The Application of qPCR Assays for the Early Detection of Toxic *Alexandrium* in Eastern Australian Waters

Thesis by

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Certificate of Original Authorship

I, Rendy Ruvindy hereby declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, In the Climate Change Cluster, Faculty of Science at the University of Technology Sydney.

This thesis is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UTS or any other educational institution. Any contribution made to the research by others, with whom I have worked at UTS or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged. This research is supported by the Australian Government Research Training Program.

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List of Publications

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Abstract

Harmful algal blooms that produce Paralytic Shellfish Toxins (PSTs) are prevalent and affect shellfish harvesting areas worldwide. PSTs have caused shellfish harvesting closures and product recalls, resulting in economic losses, as well as brand damage and damage to the wider economy including the tourism industry.

In Tasmania, it is known that four PST producing species co-occur, comprising *Alexandrium catenella*, *A. pacificum*, *A. australiense* and *Gymnodinium catenatum*. In particular, of these, three species are morphologically almost identical, the species of the former *Alexandrium tamarense* species complex (*A. catenella*, *A. pacificum*, and *A. australiense*), which cannot be differentiated using light microscopy. Therefore, phytoplankton monitoring using light microscopy and total PST in shellfish using High Performance Liquid Chromatography (HPLC) may not be sufficient to allow for an early warning with enough time to take appropriate shellfish harvesting management decisions.

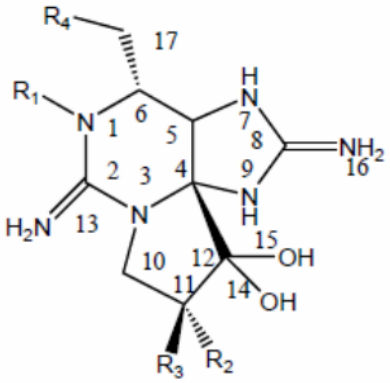
In this thesis, quantitative Polymerase Chain Reaction (qPCR) assays are investigated as an in-field early warning system, as well as a tool for long-term risk assessment of PST-associated harmful algal blooms. A commercial on-farm pipeline based on the collection and filtration of water samples using a custom designed gravity filter, a cell lysis, and a qPCR assay based on *sxtA4* was also developed and validated. QPCR assays based on ribosomal DNA (rDNA) 'barcoding' regions and an assay based on a gene associated with PST biosynthesis (*sxtA4*) were found to be generally specific, sensitive and efficient. The efficacy of an rDNA-based assay for cyst quantification was demonstrated, showing potential for its use as a long-term risk assessment tool for a new harvest area. However, qPCR assays based on rDNA gene regions were found to overestimate cell abundances. An analysis of rDNA copy number variation among strains of species of *Alexandrium* showed a variation of up to 3-5 orders of magnitude within a species, and was correlated significantly with genome size, which also varied within a species. An analysis of the variation in genomic copies of *sxtA4* genes showed variation as well, however this was of a lesser degree, of up to one order of magnitude. A positive correlation was found between *sxtA4* copies per cell and the total PST produced per cell, showing that the dosage effect may contribute to the regulation of PST biosynthesis.

Chapter 1: Paralytic Shellfish Toxin (PST) production by marine dinoflagellates, and the development and implementation of molecular genetic detection

1.1. Paralytic Shellfish Toxins

Paralytic Shellfish Toxins (PSTs), which are also known as saxitoxin (STX) and its analogues, are a group of neurotoxic alkaloid compounds responsible for a syndrome called Paralytic Shellfish Poisoning (PSP). The molecular formula of STX, the 'parent compound' of the PSTs, is $C_{10}H_{17}N_7O_4$ (Molecular Weight = 299), and it consists of a 3,4-propinoperhydropurine tricyclic system (1). PSTs are structurally diverse (Figure 1. 1), and 58 analogues have been documented (1, 2). Certain species of cyanobacteria are responsible for PST production in freshwater environments (3) whereas certain species of dinoflagellates, which are eukaryotic and in no way related to cyanobacteria, are responsible for their production in marine environments. (4-6). Each strain of PST-producing dinoflagellates produces a combination of different analogues of PSTs, and therefore can be said to have a unique toxin profile ((1) and references therein).

A combination of different side groups determines the type of the STX analogue (Figure 1. 1), and influences its reactivity and toxicity. The group with carbamoyl group ($OCONH_2$) at the R4 (carbamate group) possess the highest toxicity compared to other groups. Within the carbamate group, STX is the most toxic, followed by neosaxitoxin (NEO) and gonyautoxins 1 and 3 (GTX1, GTX3) (7). A group of toxins with the carbamoyl structure is replaced with H are called decarbamoyl toxins. Another group is called N-sulphocarbamoyl saxitoxin and has the lowest toxicity compare to the other groups, characterised by the presence of SO_3^- (8).



Division	Name [a]	R1	R2	R3	R4
Carbamate	STX	H	H	H	$OCONH_2$
	NeoSTX	OH	H	H	$OCONH_2$
	GTX1	OH	OSO_3^-	H	$OCONH_2$
	GTX2	H	OSO_3^-	H	$OCONH_2$
	GTX3	H	H	OSO_3^-	$OCONH_2$
	GTX4	OH	H	OSO_3^-	$OCONH_2$
N-sulfocarbamoyl	GTX5 (B1)	H	H	H	$OCONHSO_3^-$
	GTX6 (B2)	OH	H	H	$OCONHSO_3^-$
	C1	H	OSO_3^-	H	$OCONHSO_3^-$
	C2	H	H	OSO_3^-	$OCONHSO_3^-$
	C3	OH	OSO_3^-	H	$OCONHSO_3^-$
	C4	OH	H	OSO_3^-	$OCONHSO_3^-$
Decarbamoyl	dcSTX	H	H	H	OH
	dcNeoSTX	OH	H	H	OH
	dcGTX1	OH	OSO_3^-	H	OH
	dcGTX2	H	OSO_3^-	H	OH
	dcGTX3	H	H	OSO_3^-	OH
	dcGTX4	OH	H	OSO_3^-	OH
Deoxydecarbamoyl	doSTX	H	H	H	H
	doGTX2	H	H	OSO_3^-	H
	doGTX3	H	OSO_3^-	H	H

Figure 1. 1. The chemical structure of saxitoxin and derivatives produced by marine dinoflagellates (8). STX is the abbreviation of saxitoxin and GTX is for gonyautoxin.

STX and its derivatives are known to target the sodium channel, a voltage-gated ion channel protein consisting of one principal alpha subunit (220-260 kDa) and one to three smaller (33-36 kDa) beta subunits (9), inhibiting channel conductance and causing neuronal activity blockage (10). Unlike most neurotoxins, STX also blocks potassium and calcium channels through different mechanisms (11, 12), which may contribute to its high potency. The symptoms associated with PSP include paresthesia and numbness, muscular weakness, a sensation of lightness and floating, ataxia, motor incoordination, drowsiness, incoherence and progressively decreasing ventilatory efficiency ((16) and references therein). Severe

intoxication could lead to respiratory failure and death ((16) and references therein). The fatal dose for humans is approximately 1 mg STX eq/kg body weight (1).

PSP syndrome occurs when vector organisms, which have accumulated high concentrations of PSTs, are consumed by humans. In marine habitats, these toxins can accumulate in vector organisms when they feed on PST-producing dinoflagellates, and the toxins are eventually delivered up the marine food web to higher trophic levels. Even though mussels and clams are known to be the traditional vectors for PSTs (13, 14), there are also report of gastropods, crustaceans and fish serving as vectors (15).

Shellfish, whether they are wild or grown in aquaculture farms, tend to accumulate a high level of PST during harmful algal blooms of PST-producing dinoflagellates. The impacts of PST-associated blooms in marine habitats are significant due to the cost involved in an aquaculture farm closure, mitigation costs and losses from reductions in tourism activity ((17) and references therein). For example, the Sydney rock oyster (*Saccostrea glomerata*) industry in NSW estuaries was valued at ~$65 million in 2017, showing the potential financial loss by the shellfish industry in this region. In Tasmania, the seafood industry is considered the largest in Australia (18), and the impact of a PSP event in 2012 has been economically significant(19). At the end of October 2012, Japanese import authorities (Ministry of Health, Labour and Welfare) recalled a shipment of blue mussels (*Mytilus galloprovincialis*) derived from the east coast of Tasmania due to the presence of unacceptable levels of PSTs. The direct revenue loss was estimated to be around AU$6 million (19), and including the indirect costs, the loss was estimated at ~$23 million.

1.2. Biogeography of PSTs producing dinoflagellates

Among the marine dinoflagellates, certain species of the genus *Alexandrium* ((20) and references therein), *Pyrodinium bahamense* and *Gymnodinium catenatum* (5) (Figure 1. 2) are known to synthesise PSTs. *Gymnodinium catenatum* is an unarmoured, chain-forming dinoflagellate and the only one from the genus *Gymnodinium* that produces PSTs (5). It also produces additional STX analogues that are unique for this species, identified as GC1, GC2, GC3 (21). *Gymnodinium catenatum* was first described from the Gulf of California in 1943 (25). The first documented PSP outbreak linked to *Gymnodinium catenatum* was in 1979 in Mexico (26).It is believed to have been introduced to Australia after 1973 (27), and is now

distributed around the eastern part of Tasmania (28). Several PSP cases in Tasmanian water associated with this species have been recorded in the past (5, 29).

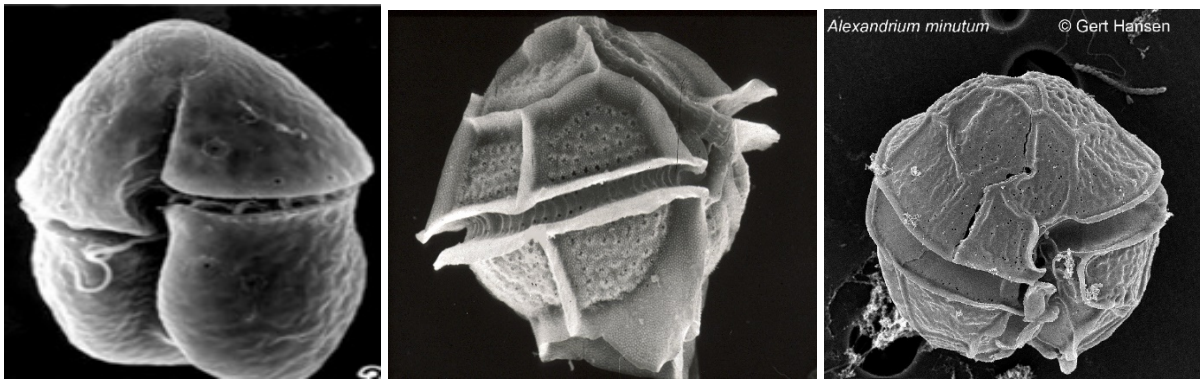


Figure 1. 2. Electron scanning microscopy pictures of (left to right) *Gymnodinium catenatum* (22), *Pyrodinium bahamense* (23) and *Alexandrium minutum* (24).

Pyrodinium bahamense is a PST-producing species that was initially identified in Papua New Guinea (30). It has a very significant economic and health impact in tropical and subtropical waters, and is responsible for the most fatalities related to PSP in these regions ((31) and references therein Blooms of this species were observed throughout the South-East Asia region since then, and the cyst appears to be prevalent in South-East Asia and Pacific embayments (32). This prevalence is likely due to its preference for a high salinity and high temperature environment (33). However, a cyst fossil study indicates that this species was also present in the waters north of Sydney around 120,000 years ago (34).

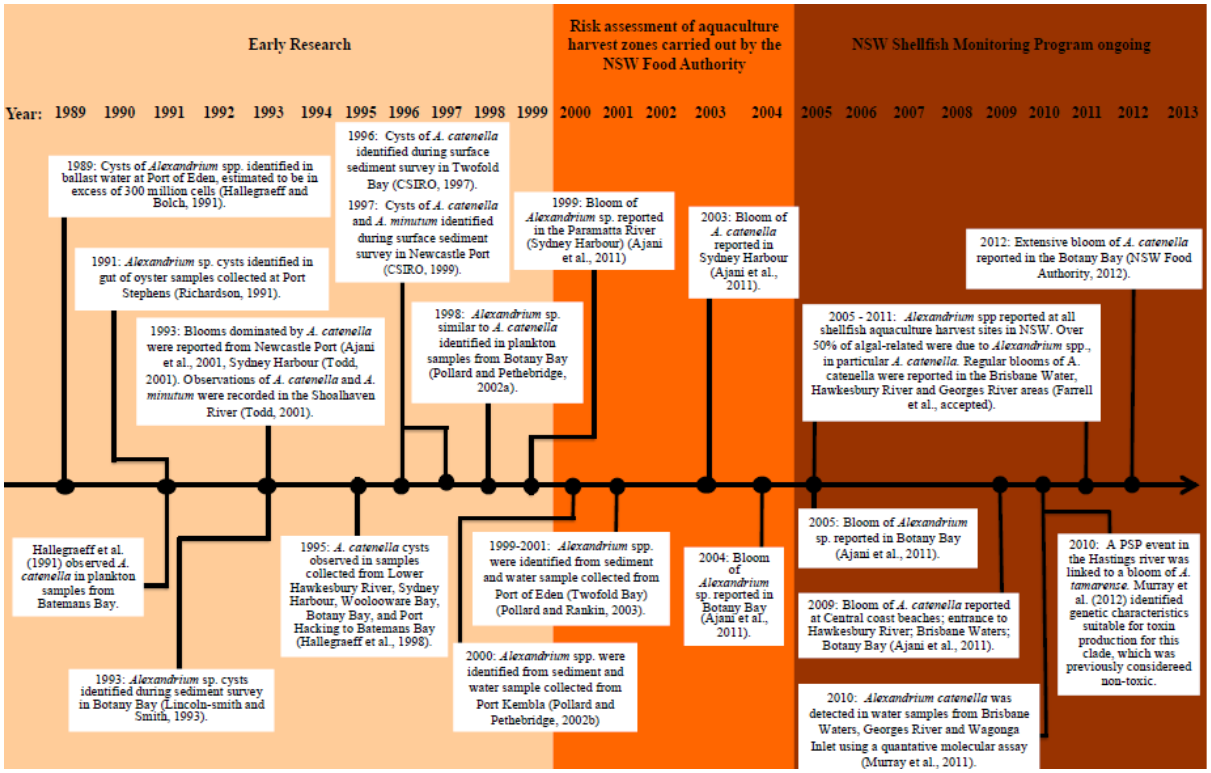


Figure 1. 3. Timeline of research in distribution and dynamics of *Alexandrium spp*. In New South Wales, Australia (35).

Of particular interest, species of the genus *Alexandrium* are known to be very cosmopolitan in their geographic distribution, as they are present in sub-arctic, temperate and tropical zones (36). This genus was described in 1960, with the description of *Alexandrium minutum* Halim in the harbour of Alexandria in Egypt (37). About a third of 31 identified *Alexandrium* species can produce PSTs (20). As a consequence, species of this genus are responsible for most of the PSP cases around the world. Several species can produce other type of toxins such as spirolides in *A. ostenfeldii* (38) and potentially goniodomins in *A. monilatum* and *A. hiranoi* (39).

