsia* 1984, 69, 185–193, doi:10.1080/00364827.1984.10420605.

22. Tangen, K. Shellfish poisoning and the occurrence of potentially toxic dinoflagellates in Norwegian waters. *Sarsia* 1983, 68, 1–7, doi:10.1080/00364827.1983.10420550.

23. Landsberg, J.H. The Effects of Harmful Algal Blooms on Aquatic Organisms. *Rev. Fish. Sci.* 2002, 10, 113–390, doi:10.1080/20026491051695.

24. Wikfors, G. A review and new analysis of trophic interactions between Prorocentrum minimum and clams, scallops, and oysters. *Harmful Algae* 2005, 4, 585–592.

25. Ogburn, D.; Callinan, R.; Pearce, I.; Hallegraeff, G.; Landos, M. Investigation and Management of a Major Oyster Mortality Event in Wonboyn Lake, Australia. In *Diseases in Asian Aquaculture*; Walker, P., Lester, R., Bondad-Reantaso, M.G., Eds.; Asian Fisheries Society: Manila, Philippines, 2005; pp. 301–309.

26. Rodriguez, I.; Alfonso, A.; Alonso, E.; Rubiolo, J.A.; Roel, M.; Vlamis, A.; Katikou, P.; Jackson, S.A.; Menon, M.L.; Dobson, A.; et al. The association of bacterial C9-based TTX-like compounds with *Prorocentrum minimum* opens new uncertainties about shellfish seafood safety. *Sci. Rep.* 2017, 7, 40880, doi:10.1038/srep40880.

27. Vlamis, A.; Katikou, P.; Rodriguez, I.; Rey, V.; Alfonso, A.; Papazachariou, A.; Zacharaki, T.; Botana, A.; Botana, L.; Vlamis, A.; et al. First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate Prorocentrum minimum. *Toxins* (Basel) 2015, 7, 1779–1807, doi:10.3390/toxins7051779.

28. Park, B.S.; Guo, R.; Lim, W.-A.; Ki, J.-S. Importance of free-living and particle-associated bacteria for the growth of the harmful dinoflagellate Prorocentrum minimum: Evidence in culture stages. *Mar. Freshw. Res.* 2018, 69, 290, doi:10.1017/MF17102.

29. Mäki, A.; Salmi, P.; Mikkonen, A.; Kremp, A.; Tiirola, M. Sample Preservation, DNA or RNA Extraction and Data Analysis for High-Throughput Phytoplankton Community Sequencing. *Front. Microbiol.* 2017, 8, 1848, doi:10.3389/fmicb.2017.01848.

30. Sellner, K.G.; Doucette, G.J.; Kirkpatrick, G.J. Harmful algal blooms: Causes, impacts and detection. *J. Ind. Microbiol. Biotechnol.* 2003, 30, 383–406, doi:10.1007/s10295-003-0074-9.

31. Medlin, L. Molecular tools for monitoring harmful algal blooms. *Environ. Sci. Pollut. Res.* 2013, 20, 6683–6685, doi:10.1007/s11356-012-1195-3.

32. Letterova, M.I.; Budvytiene, I.; Sandlund, J.; Färnert, A.; Banai, N. Simple Real-Time PCR and Amplicon Sequencing Method for Identification of Plasmodium Species in Human Whole Blood. *J. Clin. Microbiol.* 2015, 53, 2251–2257, doi:10.1128/JCM.00542-15.

33. Medlin, L.; Orozco, J. Molecular Techniques for the Detection of Organisms in Aquatic Environments, with Emphasis on Harmful Algal Bloom Species. *Sensors* 2017, 17, 1184, doi:10.3390/s17051184.

34. Kudela, R.M.; Howard, M.D.A.; Jenkins, B.D.; Miller, P.E.; Smith, G.J. Using the molecular toolbox to compare harmful algal blooms in upwelling systems. *Prog. Oceanogr.* 2010, 85, 108–121, doi:10.1016/j.pocean.2010.02.007.