The *Alexandrium* species previously classified as *Alexandrium tamarense* (M.Lebour) Balech species complex appear to have caused the most significant ecological and economic impacts of marine PST producing species (20). This group consist of *A. catenella* (Group I, formerly *A. fundyense*), *A. mediterraneum* (Group II), *A. tamarense* (Group III), *A. pacificum* (Group IV, formerly *A. catenella*), and *A. australiense* (Group V) (40). Of these species, *A. catenella* and *A. pacificum* have been confirmed to produce paralytic shellfish toxin whereas *A. tamarense* and *A. mediterraneum* is known to be non-toxic (40-44). On the other hand, two strains of *A. australiense* were found to be non-toxin producing (ATCJ33 and ATEB01) and

one was identified as toxic (ATNWB01) (45, 46). Of all the toxic species in the *Alexandrium tamarense* complex that are prevalent in Tasmanian waters, *A. catenella* showed the highest toxicity, followed by *A. pacificum* and a strain of *A. australiense* (47).

Of the species in *Alexandrium tamarense* complex, *A. pacificum* appears to be one of the most dominant in NSW water (36) and the history of the characterisation in NSW dated back since 1989 (Figure 1. 3). In NSW waters between July 2005-December 2011, *Alexandrium* species were detected at all monitored sites along the NSW coastline (36). Species detected included toxic species *A. pacificum*, *A. minutum*, *A. ostenfeldii*, and *A. tamarense*, and non-toxic species *A. fraterculus*, *A. margalefi*, and *A. pseudogonyaulax* (36). The abundance of *A. catenella* was the highest based on the total study period, whereas *A. fraterculus* was the least abundant compare to the others (36). *Alexandrium pacificum* was the main cause of uptake of PST into shellfish in NSW during the study period (35). It frequently led to harvesting area closures in Brisbane Water, Hawkesbury River, and Georges River (36). *Alexandrium tamarense* were observed in relatively low density between Richmond and Wonboyn, and was linked to bloom events at Bellinger, Hastings Rivers, and Wallis Lake (36). *Alexandrium minutum* was distributed along the NSW coast, and was responsible for PSP events in Hawkesbury River in 2007 and Port Stephens in 2009, causing in harvest closures (36). *Alexandrium ostenfeldii* was also distributed along the NSW coast, and responsible for a confirmed PSP case in Twofold Bay in 2007 (36).

A. catenella has become prevalent in eastern Tasmania waters after 2012 (47). In 2012, a large-scale bloom of a species of *Alexandrium* was detected on the east coast of Tasmania, and was dominated by *A. catenella* (48). This species also proliferated in subsequent years, particularly in 2013/2014 (48). Along the eastern coast of Tasmania, *A. australiense* and *A. pacificum* were present, whereas *A. ostenfeldii*, a toxic species, was sporadically detected in eastern and south-easterrn coasts of Tasmania (Bolch et al, unpublished). In northern coast, only *A. australiense* was available so far (Bolch et al, unpublished).

1.3. Regulation and genetics of PST Biosynthesis in *Alexandrium* species

1.3.1. External factors

The toxin content and profile of *Alexandrium* species appears to be affected by biotic and abiotic factors. For example, salinity affected the toxin concentration produced by each *A. ostenfeldii* and *A. peruvianum* strains (49). Other factors such as temperature (50, 51), nutrient limitations (50, 52, 53), nutrient supplementation (54), grazer presence (55), and intracellular arginine concentration (50, 53) are known to affect the toxin productivity.

1.3.2. Dinoflagellate genetics

Dinoflagellates share a number of unique nuclear characteristics, which includes having among the largest known genomes in eukaryotes (56, 57). As summarised on Table 1. 1, the genome size of *Alexandrium* species varies from 21.8 pg per haploid cell in *A. andersonii* to more than 100 pg in *Alexandrium tamarense* species complex (58). This poses a significant challenge for studies of the genomics of dinoflagellates, since generation of whole sequences is very difficult and expensive. Thus, many studies in dinoflagellate genomics have used transcriptomic sequencing to obtain information about dinoflagellate gene content and regulation.

Table 1. 1. Previously Published Genome Size of *Alexandrium* spp (59)

Species	Strain	Genome size (Gbp cell^{-1})	Reference
Alexandrium affine	PA8V	96.04 ± 4.01[a]	Figueroa et al. (2014)[f]
A. andersonii	SZN-12	21.52 ± 0.59[a]	Figueroa et al. (2014)[f]
A. andersonii	CCMP2222	21.32[a]	LaJeunesse et al. (2005)[f]
A. insuetum	CCMP2082	30.12[a]	LaJeunesse et al. (2005)[f]
A. lusitanicum	CCMP1888	30.32[a]	LaJeunesse et al. (2005)[f]
A. margalefii	VGO661	165.3 ± 3.14[a]	Figueroa et al. (2014)[f]
A. minutum	n.r.[b]	24.74 ± 3.13[a]	Figueroa et al. (2014)[f]
A. minutum	CNR-AMIA4	11.15 ± 2.54[c]	Galluzzi et al. (2004)[f]
A. minutum	VGO577	25.13 ± 3.13[a]	Stuken et al. (2015)[f]
A. minutum	VGO874	28.36 ± 3.33[a]	Stuken et al. (2015)[f]
A. minutum	VGO650	25.92 ± 1.66[a]	Stuken et al. (2015)[f]
A. minutum	VGO651	26.31 ± 3.42[a]	Stuken et al. (2015)[f]
A. ostenfeldii	n.r.[b]	112.5[a]	Kremp et al. (2009)[f]
A. tamarense	CCMP1598	101.2[a]	LaJeunesse et al. (2005)[f]
A. tamarense	n.r.[b]	200[d]	Hackett et al. (2005)
A. tamarense	n.r.[b]	63.28 ± 7.53[a]	Figueroa et al. (2014)[f]

Expressed sequence tag (EST) and transcriptomics analyses have generated a lot of information regarding the gene regulation in dinoflagellates. In general, in transcriptomic sequencing of dinoflagellates, it has been found that ~50% of genes do not have a homologue

in public databases (60, 61). The genome also appears to be dynamic, with extensive gene transfer between the plastids and the nucleus via horizontal gene transfer (56). The plastid gene transcripts are polyuridinylated at the 3' end, and nuclear mRNA are SL *trans* spliced at the 5' end (62, 63).

Other studies had found that only about 20% of dinoflagellate genes appear to be regulated via significant (hours/days) changes in their transcription rate (64-69). This suggests that other mechanisms, such as post-transcriptional regulation, may be more important in gene expression regulation in dinoflagellates. It also appear that toxin quantities in *A. minutum* are generally not well-correlated with biosynthetic gene transcript abundance (70).

The presence of genes in multi-copy arrays appears to be a common feature in dinoflagellate genomes, as gene copy number quantifications of ribosomal DNA (71), luciferase (72), actin (56), and mitotic cyclin (73) genes indicate the presence of large copy numbers of those genes. The recycling of cDNA, which is the mechanism of re-integrating processed mRNA into the dinoflagellate genome (74), and gene or segmental/whole genome duplications (75) are plausible contributing factors to the extensive duplication of dinoflagellate genes.

Genomic copy number variation (CNV) is present in eukaryotes and responsible for phenotypic variations (76-80). The rRNA genes are present in multiple copies in *Alexandrium* species, with the number varying from 189,570 ± 99,558 to 2,489,800 ± 550,967 in *A. pacificum* (65). This variability in gene copy number of rRNA genes in *Alexandrium* spp. (65) may be related to the fitness and evolutionary ecology due to their link to growth and development (81). More importantly, CNV is known to allow for increases in gene expression (82) regulated through positive selection (76). CNV may be involved in dinoflagellate gene regulation through a "gene dosage effect" mechanism, in which the number of copies of a gene in an organism's genome directly related to the amount of expressed gene product.

A study has been done in *A. taylorii* and *A. pacificum*, in which variations up to two orders of magnitude in ribosomal gene copy number compromised the level of accuracy of qPCR counts of target cells (65). Another study in Mediteranean samples suggest that the number from microscopy and qPCR counts had significant differences ($p<0.01$) (71). Therefore, it is important not to assume that the gene copy number is stable accross the strain and species,

and it may be crucial to obtain comprehensive gene copy number variation prior to the quantification.

1.3.3. PST biosynthesis and gene regulation

STX biosynthesis genes were initially identified in cyanobacteria species *Cylindrospermopsis raciborskii* T3(3), then *Anabaena circinalis*, *Alphanizomenon* sp (83) and *Lyngbya wollei*(84). The STX biosynthesis genes characterisation in cyanobacteria species has resulted in the identification of 14 common genes (*sxtA*-*sxtI*, *sxtP*-*sxtS* and *sxtU*) in five cyanobacterial genera, termed "core genes"(85). Of these genes, 8 of them (*sxtA*, *sxtB*, *sxtD*, *sxtG*, *sxtH/T*, *sxtI*, *sxtS* and *sxtU*) are considered to be directly related to the synthesis process (3). From previous studies on *C. raciborskii* T3, the *sxtA*-regulated enzyme is known to initiate the reaction that produces STX (3). It has four catalytic domains namely SAM (S-adenosylmethionine)-dependent methyltransferase (A1), GCN5-related N-acetyltransferase (A2), acyl carrier protein (A3), and class II aminotransferase (A4) (3). The *sxtA1-3* sequences show similarity with deltaproteobacterium sequences and *sxtA4* shows high similarity to actinobacteria, suggesting that this gene was obtained through lateral gene transfer (68).

Several attempts have been made to delineate the genetic basis of STX biosynthesis in dinoflagellates (64, 86-89). Despite these efforts, only after the emergence of Next Generation Sequencing (NGS) platforms were that the homologs of saxitoxin biosynthesis genes in dinoflagellate discovered (45). Complementary DNA (cDNA) libraries were constructed from the transcripts of several strains of *Alexandrium* sp and *Gymnodinium catenatum* (45). Sequence comparison analysis between *C.raciborskii* T3 and assembled *A. fundyense* and *A. minutum* libraries showed the presence of core genes *sxtA*, *sxtG*, *sxtB*, *sxtF/M*, *sxtH/T*, *sxtI*, *sxtR* and *sxtU* in both libraries. Homologues of *sxtC*, *sxtD*, and *sxtE* were not found in either library, while *sxtS* was only found in the library of *A. minutum* (45). Recently, *sxtO* and *sxtZ* were found in *A. catenella* (90).

The transcripts of genes encoding STX biosynthesis contains poly-A tails at the 3'end and a 22 bp spliced leader sequences at the 5'end (45). These indicate that the genes are encoded in nuclear genomes and further dismiss the hypothesis that the toxin synthesis is a result of bacteria (45). The GC content of the *Alexandrium sxtA* transcripts was higher than its cyanobacterial homologue (45). Its mRNA transcripts are monocistronic, while the cyanobacteria version is policistronic (10). Additionally, the transcripts are present in multiple copies in the nuclear genome (77, 85).

Two types of transcripts were recognised for *sxtA*. Long transcripts contain *sxtA1-A4* domains and short transcripts contain *sxtA1-A3* domains (45, 91). The genes *sxtA1* and *sxtA4* exist in the analysed STX-producing dinoflagellates, and are absent in non-toxic strains with the exception of several supposedly non-toxic strain (42, 92). Also, it appears that the domain A4 is important for STX synthesis in dinoflagellates (45) and cyanobacteria counterparts (3).

The second characterised gene in dinoflagellates is *sxtG*, which is putatively responsible for the second step in saxitoxin biosynthesis. Amidinotransferase *sxtG* utilise the product of the protein encoded by *sxtA* gene, incorporating amidino groups from second arginine molecules into saxitoxin intermediate in cyanobacteria biosynthesis pathway (3). Same as *sxtA*, the typical features for dinoflagellate transcripts such as spliced leader sequence and eukaryotic poly(A) tails also applies to *sxtG* (2). This gene also shows bacterial origin but higher GC content than the cyanobacteria homolog. However, unlike *sxtA*, it also present in non-toxic *Alexandrium* (93).

One study has found that there is a link between the copy numbers of *sxtA* and the quantity of synthesised toxin, which may be caused by a dosage effect (77). Less toxic strains appear to possess a lower number of *sxtA* gene copies in 15 identified strains of *A. minutum* (77). Interestingly, a big difference between *sxtA4* copy number in *A. pacificum* (previously *A. catenella*) *(*100-200 copies) (42, 45) and *A. minutum* (<15 copies) (77) did not translate to more toxin produced in *A. pacificum* (77). It has been postulated that this may be due to the larger genome size in *A. pacificum* associated with ploidy differences, which implies that *sxtA4* copy number may not be a positively selected trait but a result of genome dynamics (77).

1.4. Molecular methods as a potential mitigation and regulation tool

Current early warning systems for the potential for harmful algal blooms producing PST, used by shellfish monitoring authorities, rely on the morphological identification and quantification using light microscopy of seawater samples taken from estuary growing areas. However, morphotype-based identification for species belong to *Alexandrium tamarense* species complex is not possible, as these species are cryptic, meaning that different species can exhibit the same morphological features (40). The morphological characteristics used for

species identification such as cell shape, apical pore complex (APC) geometry, ventral pore presence or absence, and chain forming tendency could also be affected by environmental and culture condition (94). The use of molecular genetic tools and a platform such as qPCR (quantitative polymerase chain reaction) could provide an alternative solution to these issues.

Figure 1. 4. Portable qPCR platforms from various manufacturers, suitable for lab or on-field application (From upper left to right: Mini8 (Coyote Bioscience USA Inc), Mic (Bio Molecular Systems), Liberty16 (Ubiquitome Limited), MyGoMini (IT-IS Life Science)

There are several advantages of utilising qPCR for phytoplankton identification. One is the capacity to process many multiple samples in a high throughput manner (71). A further advantage is that the amount of training involved in operating qPCR equipment is comparatively negligible, especially compared to the years of training and experience required to reliably identify marine dinoflagellates using light microscopy.

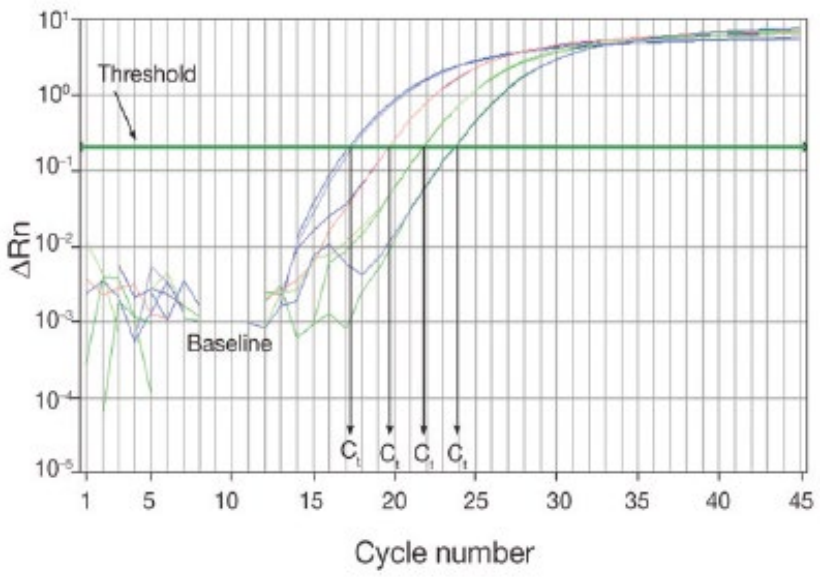


Figure 1. 5. The peaks from qPCR amplification showing the baseline and quantification cycle (C_q) (Applied Biosystems).