35. Murray, D.C.; Coghlan, M.L.; Bunce, M. From Benchtop to Desktop: Important Considerations when Designing Amplicon Sequencing Workflows. *PLoS ONE* 2015, 10, e0124671, doi:10.1371/journal.pone.0124671.
36. Stern, R.F.; Horak, A.; Andrew, R.L.; Coffroth, M.-A.; Andersen, R.A.; Küpper, F.C.; Jameson, I.; Hoppenrath, M.; Véron, B.; Kasai, F.; et al. Environmental barcoding reveals massive dinoflagellate diversity in marine environments. *PloS ONE* **2010**, *5*, e13991, doi:10.1371/journal.pone.0013991.

37. Galluzzi, L.; Bertozzini, E.; Penna, A.; Perini, F.; García, E.; Magnani, M. Analysis of rRNA gene content in the Mediterranean dinoflagellate Alexanderum catenella and Alexandrium taylori: Implications for the quantitative real-time PCR-based monitoring methods. *J. Appl. Phycol.* **2010**, *22*, 1–9, doi:10.1007/s10811-009-9411-3.

38. Godhe, A.; Asplund, M.E.; Härmström, K.; Saravana, V.; Tyagi, A.; Karunasagar, I. Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. *Appl. Environ. Microbiol.* **2008**, *74*, 7174–7182, doi:10.1128/AEM.01298-08.

39. Bachvaroff, T.R.; Place, A.R. From Stop to Start: Tandem Gene Arrangement, Copy Number and Trans-Splicing Sites in the Dinoflagellate Amphidinium carterae. *PloS ONE* **2008**, *3*, e2929, doi:10.1371/journal.pone.0002929.

40. Krehenwinkel, H.; Wolf, M.; Lim, J.Y.; Rominger, A.J.; Simison, W.B.; Gillespie, R.G. Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. *Sci. Rep.* **2017**, *7*, 1–12, doi:10.1038/s41598-017-1733-x.

41. Bradley, I.M.; Pinto, A.J.; Guest, J.S. Design and Evaluation of Illumina MiSeq-Compatible, 18S rRNA Gene-Specific Primers for Improved Characterization of Mixed Phototrophic Communities. *Appl. Environ. Microbiol.* **2016**, *82*, 5878–5891, doi:10.1128/AEM.01630-16.

42. Australian Microbiome–Australian Microbiome. Available online: https://www.australianmicrobiome.com/ (accessed on 9 December 2020).

43. Ajani, P.; Brett, S.; Krogh, M.; Scanes, P.; Webster, G.; Armand, L. The risk of harmful algal blooms (HABs) in the oyster-growing estuaries of New South Wales, Australia. *Environ. Monit. Assess.* **2013**, *185*, 5295–5316, doi:10.1007/s10661-012-2946-9.

44. Woelkerling, W.J.; Kowal, R.R.; Gough, S.B. Sedgwick-rafter cell counts: A procedural analysis. *Hydrobiologia* **1976**, *48*, 95–107, doi:10.1007/BF00040161.

45. Throndsen, J. Preservation and storage. In *Phytoplankton Manual*; Sournia, A., Eds.; UNESCO: Paris, France, 1978; pp. 69-74 ISBN 9231015729.

46. van de Kamp, J.; Mazzard, S. Coastal Seawater Sampling for Australian Coastal Microbial Observatory Network. In *Australian Microbiome Methods*; Bioplatforms Australia: Sydney, Australia, 2020; pp. 12–18. Available online: https://www.australianmicrobiome.com/wp-content/uploads/2021/01/AM_Methods_for_metadata_fields_18012021_V1.2.3.pdf (accessed 1 March 2019).

47. Keller, M.D.; Selvin, R.C.; Claus, W.; Guillard, R.R.L. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* **2007**, *23*, 633–638, doi:10.1111/j.1529-8817.1987.tb04217.x.

48. Jeffrey, S.W.; LeRoi, J.M. Simple procedures for growing SCOR reference microalgal cultures. In *Phytoplankton Pigments in Oceanography: Monographs on Oceanographic Methodology*; Jeffrey, S.W., Mantonua, R.F.C., Wright, S.W., Eds.; UNESCO: Paris, France, 1997; pp. 181–203 ISBN 9231032755.

49. Harwood, T.; Boundy, M.; Selwood, A.; Ginkel, R.; MacKenzie, L.; McNabb, P. Refinement and implementation of the Lawrence method (AOAC 2005.06) in a commercial laboratory: Assay performance during an Alexandrium catenella bloom event. *Harmful Algae* **2013**, *24*, 20–31, doi:10.1016/j.hal.2013.01.003.

50. Bio-Rad Laboratories Inc. T100™ Thermal Cycler. Available online: https://www.bio-rad.com/en-au/product/t100-thermal-cycler?ID=LGTWGIE8Z (accessed on 30 September 2019).

51. Handy, S.M.; Demir, E.; Hutchins, D.A.; Fortune, K.J.; Whereat, E.B.; Hare, C.E.; Rose, J.M.; Warner, M.; Farestad, M.; Cary, S.C.; et al. Using quantitative real-time PCR to study competition and community dynamics among Delaware Inland Bays harmful algae in field and laboratory studies. *Harmful Algae* **2008**, *7*, 599–613, doi:10.1016/j.hal.2007.12.018.

52. Bio-Rad Laboratories Inc. CFX96 Touch Real-Time PCR Detection System. Available online: https://www.bio-rad.com/en-au/product/cfx96-touch-real-time-pcr-detection-system?ID=LBJ1YU15 (accessed on 9 September 2019).

53. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622, doi:10.1373/clinchem.2008.112797.

54. Taylor, S.C.; Nadeau, K.; Abbasi, M.; Lachance, C.; Nguyen, M.; Fenrich, J. The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time. *Trends Biotechnol.* **2019**, *37*, 761–774, doi:10.1016/J.TIBTECH.2018.12.002.

55. Larionov, A.; Krause, A.; Miller, W. A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* **2005**, *6*, 62, doi:10.1186/1471-2105-6-62.

56. Hadziavdic, K.; Lekang, K.; Lanzen, A.; Jonassen, I.; Thompson, E.M.; Troedsson, C. Characterization of the 18S rRNA gene for designing universal eukaryotic specific primers. *PloS ONE* **2014**, *9*, e87624, doi:10.1371/journal.pone.0087624.

57. Bioplatforms Australia. *Protocol for 18S rRNA Amplification and Sequencing on the Illumina MiSeq*; Bioplatforms Australia: Sydney, Australia 2015.

58. Amaral-Zettler, L.L.; Bauer, M.; Berg-Lyons, D.; Betley, J.; Caporaso, J.G.; Ducklow, H.W.; Fierer, N.; Fraser, L.; Gilbert, J.A.; Gormley, N.; et al. *EMP 18S Illumina Amplicon Protocol*; Earth Microbiome Project. Available online: https://earthmicrobiome.org/protocols-and-standards/18ss/ (Accessed on 26 February 2021).

59. Guillou, L.; Bachar, D.; Audic, S.; Bass, D.; Berney, C.; Bittner, L.; Boutte, C.; Burgaud, G.; de Vargas, C.; Decelle, J.; et al. The Prosite Ribosomal Reference database (PR2): A catalog of unicellular eukaryote Small Sub-Unit rRNA sequences with curated taxonomy. *Nucleic Acids Res.* **2012**, *41*, D597–D604, doi:10.1093/nar/gks1160.
60. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583, doi:10.1038/nmeth.3869.

61. Croux, C.; Dehon, C. Influence functions of the Spearman and Kendall correlation measures. *Stat. Methods Appt.* **2010**, *19*, 497–515, doi:10.1007/s10260-010-0142-z.

62. Griffith, D.M.; Veech, J.A.; Marsh, C.J. cooccur: Probabilistic Species Co-Occurrence Analysis in R. *J. Stat. Softw.* **2016**, *69*, 1–17, doi:10.18637/jss.v069.c02.

63. Team, R.C. R: A Language and Environment for Statistical Computing; R Foundation: Vienna, Austria, 2019.

64. Veech, J.A. A probabilistic model for analysing species co-occurrence. *Glob. Ecol. Biogeogr.* **2013**, *22*, 252–260, doi:10.1111/j.1466-8238.2012.00789.x.

65. Smith, C.J.; Nedwell, D.B.; Dong, L.F.; Osborn, A.M. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ. Microbiol.* **2006**, *8*, 804–815, doi:10.1111/j.1462-2920.2005.00963.x.

66. Smith, C.J.; Osborn, A.M. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol. Ecol.* **2009**, *67*, 6–20, doi:10.1111/j.1574-6924.2008.00629.x.