Other advantages of using a qPCR platform include the comparatively low cost of reagent per sample (42). It is also a very sensitive technique, allowing cell quantity determination at the lower detection limit of optical microscopy (42, 95). qPCR has been widely used in clinical and environmental diagnostics ((96) and references therein), including aquaculture (97-99). With the emergence of portable qPCR machines (Figure 1. 4) that can run on battery and send the results through Wi-Fi connections, qPCR analyses can be carried out in the field.

In qPCR, the synthesised sequences in the presence of DNA intercalating dyes or sequence-specific fluorescently labelled probes will trigger the emission of fluorescence signals, which are then detected by the optical sensor in the platform. The cycle number at the point where all are in exponential amplification (quantification cycle, Cq) (Figure 1. 5) is directly correlated to the starting amount target molecules (100). A standard curve can be developed based on the known amount of target sequences by using a cloned target sequence in a plasmid, or from extracted DNA from a known number of cultured cells. The standard curve can then be used as a reference to determine the amount of target sequences in an unknown sample (100). Fluorescence can be generated with fluorescent dyes (e.g. SYBR Green, EVA Green) or fluorescence probe (e.g. TaqMan®, molecular beacons) (96).

Apart from its application as an early warning system, temporal qPCR-based monitoring can be used for the development of high-resolution long-term datasets that are useful to identify

the ecological factors contributing to the initiation of blooms of *Alexandrium* species. qPCR can also be employed to obtain information on the distribution of resting cysts of dinoflagellates. High-resolution datasets from multiple sites can be generated with qPCR combined with the physical data measurement, in order to model factors relating to the growth and proliferation of *Alexandrium* species.

Early research on the development of PCR assays for marine dinoflagellates have targeted ribosomal DNA gene regions, for their high copy number and phylogenetic information (96). The first introduced primers were a pair of genus specific primers for *Alexandrium* and for *A. minutum* (101). Following that, several other assays were developed specific for the species *A. catenella* Group I, *A. pacificum* Group IV, and *A. minutum* (102-105). Identification probes have also been developed for PST-producing *Gymnodinium catenatum* (106).

There are several disadvantages to the use of molecular genetic detection of *Alexandrium* spp. based on ribosomal RNA gene regions. Ribosomal RNA gene sequences are not directly related to PST synthesis, and consequently do not provide any information whether the detected species is toxic or non-toxic (42, 95). Another disadvantage is that new toxic species that are introduced to the monitored area may not be detected if the screening is only carried out for known local species.

The use of assays designed to target genes related to toxin biosynthesis can directly measure the potential for toxin production in field samples in which mixed community of toxic and non-toxic strains co-exist. A qPCR assay targeting the *sxtA4* sequence has been developed and tested in samples from NSW estuaries (42), New Zealand (91), Finland (107) Yellow Sea, China (108), Spain and Norway (109). Assays targeting *sxtA1* and *sxtG* have also been developed and used for field-based quantification of samples from Italy (110). In the Yellow Sea, China, the assay showed high specificity to detect PST-associated microalgae, and high representation of the abundance and distribution of the toxic *A. fundyense* and *A. pacificum* during the sampling season (111). Nonetheless, weak correlation was found between toxin quantification and *sxtA4*-based qPCR assay implied that the assay is not accurate enough to reflect the toxicity of the samples (108). As a possible solution, the combination of toxin analysis and qPCR-based method could be used for algae bloom monitoring.

The focus of this research is the improvement of monitoring methodology to mitigate the impact of PST-associated bloom produced by dinoflagellates in marine habitats, with

potentially severe impact on marine aquaculture industry. In particular, *Alexandrium* species have been responsible for the majority of the incidences of PSTs in shellfish in Australia, New Zealand, and other shellfish harvest areas around the world. This thesis is focused on the detection of *Alexandrium* species producing PSTs using qPCR. For these reasons assays were designed to target toxin synthesis genes of *sxtA4* domain (42). This *sxtA4*-based commercial assay termed DinoDtec, incorporates a molecular TaqMan minor groove binding assay along with several species-specific based assays for species prominent in Australian and New Zealand waters. Chapter 2 focuses on development and validation studies utilising the species-specific assays for *A. catenella*, *A. pacificum*, *A. australiense*, and *A. ostenfeldii*. In Chapter 3, the copy numbers of *sxtA4* and ribosomal genes in species of *A. catenella*, *A. pacificum*, *A. australiense*, and *A. minutum* were quantified and inter-strain and inter-species variation will be discussed. The development and validation of standardised on-farm pipeline will be described in Chapter 4. Lastly, the application of DinoDtec pipeline as an early warning system for a mussel farm in Tasmania, as well as spatial and cross-depth bloom profiling is discussed in Chapter 5.

Chapter 2: qPCR assays for the detection and quantification of multiple Paralytic Shellfish Toxin-producing species of *Alexandrium*

Authorship Declaration

By signing below I confirm that for the paper titled "qPCR Assays for the Detection and Quantification of Multiple Paralytic Shellfish Toxin-Producing Species of *Alexandrium*" and published by the Frontiers in Microbiology journal, that:

Rendy Ruvindy: Conceived the idea, performed the field sampling and qPCR experiments, analysed the data and prepared and reviewed the manuscript.

Christopher J. Bolch: Conceived the idea, isolated, maintained and contributed dinoflagellate cultures used in the project, edited and reviewed draft and submitted versions of the manuscript.

Lincoln MacKenzie: Conceived the idea, performed the field sampling and qPCR experiments, analysed the data, and reviewed the manuscript.

Kirsty F. Smith: Conceived the idea, performed the field sampling and qPCR experiments, analysed the data and wrote and reviewed the manuscript sections.

Shauna A. Murray: Conceived the idea, analysed the data, and reviewed the manuscript.

Production Note:
Signature removed prior to publication.

Rendy Ruvindy

Production Note:
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Christopher JS Bolch

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Signature removed prior to publication.

Shauna A. Murray

14/02/2019

2.1. Abstract

Paralytic shellfish toxin producing dinoflagellates have negatively impacted the shellfish aquaculture industry worldwide, including in Australia and New Zealand. Morphologically identical cryptic species of dinoflagellates that may differ in toxicity, particularly species of the former *Alexandrium tamarense* species complex, co-occur in Australia, as they do in multiple regions in Asia and Europe. To understand the dynamics and the ecological drivers of the growth of each species in the field, accurate quantification at the species level is crucial. We have developed the first quantitative polymerase chain reaction (qPCR) primers for *A. australiense*, and new primers targeting *A. ostenfeldii*, *A. catenella*, and *A. pacificum*. We showed that our new primers for *A. pacificum* are more specific than previously published primer pairs. These assays can be used to quantify planktonic cells and cysts in the water column and in sediment samples with limits of detection of 2 cells L^{-1} for the *A. catenella* and *A. australiense* assays, 2 cells L^{-1} and 1 cyst mg^{-1} sediment for the *A. pacificum* assay, and 1 cells L^{-1} for the *A. ostenfeldii* assay, and efficiencies of > 90%. We utilised these assays to discriminate and quantify co-occurring *A. catenella*, *A. pacificum* and *A. australiense* in samples from the east coast of Tasmania, Australia.

2.2. Introduction

Of all Paralytic Shellfish Toxin (PSTs) producing dinoflagellates, species of the genus *Alexandrium* are the most widely distributed around the globe, with planktonic cells found from sub-Arctic regions to the tropics (111-117). In Australia, past surveys of *Alexandrium* species indicate the presence of *Alexandrium pacificum* and *Alexandrium australiense* along the coast of the states of New South Wales (NSW) and Victoria, down to Port Lincoln in South Australia, including the east coast of Tasmania (28, 36, 118, 119). Planktonic cells of *Alexandrium catenella* (previously known as *Alexandrium fundyense*, Group 1 genotype (120)) on the other hand, have only been detected in Tasmania, along the east coast down to the western bays of the Tasman peninsula (47, 119). *Alexandrium ostenfeldii* has been identified across estuaries in NSW (36). In New Zealand, *A. australiense* and *A. catenella* have not been observed to date. However, in the Bay of Plenty in the North Island of New Zealand, the two PST producing specices *A. pacificum* and *A. minutum* bloom regularly, especially after summer storms (121, 122).

As well as living as planktonic cells, *Alexandrium* spp. undergo sexual reproduction, which results in the formation of resting cysts in coastal sediments (123). These cysts can germinate at the right environmental conditions and become the precursor for a bloom (124, 125). The cysts of *A. ostenfeldii* and *A. pacificum* have also been detected in several sampling locations in New Zealand, and Tasmania and New South Wales states in Australia (122, 126-128).

PSTs produced by *Alexandrium* species cause the human illness Paralytic Shellfish Poisoning (PSP), posing a serious public health and economic threat to local communities worldwide (129). It also affects the shellfish aquaculture industry in New Zealand and Australia, which is a significant contributor to the local economy, valued at approximately USD 188 million in New Zealand and USD 100.9 million in Australia (130, 131). Blooms of PST-producing species have been recorded in shellfish intensive areas in Australia and New Zealand (44, 132-134), resulting in product recall and farm closures. In New Zealand, blooms of *A. pacificum* started to impact the important aquaculture region in Queen Charlotte Sound in 2011, with the numbers reaching 1.3×10^5 cells $L^{-1}$and toxicity of 17 mg STX equivalents kg^{-1} in the mussel species *Perna canaliculus,* within a month after the first cell was observed (122). In December 2012, a poisoning incident in the Bay of Plenty was caused by an *A. minutum* bloom (122). Currently, the aquaculture industry is under development in this region, and the inherent risk of *Alexandrium* blooms is an important consideration. An

improved understanding of the ecology of *A. pacificum* and *A. minutum* will aid in assessing the risk of PST blooms in this region.

While PST-producing blooms of *A. pacificum* and *A. catenella* have been recorded in Australia and New Zealand (47, 122, 132, 133), the species *A. australiense* and *A. ostenfeldii* also occur (36, 46, 135). Little is known regarding the dynamics and environmental drivers underlying blooms of each species in this region. In particular cases, more than one species can co-occur during a bloom (47). Co-occurrences of cryptic *Alexandrium* species during blooms have also been reported in the UK (136, 137), Ireland (138), the Mediterranean (139) and Korea (140). Factors such as seasonality, temperature, rainfall, and parasitism that can influence the growth, development and decline of blooms of *Alexandrium* species can be species-specific, and therefore, accurate quantification at the species level is vital (41, 43, 44, 46).

In general, the few previous studies of *Alexandrium* in Australia have used light microscopy to identify species (28, 36, 118). One of the crucial limitations of this method is its inability to discern species with morphological features that are not distinguishable, or overlap, which is the case for *A. pacificum*, *A. catenella*, and *A. australiense*. While these species are not distinguishable based on their morphology, they have been identified as part of the former *A. tamarense* species complex and can be distinguished by clear genetic differences (40, 141-143). For cyst identification, the use of optical microscopy may also not be the most reliable method, as the cyst morphology can be very similar for species within the genus *Alexandrium* (144), and species confirmation is required through germination. Therefore, a method that is also able to detect and identify the species of *Alexandrium* cysts will be useful.

The development of molecular genetic methods to detect and enumerate harmful algal bloom forming species, such as microarrays, next generation sequencing, and quantitative Polymerase Chain Reaction (qPCR) has been rapid (40, 143, 145, 146). Apart from their sensitivity, molecular methods allow for high throughput sample analysis. Platforms such as qPCR have grown in popularity due to their sensitivity and specificity (96), as well as their speed and lower cost in comparison to light microscopy-based identification (147). Species-specific qPCR assays have been developed to identify cysts and motile cells of *A. catenella, A. pacificum, and A. ostenfeldii* utilising ribosomal gene regions as targets (102, 104, 148). However, no assay has yet been developed for *A. australiense*, and the specificity of previously published assays has not been thoroughly tested in the laboratory. The only

published assay for detecting *A. ostenfeldii* was not ideal, as it did not show a strong correlation with cell counts through other methods (R^2=0.44, (148)).

An assay based on a functional gene target involved in PST biosynthesis (*sxtA4*) has been developed (42, 108, 110). This method has shown high levels of efficiency and specificity to PST producing dinoflagellates and has also been trialled in shellfish meat as an indicator of meat toxicity (149). The qPCR assay based on *sxtA* outperformed qPCR methods targeting rDNA in several ways, such as the consistency of results among sites (42, 108, 110) and is therefore useful as a monitoring tool. However, it does not allow for the identification of individual species, which is required in order to investigate the ecological parameters related to bloom formation of individual species.

In this study, we developed and analysed the specificity and the efficiency of qPCR species-specific assays targeted at the species known to be present in Australian and New Zealand waters: *A. catenell*a, *A. pacificum*, *A. ostenfeldii,* and for the first time *A. australiense*. For the *A. catenella* and *A. pacificum* assays, the specificity and sensitivity were compared to previously published assays. The performance of the qPCR species-specific assays in relation to light microscopy cell and cyst counts was also assessed, to explore the possibility of using this assay for the detection of sediment cysts.

2.3. Materials and methods

2.3.1. *Alexandrium* culture conditions

Alexandrium cultures were grown at 35 PSU (Practical Salinity Unit) salinity, light was provided on a 12h/12h dark/light cycle at a photon irradiance of 100 µmol photons m^2s^{-1} (Table 2. 1). Strains used in this study were provided by the Cawthron Institute Culture Collection of Micro-algae, Australian National Algae Culture Collection, and University of Tasmania (Isolated by Chris Bolch and Miguel de Salas).

2.3.2. Development of qPCR assays

Ribosomal sequences from the large subunit 28S of four *Alexandrium* species were examined for regions of similarity and difference. Comprehensive alignment was constructed using all examined species from all available strains. Regions of similarity were defined by eye. Informative sequence differences were identified based on conserved regions from each species, and primers were designed at these locations. Five sets of primers and probes intended to be specific for each of the five *Alexandrium* target groups were designed using

Geneious R11 software. Probes were labelled with 6 FAM (6-carboxyfluorescein) to the 5'-end of the sequences, and BHQ1 (Black Hole Quencer) was attached to the 3'-end as a quencher (Integrated DNA Technologies, Iowa, USA).

Primer specificity tests were carried out by running qPCR reactions against DNA templates from *Alexandrium catenella*, *A. pacificum*, *A. australiense*, *A. minutum*, and *A. ostenfeldii* (Table 2. 3). At the exponential phase, a total of 60,000 – 75,000 cells were harvested in triplicate by centrifugation using Gyrozen 1580R centrifuge at 1,000g for 10 min (LaboGene, Bjarkesvej, Denmark). FastDNA Spin kit for Soil (MP Biomedicals, Solon, OH) was used to extract the DNA from the cultures according to manufacturer's protocol. Samples were eluted in 80 µL of elution buffer and stored at -20ºC until further analysis. The quantity and quality of the DNA was measured by Nanodrop ND-1000 (Thermofisher Scientific, MA, USA).