67. Zhu, X.; Zhen, Y.; Mi, T.; Yu, Z. Detection of Prorocentrum minimum (Pavillard) Schiller with an Electrochemiluminescence-Molecular Probe Assay. *Mar. Biotechnol. (NY)* **2012**, doi:10.1007/s11012-012-9431-x.

68. Silva, E.S.; Sousa, I. Experimental work on the dinoflagellate toxin production. *Arq. Inst. Nac. Saude* **1981**, *6*, 381–387.

69. Chen, Y.Q.; Gu, X. An ecological study of red tides in the East China Sea. In *Toxic Phytoplankton Blooms in the Sea*; Smarya, T.J., Shimizu, Y., Eds.; Elsevier: Amsterdam, The Netherlands, 1993; pp. 217–221.

70. Grzebyk, D.; Berland, B. Influences of temperature, salinity and irradiance on growth of *Protroccus minimum* (Dinophyceae) from the Mediterranean Sea. *J. Plankton Res.* **1986**, *18*, 1837–1849, doi:10.1093/plankt/18.10.1837.

71. Murray, D.C.; Bunce, M.; Cannell, B.L.; Oliver, R.; Houston, J.; White, N.E.; Barrero, R.A.; Bellgard, M.I.; Haile, J. DNA-Based Faecal Dietary Analysis: A Comparison of qPCR and High Throughput Sequencing Approaches. *PLoS ONE* **2011**, *6*, e25776, doi:10.1371/journal.pone.0025776.

72. Ruivindry, R.; Bolch, C.J.; MacKenzie, L.; Smith, K.F.; Murray, S.A. qPCR Assays for the Detection and Quantification of Multiple Paralytic Shellfish Toxin-Producing Species of Alexandrium. *Front. Microbiol.* **2018**, *9*, 3153, doi:10.3389/fmicb.2018.03153.

73. Svec, D.; Tichopad, A.; Novosadova, V.; Pfaffl, M.W.; Kubista, M. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomol. Detect. Quantif.* **2015**, *3*, 9–16, doi:10.1016/j.bdq.2015.01.005.

74. Kontonis, E.J.; Reed, F.A. Evaluation of Real-Time PCR Amplification Efficiencies to Detect PCR Inhibitors. *J. Forensic Sci.* **2006**, *51*, 795–804, doi:10.1111/j.1556-4029.2006.00182.x.

75. Murray, S.; Flo Jørgensen, M.; Ho, S.Y.W.; Patterson, D.J.; Jermiin, L.S. Improving the Analysis of Dinoflagellate Phylogeny based on rDNA. *Protist* **2005**, *156*, 269–286, doi:10.1016/j.protis.2005.05.003.

76. Litaker, R.W.; Vandersea, M.W.; Kibler, S.R.; Reece, K.S.; Stokes, N.A.; Lutzoni, F.M.; Yonish, B.A.; West, M.A.; Black, M.N.D.; Tester, P.A. Recognizing dinoflagellate species using ITS rRNA sequences. *Biomol. Detect. Quantif.* **2015**, *3*, 315–353, doi:10.3389/fmicb.2018.03153.

77. Andree, K.B.; Fernández-Tejedor, M.; Elandaloussi, L.M.; Quijano-Scheggia, S.; Sampedro, N.; Garcés, E.; Camp, J.; Diogène, J. Quantitative PCR coupled with melt curve analysis for detection of selected Pseudo-nitzschia spp. (Bacillariophyceae) from the northwestern Mediterranean Sea. *Appl. Environ. Microbiol.* **2011**, *77*, 1651–1659, doi:10.1128/AEM.01978-10.

78. Winder, L.; Phillips, C.; Richards, N.; Ochoa-Corona, F.; Hardwick, S.; Vink, C.J.; Goldson, S. Evaluation of DNA melting analysis as a tool for species identification. *Methods Enol. Ecol.* **2011**, *2*, 312–320, doi:10.1111/j.2041-210X.2010.00079.0.10.1111/(ISSN)2041-210X.MICRO17.

79. Bustin, S.; Huggett, J. qPCR primer design revisited. *Biomol. Detect. Quantif.* **2017**, *14*, 19–28.

80. Kress, W.J.; Garcia-Robledo, C.; Uriarte, M.; Erickson, D.L. DNA barcodes for ecology, evolution, and conservation. *Trends Ecol. Ecol.* **2015**, *30*, 25–35, doi:10.1016/j.tree.2014.10.008.

81. Godhe, A.; Cusack, C.; Pedersen, J.; Andersen, P.; Anderson, D.M.; Bresnan, E.; Cembella, A.; Dahl, E.; Diercks, S.; Elbrächter, M.; et al. Intercalibration of classical and molecular techniques for identification of Alexandrium fundyense (Dinophyceae) and estimation of cell densities. *Harmful Algae* **2007**, *6*, 56–72, doi:10.1016/j.hal.2006.06.002.

82. Le Bescot, N.; Mahé, F.; Audic, S.; Dimier, C.; Garet, M.J.; Poulain, J.; Wincker, P.; de Vargas, C.; Siano, R. Global patterns of pelagic dinoflagellate diversity across protist size classes unveiled by metabarcoding. *Environ. Microbiol.* **2016**, *18*, 609–626, doi:10.1111/1462-2920.13039.

83. Smith, K.F.; Kohl, G.S.; Murray, S.A.; Rhodes, L.L. Assessment of the metabarcoding approach for community analysis of benthic-epiphytic dinoflagellates using mock communities. *New Zealand J. Mar. Freshw. Res.* **2017**, *51*, 555–576, doi:10.1080/00283303.2017.1298632.

84. Hong, S.; Bunge, J.; Leslin, C.; Jeon, S.; Epstein, S.S. Polymerase chain reaction primers miss half of RNA viral diversity. *ISME J.* **2009**, *3*, 1365–1373, doi:10.1038/isemj.2009.89.

85. Ajani, P.A.; Hallegren, G.M.; Allen, D.; Coughlan, A.; Richardson, A.J.; Armand, L.K.; Ingleton, T.; Murray, S.A. Establishing Baselines: Eighty Years of Phytoplankton Diversity and Biomass in South-Eastern Australia. In *Oceanography and Marine Biology*; CRC Press: Boca Raton, FL, USA, 2016; pp. 395–420.
86. Ajani, P.A.; Allen, A.P.; Ingleton, T.; Armand, L.K. A decadal decline in relative abundance and a shift in microphytoplankton composition at a long-term coastal station off southeast Australia. *Limnol. Oceanogr.* 2014, 59, 519–531, doi:10.4319/lo.2014.59.2.0519.

87. Macherioutou, L.; Guillini, K.; Bezerra, T.N.; Tytgat, B.; Nguyen, D.T.; Phuong Nguyen, T.X.; Noppe, F.; Armenteros, M.; Boufahja, F.; Rigaux, A.; et al. Metabarcoding free-living marine nematodes using curated 18S and CO1 reference sequence databases for species-level taxonomic assignments. *Ecol. Evol.* 2019, 9, 1211–1226, doi:10.1002/ece3.4814.

88. Boers, S.A.; Jansen, R.; Hays, J.P. Understanding and overcoming the pitfalls and biases of next-generation sequencing (NGS) methods for use in the routine clinical microbiological diagnostic laboratory. *Eur. J. Clin. Microbiol. Infect. Dis.* 2019, 38, 1059–1070, doi:10.1007/s10096-019-03520-3.

89. Pochon, X.; Bott, N.J.; Smith, K.F.; Wood, S.A. Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests. *PLoS ONE* 2013, 8, e73935, doi:10.1371/journal.pone.0073935.

90. Kohli, G.S.; Neilan, B.A.; Brown, M.V.; Hoppenrath, M.; Murray, S.A. Cob gene pyrosequencing enables characterization of benthic dinoflagellate diversity and biogeography. *Environ. Microbiol.* 2014, 16, 467–485, doi:10.1111/1462-2920.12275.

91. Galluzzi, L.; Penna, A.; Bertozzini, E.; Vila, M.; Garces, E.; Magnani, M. Development of a Real-Time PCR Assay for Rapid Detection and Quantification of *Alexandrium minutum* (a Dinoflagellate). *Appl. Environ. Microbiol.* 2004, 70, 1199–1206.

92. Shaw, J.L.-A. *Metagenomic Amplicon Sequencing as a Rapid and High-Throughput Tool for Aquatic Biodiversity Surveys*; University of Adelaide: Adelaide, SA, Australia, 2015.