Table 2. 1. Cultures Used for this Study

Species Name	Strain Number	Origin	Isolation Date	Isolator	Growth Temperature	Growth Media
A. pacificum	CAWD44	Tauranga, NZ	Nov-97	L.MacKenzie/J.Adamson	18	GSe
	CS300	Samchonpo, Korea	Nov-90	C.Bolch	18	GSe
A. australiense	ATCJ33			Miguel de Salas	18	GSe
	AT-YC-H			C.Bolch	18	GSe
A. catenella	TRIA-E	Triabunna, Tasmania, AU		C.Bolch	18	GSe
	AT-STH-M	St Helen, Tasmania, AU		C.Bolch	18	GSe
A. ostenfeldii	CAWD136	Big Glory Bay, NZ	Jan-04	L.Mackenzie	18	GP
	CAWD135	Big Glory Bay, NZ	Jan-04	L.MacKenzie	18	GP
A. minutum	CAWD12	Anakoha Bay, NZ	Jan-94	L.MacKenzie	18	GSe
	CS324/12	Port River, Adelaide, AUS	Nov-88	S.Blackburn/J. Cannon	18	GSe
	AMNC04	Newcastle, NSW	Aug-97	C.Bolch	18	GP

Cross-reactivity was tested using qPCR carried out on a StepOne Plus Real-Time PCR System (Thermofisher Scientific, MA, USA) platform with the following cycles: 95°C for 10 s and 35 replicates of 95°C for 15 s and 60°C for 30 s. Each 20 µL reaction contained 10 µL of Itaq Universal Probe mix (Biorad, CA, USA), 0.5 µM of each primer (Table 2. 2), 0.05 µM fluorescence probe, 1 µL template DNA (corresponding to 500-1000 cells) 1 µL BSA, and 6 µL PCR-grade water.

The amplification efficiency of each primer pair was determined through the slope of the log-linear portion of the cell-based standard curve from one strain from each species. Ten-fold serial dilution of the DNA extracts at a range between 2000 – 0.02 cells for *A. catenella*, *A. pacificum* and *A. australiense* and between 800 – 0.008 cells for *A. ostenfeldii* were used to develop the curves. Three replicate DNA extracts from each strain were used to construct the standard curves. The qPCR was carried out as described above.

2.3.3. Test of qPCR methodology for cyst quantification

A van Veen grab (320 x 350 mm sampling area) was used to obtain sediment samples in shellfish aquaculture intensive area in Opua Bay, Marlborough Sounds, New Zealand (Figure 2.1). The grab had a lid that prevented surface sediments being lost on retrieval. Sediment within the grab sample was subsampled with three 60 mm diameter cores from which the top 1 cm was sliced off and pooled to make one composite sample. From this sample, the number of cysts per m^2 of sediment in the surface sediments could be calculated. Samples were stored frozen at -20ºC after collection as freezing does not affect the integrity of dinoflagellate cysts in sediment samples (150).

Table 2. 2. Sequence of Primers and Probes Specific to *A. catenella*, *A. pacificum*, *A. australiense* and *A. ostenfeldii*

Target species	References	Primers	
		Name	Sequence
A.catenella	This publication	ACT-US-408-F	5’-ACT TGA TTT GCT TGG TGG GAG-3’
		ACT-US-645-R	5’-AAG TCC AAG GAA GGA AGC ATC C-3’
	This publication	US-412-F	5’-TGA TTT GCT TGG TGG GAG TG-3’
		US-642-R	5’-CAA GGA AGG AAG CAT CCC C-3’
		US-443-P	FAM-CTTGACAAGAGCTTTGGGCTGTG-BHQ1
	Erdner et al (2010)	AlexLSUf2	5'-GGC ATT GGA ATG CAA AGT GGG TGG-3'
		Alexgp1RevAF1	5'-GC AAG TGC AAC ACT CCC ACC AAG CAA-3'
A.pacificum	Hosoi Tanabe & Sako (2005)	catF	5’-CCT CAG TGA GAT TGT AGT GC-3’
		catR	5’-GTG CAA AGG TAA TCA AAT GTC C-3’
	This publication	ACTA-416-F	5’-TCC TCA GTG AGA TTG TAG TG-3’
		ACTA-605-R	5’-GAC AAG GAC ACA AAC AAA TAC-3’
		ACTA456-P	FAM-TTTGGCTGCAAGTGCAATAATTCTT-BHQ1
A.australiense	This publication	AusTv2-F	5’-CGG TGG GTG CAA TGA TTC-3’
		AusTv2-R	5’-GCA GGA AAA TTA CCA TTC AAG T-3’
		AusTv2-P	CACAGGTAATCAAATGTCCACATAGAAACTG
A.ostenfeldii	This publication	AO352-F	5'-AAACAGAATTGATCTACTTGGTG-3'
		AO493-R	5'-ATTCCAATGCCCACAGG-3'
		AO377-P	FAM-ATTGTTGCGTCCACTTGTGGG-BHQ1

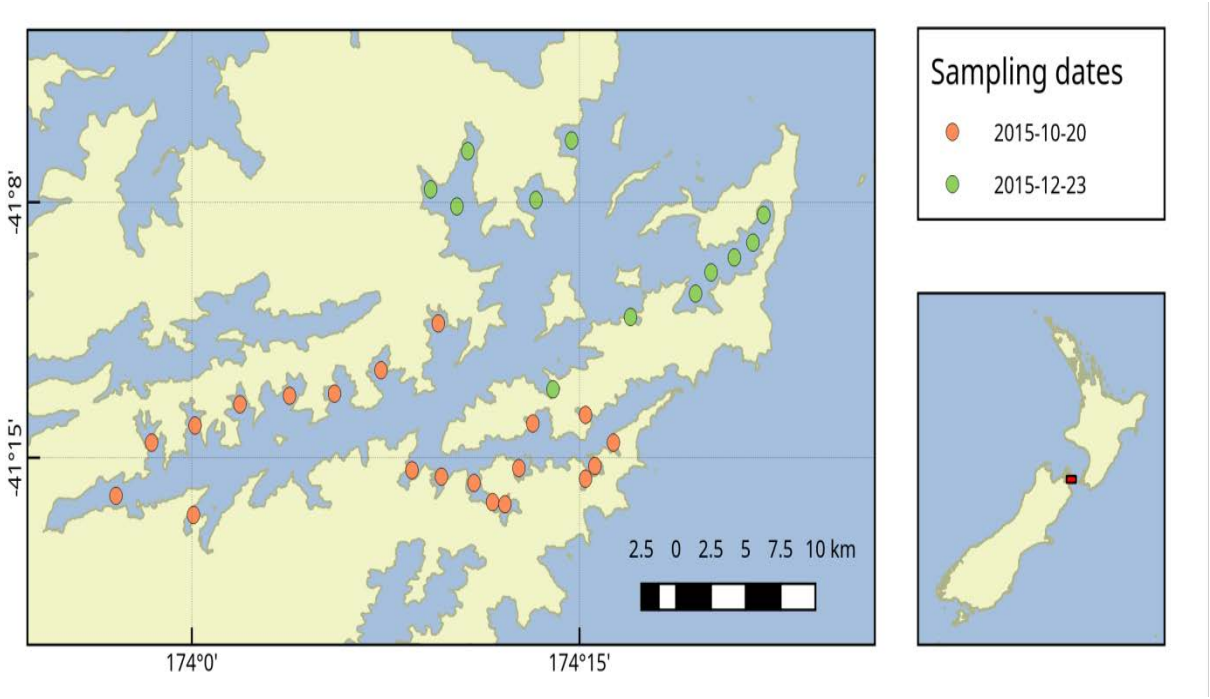


Figure 2. 1. Sediment sampling stations in Queen Charlotte Sound, Marlborough Sounds, New Zealand.

Alexandrium pacificum cysts were counted using the fluorescent staining method described by Yamaguchi (151). Briefly, this involved diluting 2 mL of the homogenised sediment sample in tap water, sonicating and prefiltering through an 80 µm mesh to remove larger particulates. The filtrate was washed through a 20 µm screen, and the deposit made up to 25 mL. A 5 mL subsample was fixed with 100 µL of glutaraldehyde, centrifuged, and the pellet resuspended in 15 mL of methanol and stored at 4°C for a minimum of 4 h (less than 4 h produces variable staining and inaccurate counts). After centrifugation and washing with distilled water, the pellet was stained for 1 h with a 2 mg mL^{-1} solution of Primuline dye (Sigma-Aldrich 206856-5G). After further washing with distilled water and resuspension in 5 mL of water, a subsample (typically 0.25 – 0.5 mL) was settled in an Utermöhl chamber and examined under UV light with an inverted microscope. Using this method, the brightly stained *A. pacificum* cysts contrasted well against the background of other particulate material in the sample and were easily identified and counted.

For qPCR analysis, 50 mg subsamples of sediment were taken for total genomic DNA extractions using Power Soil® DNA isolation kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. DNA extractions were eluted into 50 µL, quantified using a

NanoPhotometer (Implen, Munich, Germany) to check for DNA quantity and quality (260/280 ratio), and stored at -20°C until further analysis.

2.3.4. Cross depth quantification of *Alexandrium* spp

Seawater samples were collected during the peak of an *Alexandrium catenella* bloom in the shellfish farming area on the east coast of Tasmania in Spring Bay and Great Oyster Bay in August 2016 aboard the RV *Southern Cross* (**Figure 2. 2**). Seawater samples of 3 L were collected at multiple depths with Niskin bottles on the same location and filtered with 8 µm polycarbonate filters in triplicates and stored at -20°C until the DNA extraction. DNA extraction was then carried out with FastDNA for Soil kit according to manufacturer's protocol (MPBiomedical, USA) and stored at -20°C until analysis.

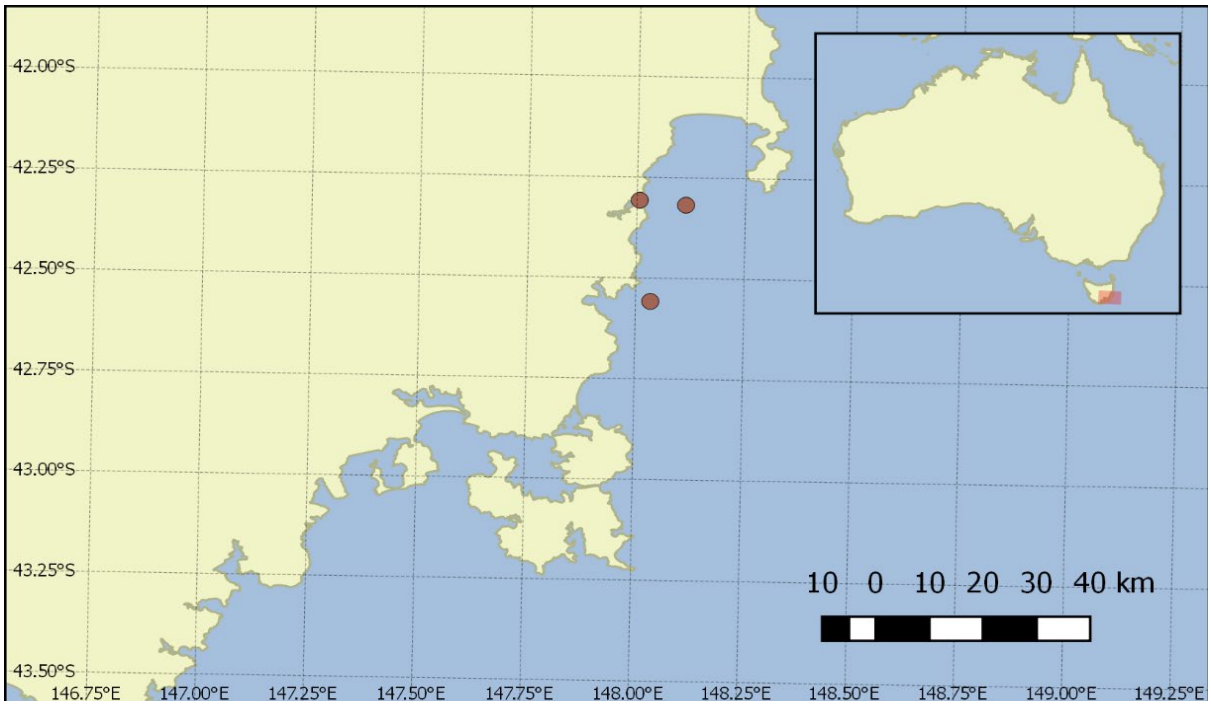


Figure 2. 2. Sample collection points in Great Oyster Bay and Spring Bay, Tasmania.

One L of water was stored in Lugol's iodine at 4°C until *Alexandrium* cell concentrations were determined. The samples were settled and total *Alexandrium* cells were counted using an inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.3.5. Bloom dynamics comparative analysis between light microscopy and qPCR

During a bloom of *A. pacificum* in Opua Bay (Queen Charlotte Sound, the Marlborough Sounds, New Zealand) samples were collected for both light microscope counts and qPCR analysis. Discrete grab samples were collected from 6 m depth. Samples for microscope cell

counts (100 mL) were preserved in Lugol's iodine, and a subsample of 10 mL was settled in an Utermöhl chamber and examined with an inverted microscope (CK41, Olympus, Wellington, New Zealand). For qPCR analyses 100 mL of seawater was filtered (Durapore membrane filters, 0.45 μm, Millipore, USA). Genomic DNA was extracted from the filters using Power Soil DNA extraction kits as described above. The genomic DNA samples were then analysed using the *A. pacificum* qPCR assay as described above. To create a standard curve for the qPCR analyses, DNA was extracted from replicate samples of known numbers of cells of the CAWD44 strain of *A. pacificum*. Cell concentrations of culture were estimated during exponential growth phase using Utermöhl chambers and an inverted microscope. Replicates samples consisting of 150,000 cells were filtered (Durapore membrane filters, 0.45 μm) and extracted using Power Soil DNA extraction kits (Qiagen, Hilden, Germany) as described above. These extractions were serially diluted and used to generate a standard curve of known cell number per reaction versus Cq data.

2.4. Results

2.4.1. *Alexandrium* sp. species-specific assays specificity test

In this study, we designed two primer pairs and a probe for *A. catenella*, and one primer pair and probe each for *A. catenella*, *A. australiense* and *A. ostenfeldii* species (Table 2. 2). The specificity of our assays and previously published assays was tested. Except for a previously published *A. pacificum* assay (102), which also positively amplified the strain of *A. australiense*, all the tested assays were specific to the target species (Table 2. 3). Further BLAST (Basic Local Alignment Tool) nucleotide analysis of the published *A. pacificum* catF/catR primers resulted in 100% match of the reverse primer with *A. australiense* strain ATCJ33 (KJ879221.1) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

Table 2. 3. Specificity of *Alexandrium* qPCR Assays

Species	Assays	*A.pacificum*		*A.australiense*		*A. catenella*		*A. ostenfeldii*	*A.minutum*	
		CAWD44	CS300	ATCJ33	AT-YC-H	TRIA-E	AT-STH-M	CAWD135	CAWD12	CS324
A. catenella	ACT-US-408-F/ACT-US-645-R	-	-	-	-	+	+	-	-	-
	US-412-F/US-642-R	-	-	-	-	+	+	-	-	-
	AlexLSUf2/Alexgp1RevAF1	-	-	-	-	+	+	-	-	-
A. pacificum	catF/catR	+	+	+	-	-	-	-	-	-
	ACTA-416-F/ACTA-605-R	+	+	-	-	-	-	-	-	-
A. australiense	AusTv2-F/AusTv2-R	-	-	+	+	-	-	-	-	-
A. ostenfeldii	AO352-F/AO493-R	-	-	-	-	-	-	+	-	-

[a] +, target sequence amplified ; -, target sequence not amplified

2.4.2. *Alexandrium* sp species-specific assays efficiency assessment

The amplification efficiency of the assays was measured based on the standard curve slope, developed through serial dilution of DNA extracts from known cell numbers. The reaction efficiencies for all tested assays were higher than 90%. Coefficient of determination of all standard curves were higher than 0.99. The amplification efficiency of the assay targeting *A. ostenfeldii* was lower than assays specific for *A. catenella*, *A. pacificum*, and *A. australiense* (Figure 2. 3). The limits of detection were 2 cells L^{-1} for the *A. catenella* and *A. australiense* assays, 2 cells $L^{-1}$and 1 cyst mg^{-1} sediment for the *A. pacificum* assay, and 1 cells L^{-1} for the *A. ostenfeldii* assay.

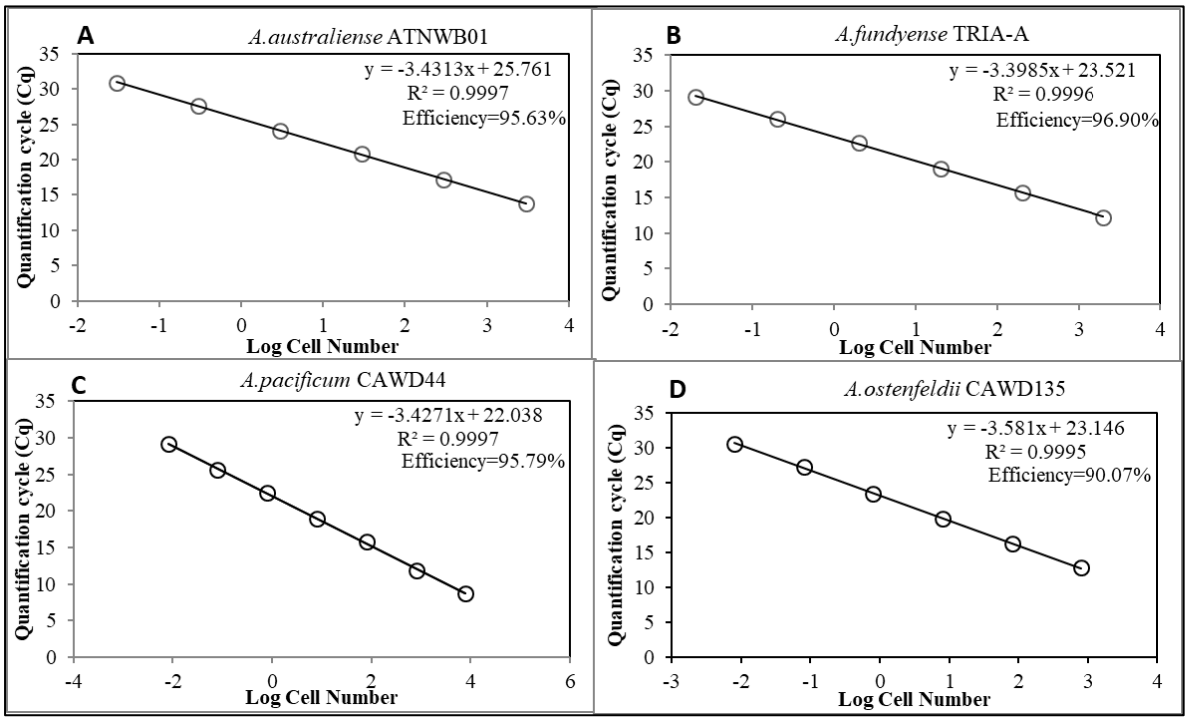


Figure 2. 3A-D. Standard curves of species-specific assays for *Alexandrium* spp, presenting the quantification cycle (y-axis) versus the known cell number in log scale (x-axis). (A) *A.australiense* strain ATNWB01 standard curve. (B) *A.catenella* strain TRIA-A standard curve. (C) *A.pacificum* strain CAWD44 standard curve. (D) *A.ostenfeldii* strain CAWD135 standard curve.

The *A. pacificum* assay (ACTA-416-F/ACTA-605-R) was tested with DNA extracts from cysts (Figure 2. 4). The standard curve's efficiency developed from the cysts was sufficiently high (90.53%), and the coefficient of determination was also relatively high (0.8507).

2.4.3. Comparison between light microscopy and qPCR

The quantity of *A. pacificum* cysts per gram of sediment samples from the same source were quantified using both light microscopy and qPCR. The coefficient of determination (Pearson $R^2 = 0.8578$) shows a high correlation between the value obtained from light microscopy and qPCR (Figure 2. 5).

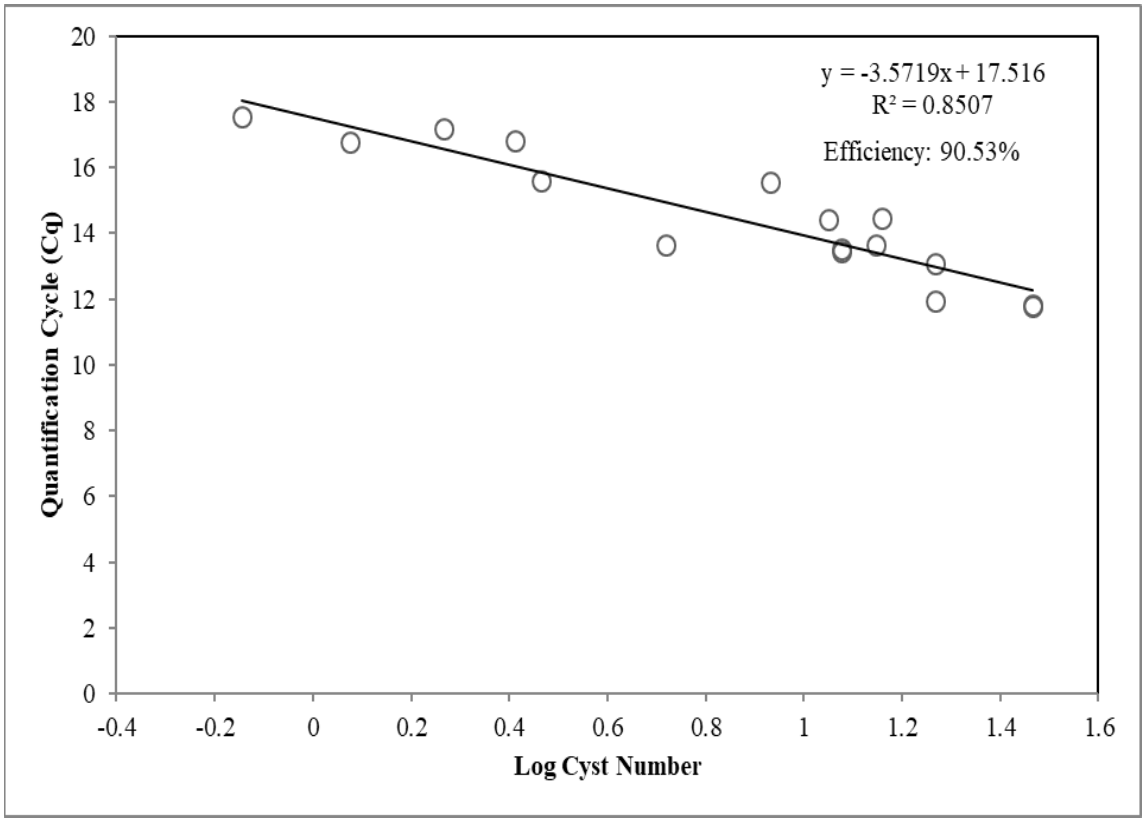


Figure 2. 4. Standard curve presenting the qPCR quantification cycle value (y- axis) versus the diluted number of *A. pacificum* cysts in log scale.

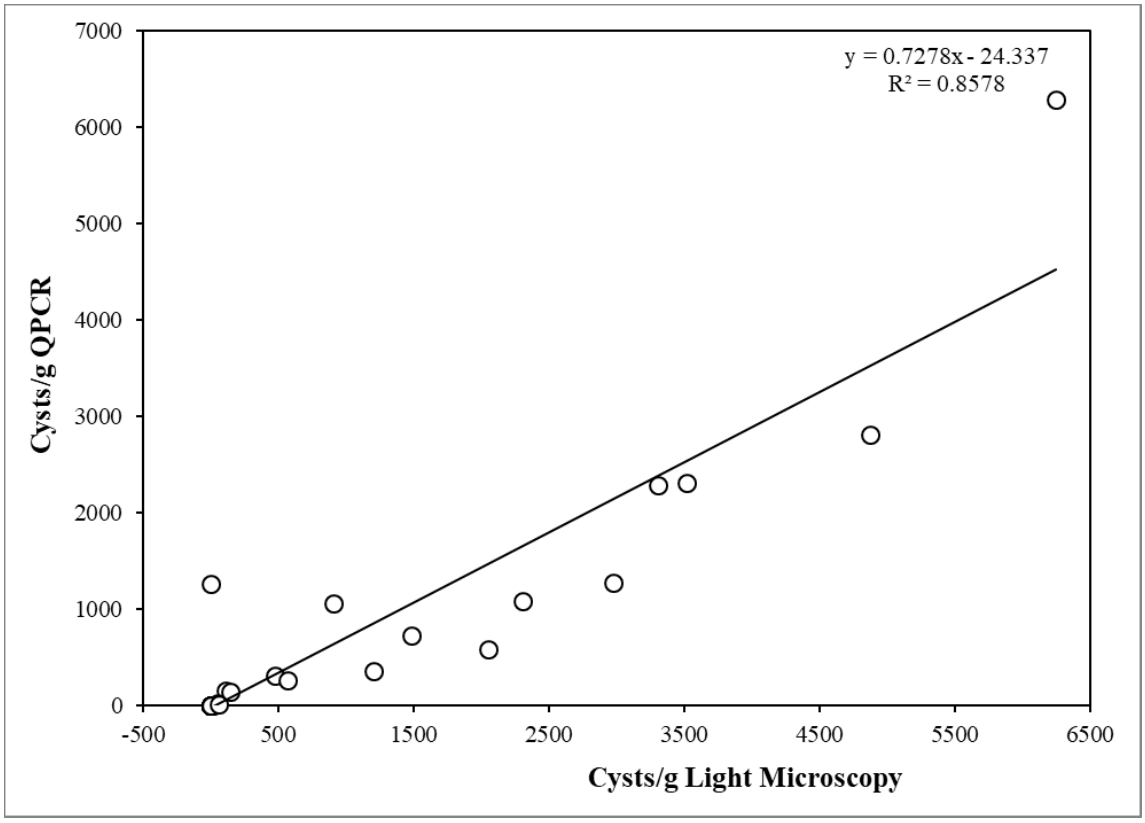


Figure 2. 5. Linear regression of *A. pacificum* cyst quantification from sediments with light microscopy and qPCR.

The planktonic cell count using light microscopy was compared with the quantification using qPCR approach in samples from Opua Bay (Figure 2. 6A). The cell number fluctuation observed with the light microscopy and qPCR methods are comparable, especially in low cell number (below 5000). On the date of 2nd Dec 2015, about 100 cells were detected using light microscopy and 5.4×10^3 cells were detected with qPCR. On 2nd Mar 2016, 1.4×10^4 cells were detected by light microscopy and 2.4×10^4 cells were detected by qPCR. During times of the highest cell concentration, the qPCR assay detected more cells than light microscopy. The results from the Bland-Altman analysis (Figure 2. 6B) supported this observation, showing less agreement between two methods in the high cell number phase (above 5000 cells L^{-1}).

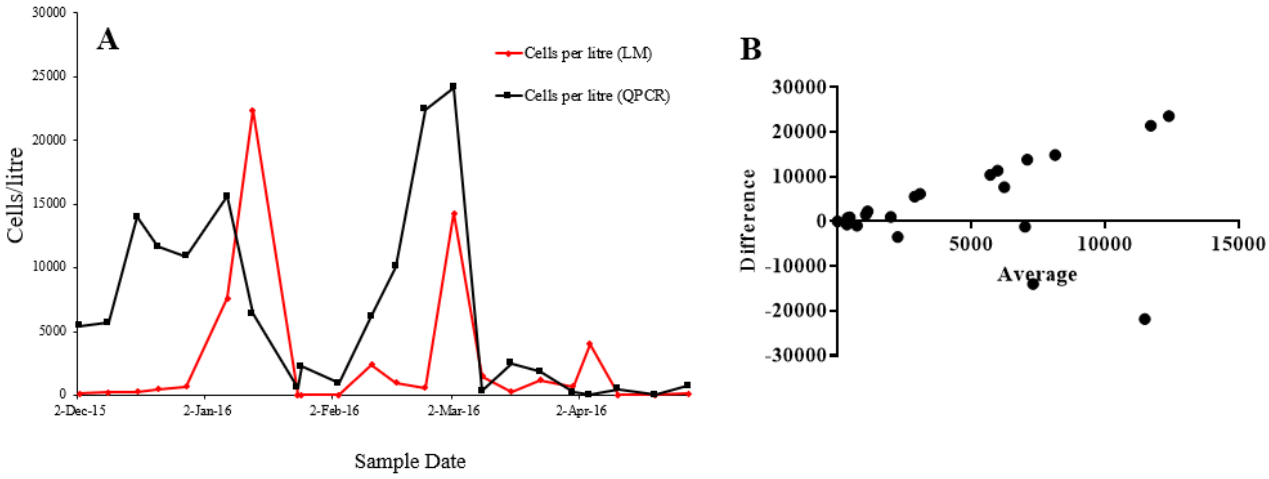


Figure 2. 6. (A) Cellquantification with light microscopy (red line) and qPCR method (black line) throughout the *A.pacificum* bloom in Opua Bay, Marlborough Sound. (B) Bland-Altman measurement of agreement between cell quantification using light microscopy and species-specific qPCR assay.