93. Penna, A.; Galluzzi, L. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. *Environ. Sci. Pollut. Res. Int.* 2013, 20, 6851–6862, doi:10.1007/s11356-012-1377-z.

94. Paxinos, R. A rapid Utermöhl method for estimating algal numbers. *J. Plankton Res.* 2000, 22, 2255–2262, doi:10.1093/plankt/22.12.2255.

95. Murray, S.A.; Ruvindy, R.; Kohli, G.S.; Anderson, D.M.; Brosnahan, M.L. Evaluation of sxtA and rDNA qPCR assays through monitoring of an inshore bloom of *Alexandrium catenella* Group I. *Sci. Rep.* 2019, 9, 14532, doi:10.1038/s41598-019-51074-3.

96. Engesmo, A.; Strand, D.; Gran-Stadniczko, S.; Edvardsen, B.; Medlin, L.K.; Eikrem, W. Development of a qPCR assay to detect and quantify ichthyotoxic flagellates along the Norwegian coast, and the first Norwegian record of *Fibrocapsa japonica* (Raphidophyceae). *Harmful Algae* 2018, 75, 105–117, doi:10.1016/j.hal.2018.04.007.

97. Loveless, A.M. A multi-dimensional receiving water quality model for Botany Bay (Sydney, Australia). In Proceedings of the 18th World IMACS / MODSIM Congress; Cairns, Australia, 13–17 July 2009; pp. 4170–4176.

98. DECCW. *Towra Point Nature Reserve Ramsar Site*; DECCW: Sydney, Australia, 2010.

99. Ajani, P.; Ingleton, T.; Pritchard, T.; Armand, L. Microalgal Blooms in the Coastal Waters of New South Wales, Australia. *Proc. Linn. Soc. New South. Wales* 2011, 133, 15–32.

100. Fu, F.X.; Zhang, Y.; Warner, M.E.; Feng, Y.; Sun, J.; Hutchins, D.A. A comparison of future increased CO2 and temperature effects on sympatric *Heterosigma akashiwo* and *Prorocentrum minimum*. *Harmful Algae* 2008, 7, 76–90, doi:10.1016/j.hal.2007.05.006.

101. Skarlato, S.; Filatova, N.; Knyazev, N.; Berdieva, I.; Telesh, I. Salinity stress response of the invasive dinoflagellate *Prorocentrum minimum*. *Estuar. Coast. Shelf Sci.* 2018, 211, 199–207, doi:10.1016/j.ecss.2017.07.007.

102. Trombeta, T.; Vidussi, F.; Mas, S.; Parin, D.; Simier, M.; Mostajir, B. Water temperature drives phytoplankton blooms in coastal waters. *PLoS ONE* 2019, 14, doi:10.1371/journal.pone.0214933.

103. Collos, Y. Time-lag algal growth dynamics: Biological constraints on primary production in aquatic environments. *Mar. Ecol. Prog. Ser.* 1986, 33, 193–206.

104. Reguera, B.; Velo-Suárez, L.; Raine, R.; Park, M.G. Harmful Dinophysis species: A review. *Harmful Algae* 2012, 14, 87–106, doi:10.1016/j.hal.2011.10.016.

105. Anderson, D.M.; Alpermann, T.J.; Cembella, A.D.; Collos, Y.; Masseret, E.; Montresor, M. The globally distributed genus *Alexandrium*: Multifaceted roles in marine ecosystems and impacts on human health. *Harmful Algae* 2012, 14, 10–35, doi:10.1016/j.hal.2011.10.012.

106. Gao, Y.; Yu, R.C.; Chen, J.H.; Zhang, Q.C.; Kong, F.Z.; Zhou, M.J. Distribution of *Alexandrium fundyense* and *A. pacificum* (Dinophyceae) in the Yellow Sea and Bohai Sea. *Mar. Pollut. Bull.* 2015, 96, 210–219, doi:10.1016/j.marpolbul.2015.05.025.

107. Shin, H.H.; Li, Z.; Kim, E.S.; Park, J.-W.; Lim, W.A. Which species, *Alexandrium catenella* (Group I) or *A. pacificum* (Group IV), is really responsible for past paralytic shellfish poisoning outbreaks in Jinhae-Masan Bay, Korea? *Harmful Algae* 2017, 68, 31–39, doi:10.1016/j.hal.2017.07.006.