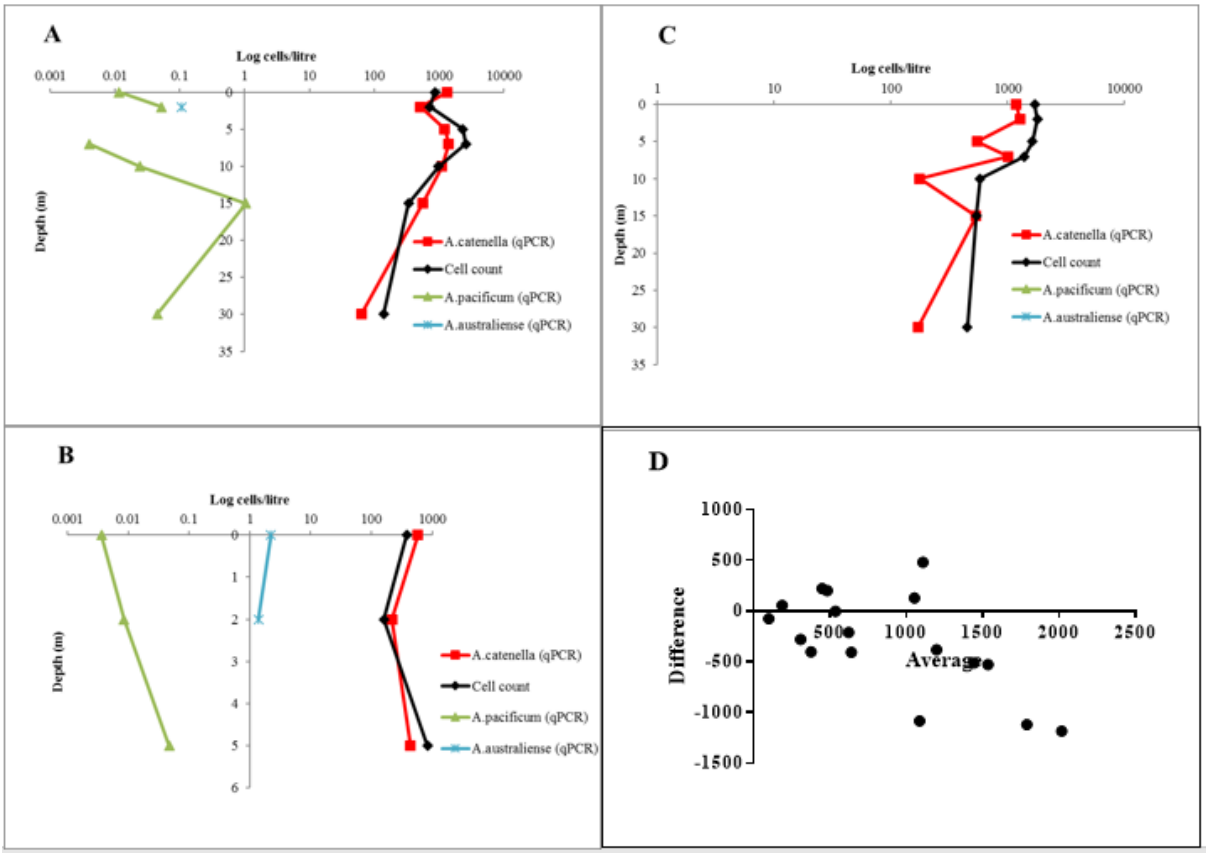


Figure 2. 7. Cross depth cell quantification using species-specific qPCR assays for *A.catenella*, *A.pacificum*, and *A. australiense*, in comparison with total *Alexandrium* count

(A). Great Oyster Bay Site 1 (B) Great Oyster Bay Site 2. (C) Spring Bay Site 3 (D) Bland-Altman measurement of agreement between species-specific *A. catenella* assays and light microscopy.

The cross-depth cell quantification of multispecies samples from Spring Bay and Great Oyster Bay, Tasmania, using light microscopy and species-specific qPCR assays suggest that the bloom was dominated by *A.catenella* (Figure 2. 7A, B, C). The quantity of cells measured by light microscopy and qPCR appeared to correlate well. Additionally, Bland-Altman analysis of combined Southern Cross samples shows that the differences between two methods are mostly around 500 cells L^{-1}, with the data points spread evenly (Figure 2. 7D).

2.5. Discussion

The co-occurrence of cryptic species of dinoflagellates can lead to difficulties in accurately enumerating the target species, which is necessary in order to establish the ecological factors driving the growth, proliferation and decline of harmful algal blooms. Rapid molecular methods have become a promising solution (105, 136, 138). Currently, the occurrence and seasonality of *A. australiense* blooms in Australia or elsewhere have never been reported. *A. australiense* can be weakly toxic or non-toxic (46), as two of the four strains for which PSTs have been measured have shown very low levels of PSTs. Understanding the bloom dynamics and environmental drivers may potentially reveal the factors underlying intra-species toxicity variation. This study described the first method for determining the abundance of *A. australiense*, with sensitivity up to one cell and efficiency of 95.63% (Figure 2. 3A). The assay was also specific to this species, when tested against DNA extracts from *A. catenella*, and *A. pacificum*.

The specificity test (Table 2. 1) indicated that our *A. pacificum* assay is more specific than a previously published assay (102). Further BLAST analysis of the published primer pair revealed an overlap between *A. australiense* long subunit sequences with the reverse primer. This shows that we could find not just morphological, but also genetic sequence overlaps in cryptic species, emphasising the importance of cross-specificity tests against species from this complex. All the assays showed high efficiency (Figure 2. 3), and low limits of detection: 2 cells L^{-1} for *A. catenella*, *A. pacificum*, *A. australiense* assays, and 2 cells L^{-1} for *A. ostenfeldii* primers. The efficiency of the *A. ostenfeldii* assay is lower (90.07%), when compared to other tested assays (above 95%). However, it is still within the desirable range (90-100%) (148).

Cyst are usually found in sediment as dormant cell and can persist for decades (152). The detection and enumeration of cysts can provide information to determine the potential risk of toxic blooms in a certain area, and possibly the scale of it. Our results strongly indicated that quantification using qPCR is comparable to a widely used auto fluorescence methodology (151). One of the advantages of the qPCR method is its relative simplicity and high-throughput capacity, allowing simultaneous quantification of cysts across different samples. However, phenolic substances and humic acids commonly found in sediment samples could inhibit amplification reactions (153), lowering the efficiency of the qPCR reaction. This is shown by the lower standard curve efficiency when quantifying diploid cysts compared to haploid planktonic cells (Figure 2. 3C & Figure 2. 4). The extraction protocol and the PCR conditions we used appear to be optimal, as can be shown from the above 90% efficiency (Figure 2. 4).

The assays that we have designed for *A. catenella, A. pacificum*, and *A. australiense* were used jointly in order to characterise the species responsible for a bloom event in Tasmania, Australia (Figure 2. 7ABC). In this case, the 2016 bloom on the east coast of Tasmania was dominated by *A. catenella*, while *A. pacificum* and *A australiense* were also detected in the background. This pattern is consistent with the other samples analysed in 2015 in the east coast of Tasmania (47). Quantification of an *A. pacificum* bloom over time suggests that the results of the qPCR assays were comparable to those of the microscope cell count in terms of tracking the patterns over time of the bloom development and collapse (Figure 2. 6A). Nonetheless, we found that the qPCR methods based on rRNA genes overestimated the absolute cell abundance compared to the count using light microscopy (Figure 2. 6A & B). The overestimation of *Alexandrium* cell abundances using species-specific qPCR assays based on ribosomal RNA genes has been previously reported (154). Ribosomal RNA gene copy numbers between strains can be within orders of magnitude (65, 107). It is possible that the copy number of the strain used for the standard curve was very different to that of the environmental strains. Consequently, the difference was amplified when the cell number increased. Another Bland-Altman analysis of the Tasmanian bloom samples (Figure 2. 7D) shows that there is lesser overestimation when local strain is used for qPCR quantification. Thus, the use of multiple local strains to develop the standard curve may be able to correct the impact of the variation in copy number of the target gene. Alternatively, when species-specific information is not the crucial need for a study, the use of an assay based on functional gene target related to PST synthesis (eg; *sxtA4*) (42, 108, 110) can be an

alternative, as the copy number variation among strains appears to be far less for this gene region than that of rRNA genes (42, 107).

In conclusion, we describe the assay based on qPCR for distinguishing the cryptic species *A. australiense* from potential co-occurring, morphologically identical species *A. pacificum* and *A. catenella*, as well as the toxic species *A. ostenfeldii*. We show that this method can be used to estimate abundance of these potentially PST-producing dinoflagellates in mixed samples. We have also shown that the methodology can be used to quantify cysts with comparable precision to current microscopy-based method.

Chapter 3. The significance of genomic copy number variation in paralytic shellfish toxin producing *Alexandrium*: *sxtA* and rRNA genes

3.1. Abstract

The commercial and public health impacts of Paralytic Shellfish Toxin (PST)- producing *Alexandrium* spp. are significant. As a consequence, molecular genetic methods targeting molecular barcoding genes, such as regions of rRNA, have been developed. For accurate quantification of species using a gene, it is crucial to understand the variability in genomic copy numbers among strains or species. For functional genes, genomic copy numbers may be particularly relevant, due to the dosage effect, by which genes that are present in more genomic copies may be transcribed more frequently, leading to greater activity. Currently, little is known regarding the copy number variation of ribosomal RNA genes and the PST-biosynthesis gene *sxtA4* in species of *Alexandrium*. In this study, we quantified the copy number of *sxtA4* and ribosomal RNA genes to examine inter- and intra-species variability. We measured the total PST cell^{-1} and determined its relationship with *sxtA4* copies cell^{-1} to investigate the presence of a dosage effect. Finally, we measured the genome sizes of four *Alexandrium* species (*A. catenella*, *A. pacificum*, *A. minutum*, and *A. australiense*), to determine the relationship between the size of the genome and rRNA gene copies cell^{-1}. We found that *sxtA4* copies cell^{-1} varied greatly within species, by up to approximately an order of magnitude. However, this variation was much less than the variation of rRNA gene copies cell^{-1}, which varied by 3-5 orders of magnitude, and was significantly correlated to genome size. A highly significant dosage effect of *sxtA4* on PST cell^{-1} was evident across PST-producing *Alexandrium* species. These results suggest that great caution needs to be used when attempting to quantify *Alexandrium* species using rRNA barcoding genes and that quantification using a functional gene such as *sxtA* may be more stable and have an additional benefit of being more closely correlated with PST amounts.

3.2. Introduction

Paralytic shellfish toxins (PSTs) are a group of marine biotoxins with significant public health and economic implications worldwide (1). They are synthesised by the species of marine dinoflagellates *Pyrodinium bahamense*, *Gymnodinium catenatum*, and several species of the genus *Alexandrium*. The unusual structure and large size of the dinoflagellate genomes are notable amongst eukaryotes (57). Genomes of dinoflagellate species are estimated between 2-200 Gbps per cell, which is up to 40 times larger than the human genome (58, 77, 89, 155). This results in a high number of chromosomes per cell, which can also vary greatly from 24 to 220 per cell (156). Dinoflagellate chromosomes lack the presence of nucleosomes (157), and they are permanently condensed at all stages of the cell cycle (158).

Ninety-nine percent of dinoflagellate DNA content consists of structural non-coding repeat sequences (159, 160), but the coding genes can be present in multiple tandem repeats (56, 72, 73). Mechanisms such as partial gene duplication and retroposition (74) (161, 162) contribute to copy number increase, and environmental stress may promote it (162). Certain organisms can take advantage of the abundant gene copies to help them produce more transcripts (72). This mechanism is commonly called the 'dosage effect', and it has been found in several eukaryote organisms (76, 78, 80).

Several genes associated with PST biosynthesis have been discovered in *Alexandrium* (43, 45, 93). The transcript sequences of dinoflagellates have unique features, such as the presence of a spliced leader sequence, a higher GC-content, and eukaryotic poly-A tails (45. However, little is known regarding the gene expression regulation in dinoflagellate, particularly the way they synthesise toxins. Only 10-27 % of genes in dinoflagellates are regulated at the transcriptional level (69, 163), and the dosage effect may be active in PST-producing *Alexandrium* spp and the other PST-producing species. Therefore, the quantification of PST biosynthesis genes copy number is an essential first step to understand the regulation of toxin biosynthesis.

Gene copy number quantification is useful, not only to investigate the dosage effect, but also to standardise cell quantification by qPCR. Currently, phytoplankton cells may be quantified using light microscopy, quantitative Polymerase Chain Reaction (qPCR), and other molecular genetic methods including metabarcoding. Molecular genetic methods can be at times more accurate than light microscopy for cell identification and quantification, in particular for the

quantification of cryptic species (105, 136, 141, 164). Molecular genetic methods rely on the amplification and quantification of specific target genes, which consequently requires exact copy number quantification of a target gene per cell.

Studies about the copy number quantification in *Alexandrium* species have been carried out for *sxtA* and ribosomal LSU or SSU sequences, which are also common targets for qPCR-based detection and quantification (65, 77, 101, 104, 165). rRNA genes are present in considerable numbers in *Alexandrium* species, for example, they have been estimated previously at 189,570 ± 99,558 to 2,489,800 ± 550,967 copies cell^{-1} in *A. pacificum* (65). A previous quantification of rDNA copies cell^{-1} in *A. catenella* showed that they may be orders of magnitude lower (28,402 ± 6,152) (104) compared to *A. pacificum*. In *A. minutum* and *A. taylori*, the number of ITS rDNA copies is even lower, with 1084 ± 120.3 and 1345 ± 780 copies respectively (65, 101). The rRNA genes are among the most important housekeeping genes, directly involved in cell growth and development (81, 166).

Despite these estimates, a comprehensive understanding of inter-species and intra-species copy number variation in PST biosynthesis and rRNA genes is still lacking. In order to establish whether the dosage effect occurs for PST biosynthesis genes in *Alexandrium* species, gene copy number quantification needs to be carried out across several PST-producing species.

Here, the copy number of LSU for *A. catenella*, *A. pacificum*, and *A. australiense*, SSU for *A. minutum,* and *sxtA4* copy numbers in all four species was quantified utilising qPCR. Genome size was measured using flow cytometry, and the relationship of genome size to copy number of *sxtA4* and ribosomal RNA genes was investigated. Measurements of 14 PST variants were carried out using LC-MS to examine the dosage effect across *Alexandrium* species.

3.3. Materials and Methods

3.3.1. Culture synchronisation and harvest

All strains (Appendix A) were grown in 500 mL of GSe media (167) at 18°C. *Alexandrium* spp. were grown to exponential phase then incubated for 48 h in darkness to induce synchronisation of cell phase (168, 169). Cell quantification was carried out on Lugol's fixed cells using a Sedgewick-Rafter cell (ProSciTech, Kirman, Australia) and an inverted light microscope (Leica Microsystems, Wetzlar, Germany). Cells were harvested by centrifugation for 10 min at 1000 g. Between 60,000-75,000 cells were harvested in triplicate for copy

number quantification with qPCR. Around 100,000 cells were harvested in triplicate for genome size quantification with flow cytometry. At least 1,000,000 cells were harvested for the PST measurement with LC-MS.

3.3.2. Genome Size Measurement

After the cells were harvested by centrifugation, they were washed with a 1×PBS solution. The cells were then fixed with 1% paraformaldehyde for 10 min and then washed with 1×PBS. The cell pellets were re-suspended in 2 mL of cold methanol and stored for at least 12 hours at 4°C, allowing intracellular chlorophyll to be extracted. After the incubation, the cells were washed two times with 1xPBS and then stained for at least 3 hours in 0.1 mg mL^{-1} propidium iodide and 2 µg mL^{-1} RNAse (Merck KGaA, Darmstadt, Germany).

A CytoFLEX S (Beckman Coulter, California, US) was used with laser excitation at 488 nm to measure the genome size. Triplicates were run at medium speed (30 µL min^{-1}), and data were acquired in linear and log modes until at least 10,000 recorded events. BD™ DNA QC Particles Chicken blood cells (BD Biosciences, San Jose, USA) of 3pg DNA/nuclei were used as a standard (170). Fluorescence emission of propidium iodide was detected at 610±10 nm. The peak ratios and coefficient of variation (CVs) were quantified with CytExpert software (Beckman Coulter, California, US). The values of the CV were kept below 20%. The genome size calculation was based on the conversion factor 1 pg = 978 Mbp (171)

3.3.3. Gene Copy Number Quantification with qPCR

DNA extraction was carried out using a PowerSoil DNA Extraction kit (QIAGEN, OH, USA) according to manufacturer's instructions. Three technical replicates were extracted for each strain. Quality and quantity of DNA were determined using a Nanodrop ND-1000 instrument (Thermoscientific, Massachusetts, USA,) and Qubit 2.0 Fluorometer (ThermoFisher Scientific, Massachusetts, USA). Samples were stored at -20ºC until further analysis.

Standard curves of *sxtA4* and rDNA copy number vs quantification cycle (Cq) were developed through ten-fold serial dilution of Gblocks® (Integrated DNA Technologies, Illinois, USA), which are synthetic oligonucleotides of the target sequence with defined quantity. Copy number per µL DNA was determined by using a formula:

$$\text{copy number per µl DNA} = \frac{\text{DNA amount (ng)per µl x } 6.022 \text{ x} 10^{23}}{\text{length of fragment (124bp)x } 660 \text{ x } 10^{9}}$$

Standard curves for the assays were run on the same plate as the DNA extracts from *Alexandrium* sp strains with known cell number μL^{-1} DNA. The *sxtA4* and rDNA copy numbers μL^{-1} DNA of *Alexandrium* sp. strains were determined by plotting each strains' Cq on the standard curve. The number of copies per cell for each strain was determined by dividing the copy number μL^{-1} DNA with cell number μL^{-1} DNA.

The qPCR runs were carried out with a BioRad CFX384 Touch™ Real-Time PCR Detection System (Biorad, Hercules, California, USA). The reactions were run in triplicate for each DNA extract with temperature set up of 95°C for 10 s and 35 replicates of 95°C for 15 s and 60°C for 30 s. Melting-curve analysis was performed at the end of each cycle to confirm amplification specificity by increasing the temperature to 95°C for 10s, then to 65°C for 5s, and 95°C with ramp temperature of 0.5° C sec^{-1}. The total volume of the reactions was 10µl, with 5 µl of SybrSelect™ Mix (ThermoFisher Scientific, Massachusetts, USA), 0.5 µM of each primer, 1µl template DNA, and 3µl PCR-grade water. The samples and master mix were loaded to Hard-Shell 384-Well PCR Plates using epMotion 5075 Liquid Handling Workstations (Eppendorf AG, Hamburg, Germany). PCR-grade water was used as a No Template Control (NTC). Data analysis was carried out in CFX Manager 3.1 software by Biorad and GraphPad Prism 7.04.

3.3.4. PST Quantification

Approximately 1 million cells were harvested and freeze-dried prior to the analysis. Samples were extracted and measured according to previously published methods (134). Each sample was resuspended in 2 mL of 1 mM acetic and vortexed for 90 s. Sample tubes were placed into boiling water bath for 5 min and then cooled to room temperature. Samples were then put into an ultrasonic batch for 5 min and filtered with 0.45 µm PVDF filter (Merck Milipore). The supernatant was then passed through the Liquid Chromatography-Mass Spectrometry) LC-MS analysis, with sheath gas flow rate of 50, aux gas flow rate of 13, spray voltage (kV) of 3.5, capillary temperature of 263°C, and aux gas heater temperature of 425°C.

The PST content per cell was quantified by Liquid Chromatography-Mass Spectrometry (LC-MS) Thermo Scientific™ Q Exactive™ (ThermoFisher Scientific, Massachusetts, USA), equipped with a Thermo Scientific Accela UHPLC. The column used was Acquity UPLC BBEH Amide 130 A 1.7µm 150 x2.1 mm. Mobile phases were water: formic acid: NH_4OH (500:0.075:0.3) and acetonitrile: water: formic acid (700:300:0.1) with an injection volume of

5 µL. Toxin content was measured for ten common variants, using standards obtained from National Research Council, Certified Reference Material Program (NRC–CRM), Halifax, NS, Canada. The total PST values were obtained by adding the numbers from each ten STX equivalent".

3.4. Results

The genome size of *A. minutum* was the smallest when compared to other species, averaging around 27.02 pg (Figure 3. 1). The average genome size of *A. australiense* was the largest, 87.32 pg. The genome sizes of two other species were similar, with *A. catenella* averaging 80.04 pg and *A. pacificum* 75.26 pg. The genome sizes of *A. pacificum* strains appeared to be highly variable when compared to other species, with a coefficient of variation 29.50%. The strain with the smallest genome size ACSH02 (42.35 pg), had almost a third of the genome size of the largest strain CS798 (130.9 pg).

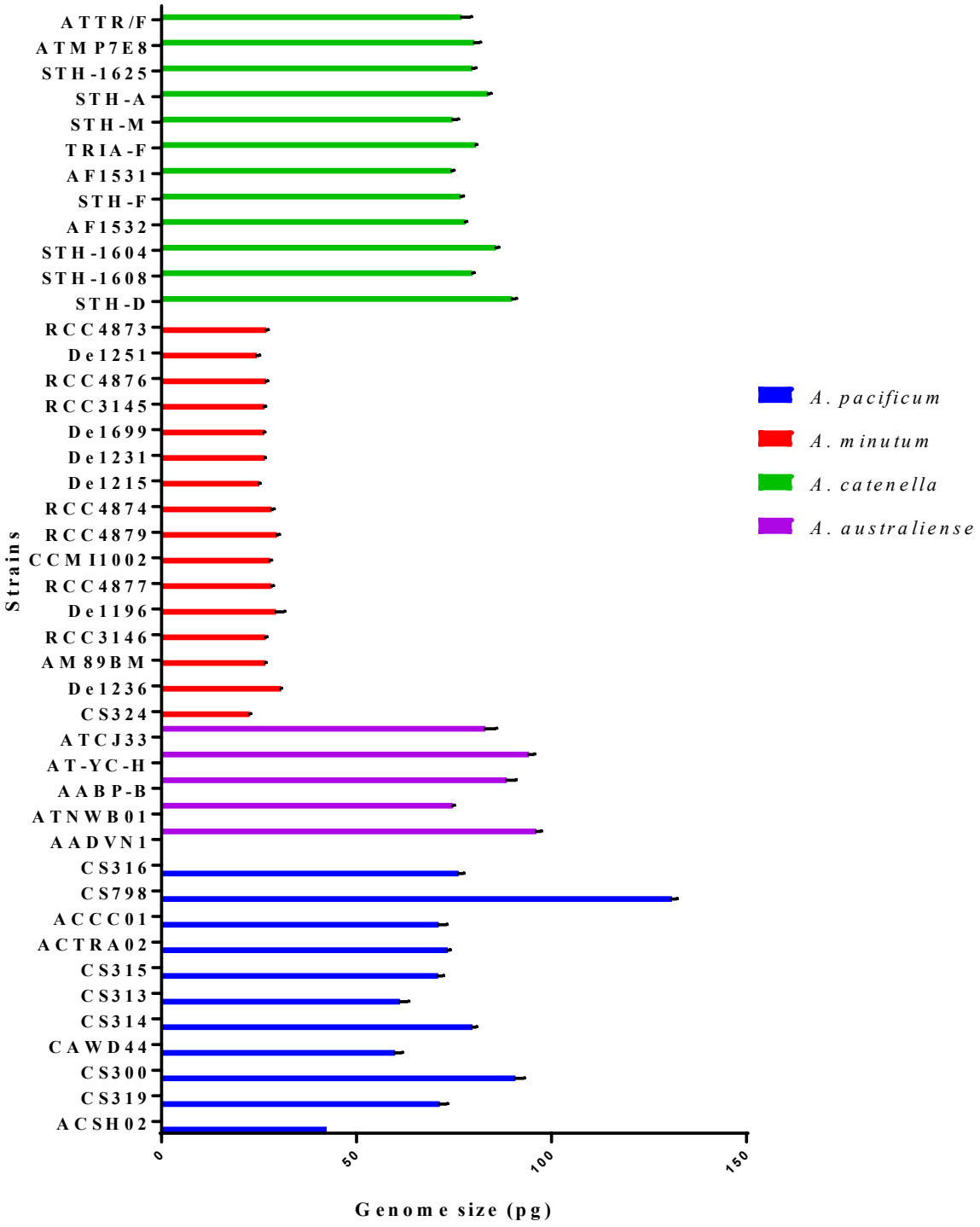


Figure 3. 1. Genome size of strains of *A. catenella*, *A. pacificum*, *A. australiense* and *A. minutum.*

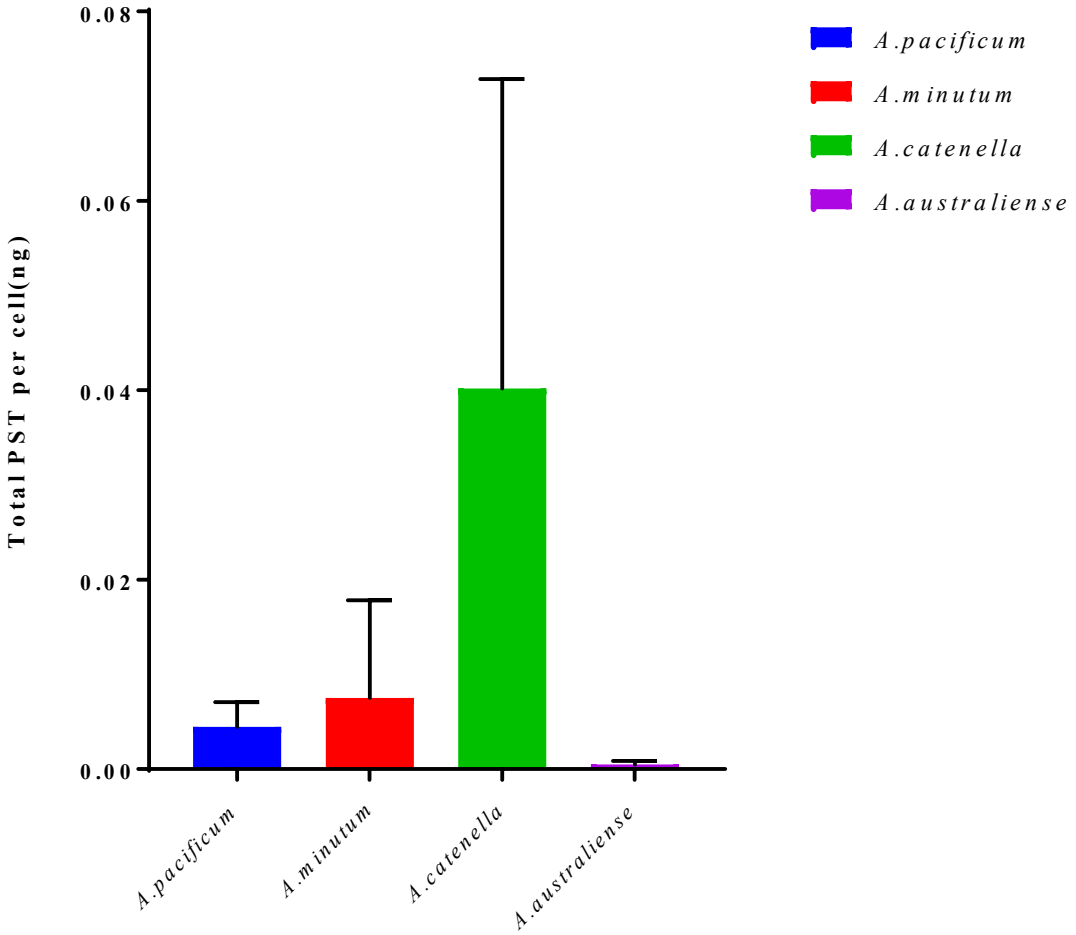
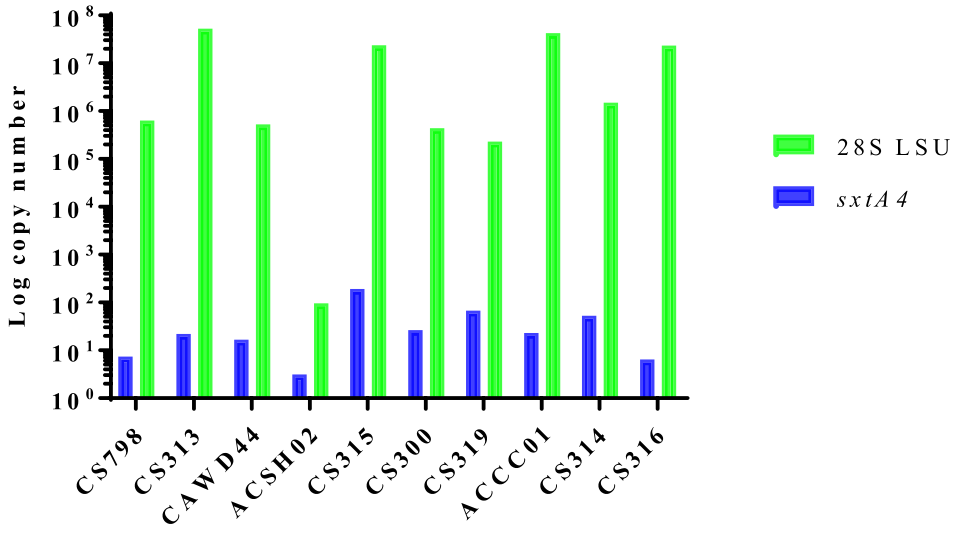
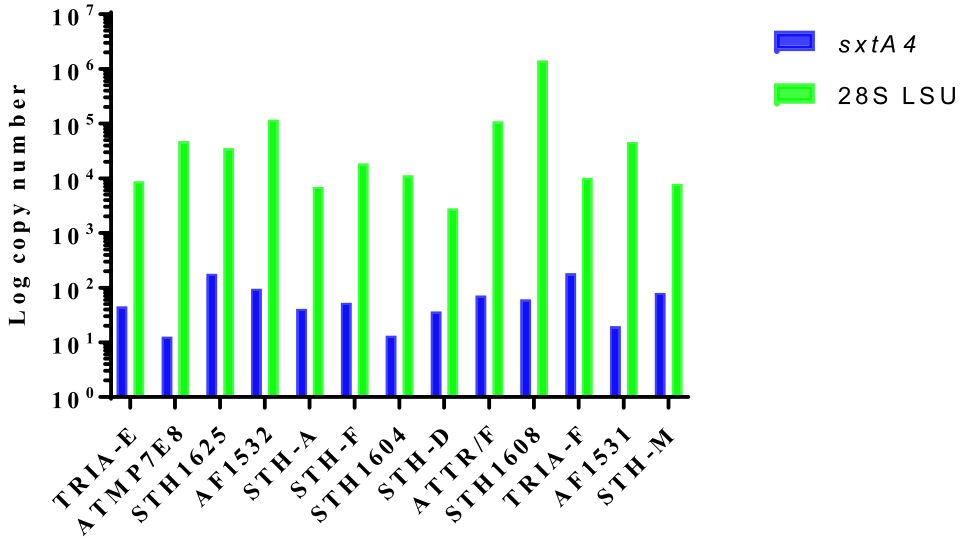


Figure 3. 2. Average of intracellular total PSTs concentration per cell across species of *Alexandrium*. The values are the mean of total PST variants per cell.

A)


Log copy number
10 8
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
CS798
CS313
CAWD44
ACSH02
CS315
CS300
CS319
ACCC01
CS314
CS316
28S LSU
sxtA4

B)


Log copy number
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
TRIA-E
ATMP7E8
STH1625
AF1532
STH-A
STH-F
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STH-D
ATTR/F
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TRIA-F
AF1531
STH-M
sxtA4
28S LSU

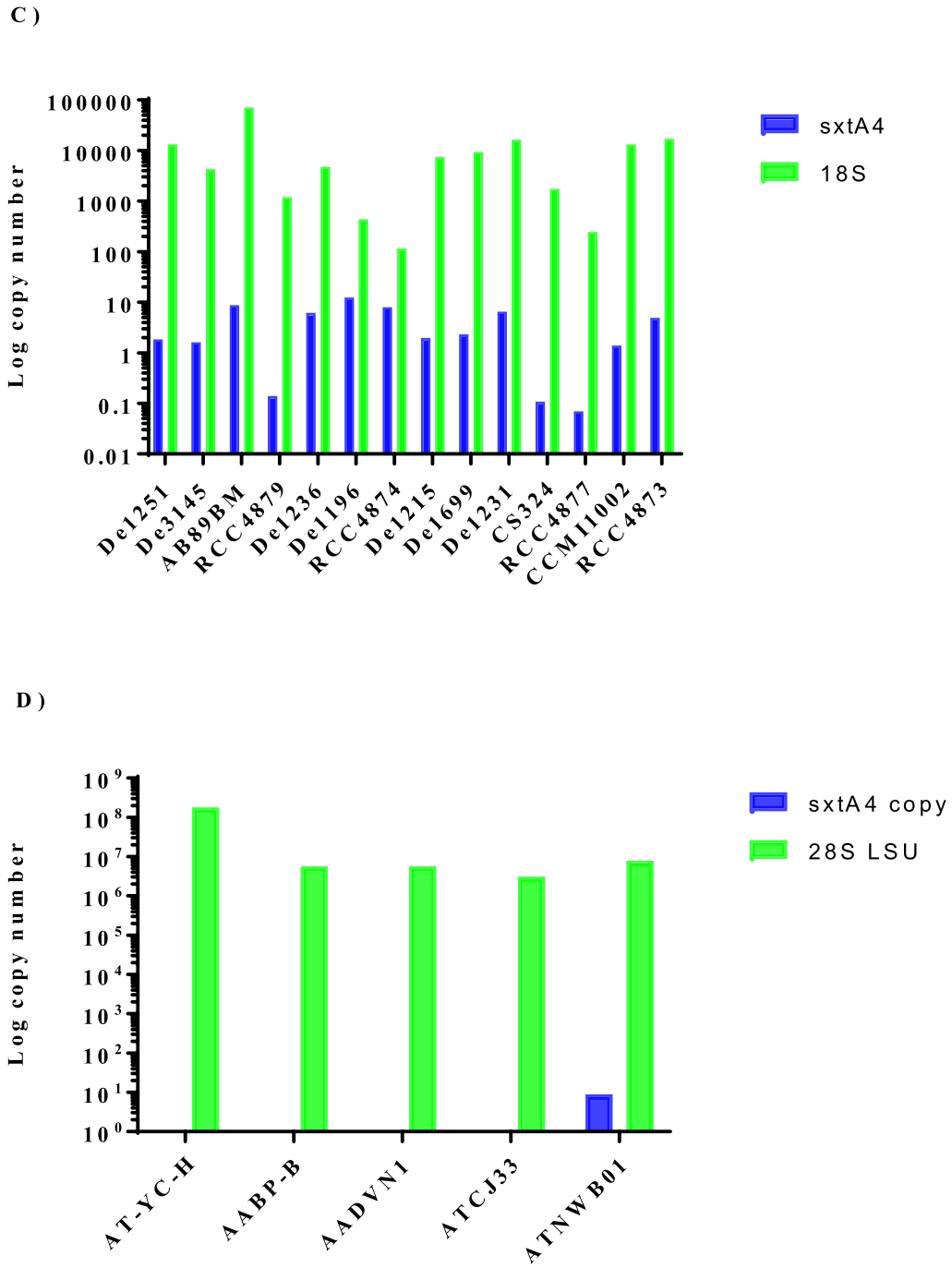


Figure 3. 3. Copy number variations of *sxtA4* (blue) and ribosomal RNA gene (green) across and within the species of *Alexandrium* in a log scale. A) *A. pacificum*. B) *A. catenella*. C) *A. minutum*. D) *A. australiense*.

The number of ribosomal RNA gene copies across the *Alexandrium* species was significantly higher than the number of *sxtA4* gene copies. Moreover, the ribosomal RNA copy number

differences across the strains of *A. catenella*, *A. pacificum*, *A. minutum* and *A. australiense* were large (Figure 3. 3A-D). This difference could reach up to five orders of magnitude across *A. pacificum* strains (Figure 3. 3A). In *A. minutum*, the difference in the ribosomal RNA gene copy numbers could reach three orders of magnitude, even though the genome sizes were similar. The copy numbers of ribosomal RNA gene across the strains of *A. australiense* were less varied compare to the other *Alexandrium* species, with the exception of strain AT-YC-H. The difference in the *sxtA4* copy numbers within species of *Alexandrium* was much lower, compared to the difference in the ribosomal RNA gene copies. The inter-strain copy number differences in *sxtA4* were highest in *A. pacificum* and *A. catenella,* with an order of magnitude differences across the strains.

From the LC-MS results, *A. catenella* produced the highest average PST-concentration per cell, followed by *A. minutum*. Furthermore, the PST-concentration per cell synthesised by *A. pacificum* appeared to be lower than *A. minutum*, but higher than *A. australiense*, which are mostly non-toxic.

Spearman correlation was used to assess the relationship between the copy number of *sxtA4* and the total PST concentration per cell of 33 strains of PSTs-producing *Alexandrium*. There was a highly significant positive correlation between *sxtA4* copies and total PSTs per cell (Spearman's rho=0.7705, $p<0.0001$). When correlation was assessed within species level, *A. australiense* had strong correlation (Spearman's rho= 0.8208), and weak for *A. pacificum* (Spearman's rho= 0), *A. catenella* (Spearman's rho= 0.1515), and *A. minutum* (Spearman rho=0.05011). From Figure 3. 4, *A. minutum* seemed to produce a higher level of PSTs per copy number of *sxtA*4 in comparison to *A. pacificum*. A strain of *A. australiense* (ATNWB01) produced PSTs on the same level as *A. minutum* strains, also with similar *sxtA4* copy number to *A. minutum* strains.

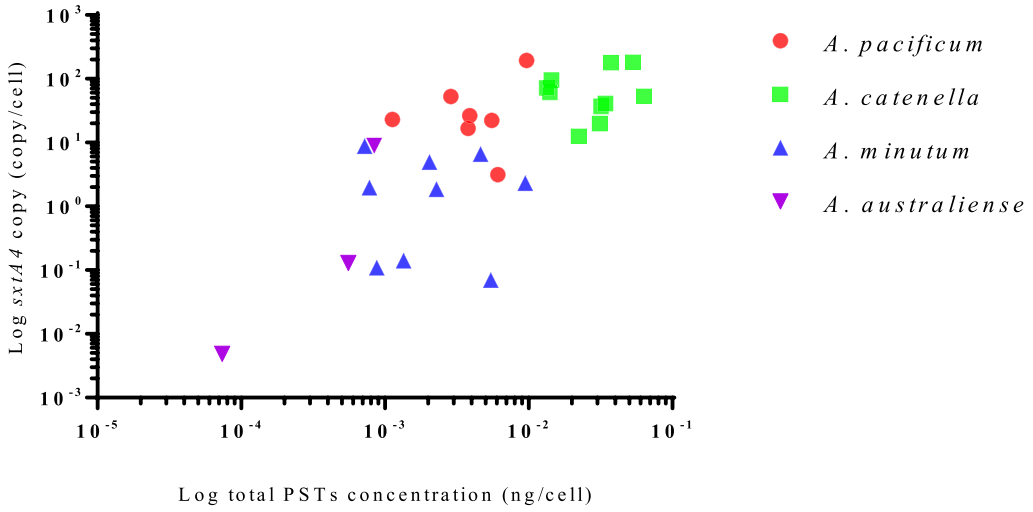


Figure 3. 4. Correlation between total PSTs concentration (ng cell^{-1}) vs *sxtA4* copy number per cell in log scale across four species of *Alexandrium.*

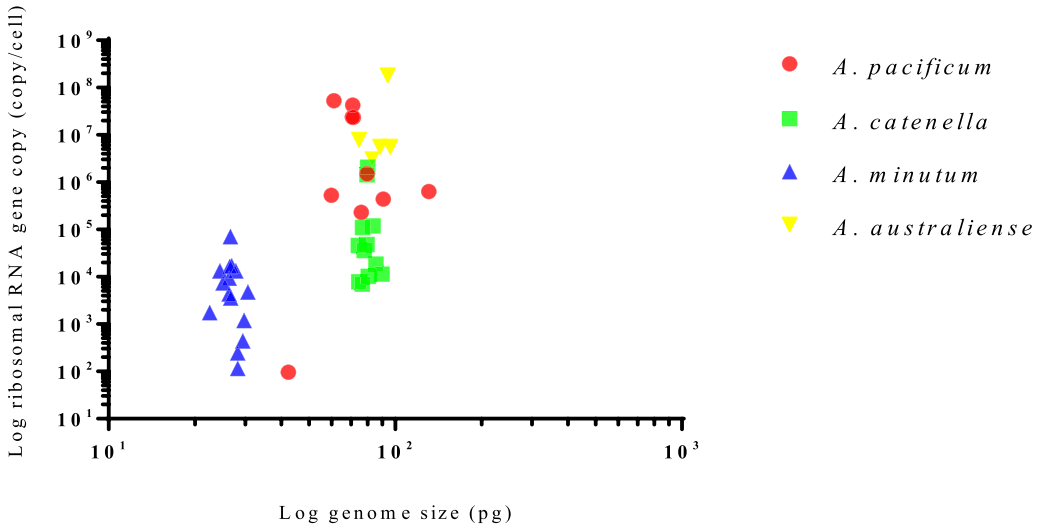


Figure 3. 5. Correlation between the genome size (pg) and ribosomal RNA gene copy number across four *Alexandrium* species.

The relationship between the genome size and ribosomal RNA gene copy number was examined for the 42 strains of *Alexandrium*, and a highly significant positive correlation was found (Spearman rho= 0.55556, p=0.0001). When correlations were drawn within species, negative correlations were found in *A. pacificum* and *A. minutum* (Spearman rho=-0.06667 and r=-0.3643 respectively), and very weak positive correlation were found in *A. catenella* and *A. australiense* (r=0.1678 and r=0.2 respectively). No correlation was found between

sxtA4 copy number and genome size across 41 strains of *Alexandrium* species (Spearman r= 0.2946, p=0.0615). *A. minutum* strains had the smallest number of ribosomal RNA gene copy numbers, and the smallest genome size compared to the other three species. While the species *A. pacificum*, *A. catenella*, and *A. australiense* had a similar genome size, differences in the number of copy of ribosomal RNA gene were evident. *A. australiense* had larger ribosomal RNA gene copy numbers compared to *A. catenella* strains. The ribosomal RNA copy numbers of *A. pacificum* strains were highly varied. One strain of ACSH02 had a very low ribosomal RNA gene copy number closer to those of *A. minutum*, and consistent with the small size of its genome. Except for strain ACSH02, the ribosomal RNA gene copy numbers of *A. pacificum* strains were higher than *A. catenella*.

3.5. Discussion

These results suggest that the intra-species variation of ribosomal RNA genes across the toxic species of *Alexandrium* are higher than previously thought. In *A. pacificum*, the variation could reach up to five orders of magnitude, and it was found to be at least one order of magnitude in all other species. qPCR-based cell quantification using ribosomal RNA gene assays may not be accurate if this copy number variation is present in environmental strains, which is likely. Quantification and community characterisation using metabarcoding is also likely to be impacted by this large variation in genomic copy numbers of this common barcoding gene, as community abundance characterisation can bias towards species with the higher copy number. As a result, the amplification will not accurately represent the relative abundance of studied organisms in the community. This issue can be exacerbated by the amplification bias prevalent in metabarcoding (172, 173), where the efficiency of each cycle in the PCR amplification is unknown.

Currently, it is unknown whether local populations of a species of *Alexandrium* may have a more similar genomic copy number of ribosomal RNA genes than strains isolated from geographically distant locations. Unlike the strains of *A. catenella* and most of *A. minutum* strains used in this study, the strains of *A. pacificum* were collected from various locations across a vast geographical span. Furthermore, the *A. pacificum* strains exhibit higher variability of ribosomal RNA gene copy number compared to *A. catenella* and *A. minutum* strains. While further study is required to establish the correlation, it is possible that geographical difference plays a role in the copy number differentiation. This may be related to the distinct environmental conditions the strains were exposed to, which may result in

distinct retroposition frequency. Most of the strains of *A. catenella* and all of *A. australiense* used in this study originated from the east coast of Tasmania, and they possess less copy number variability when compared to *A. pacificum*. Therefore, it is possible that using strains isolated from the same environmental sample to develop a standard curve will increase the quantification accuracy.

These results showed that *sxtA4* was present in lower copies compared to ribosomal RNA genes, with lower variation within species. Previous studies found about 1-10 *sxtA4* copies in *A. minutum* (77) and 100-200 copies in *A. pacificum* (42, 45). With less variation within species, it is evident that quantification using *sxtA4* will yield more accurate results than ribosomal RNA gene-based assays when applied as a standard early warning tool. Thus, *sxtA4* based assays may be a better option for regular monitoring and early-warning purposes, when indication of an oncoming toxic bloom is more important than information about species identity.

The variation in genomic copy number may be an important part of the gene expression regulation in dinoflagellates, as transcriptomic level variation is less in dinoflagellates compared to other organisms. A highly significant correlation was found in this study between *sxtA4* copies and total PST production per cell in *Alexandrium* species, suggestive of a dosage effect on PST biosynthesis due to genomic copies per cell. Intriguingly, while the copy numbers of *sxtA4* in *A.minutum* were lower than those of *A. pacificum*, total PSTs produced were higher. Previous studies also suggest that there is no correlation between higher copy number of *sxtA4* in *A. pacificum* (previously *A. catenella*) *(*100-200 copies) (42, 45) compared to *A. minutum* (1-10 copies) with the concentration of PSTs produced (77). It was postulated that the larger genome size in *A. pacificum* was associated with ploidy differences, which implies that *sxtA4* copy number may not be a positively selected trait but a result of genome dynamics (77). It is possible that there are more retrocopies in *A. pacificu*m than *A. minutum*. Retroposition inefficiencies could also mean that the integrated genes, while present in complete sequences, do not have promoter sequences at the beginning of the gene. This case is commonly called retrocopies (174). The presence of retrocopies instead of functional genes may also be the case for the strain CCMI1002 of *A. minutum*, which does not produce PST despite the presence of *sxtA4* sequence. However, this case seemed to be very rare, as the previous tests in chapter 2 confirmed the qualitative association of *sxtA4* domain with PST production in *Alexandrium* species.

It appears that several strains have less than one genomic copy per cell. While it is possible that this is due to primer inefficiency, it is also likely that retrocopies were amplified from the genomic DNA. Also, retroposition may not occur efficiently, and only part of the genes was integrated into the genome (161, 174, 175). As a result, only part of the gene sequence could be amplified.

In general, *A. minutum* is smaller than the species from *Alexandrium tamarense* species complex, and cell size is known to correlate positively with the genome size (159). This is also shown in this study with the small genome size of one *A. pacificum* strain in comparison to the other *A. pacificum* strains. Whether the smaller cell positively selects the gene copies and as a consequence possess more streamlined and more efficient genome organisation and regulation is still unknown.

A positive correlation was found in this study between genome size and the rDNA copy number across species, which is in line with earlier evidence (79). However *A. ostenfeldii*, is an exception to this trend, as it has a large genome size (115 pg per cell (169)), but a comparatively low rDNA copy number, at 2,480–4,730 copies per cell (107). In *A. minutum,* despite the large difference in their genome size, the rRNA gene copy numbers were comparable to *A. catenella*. It is possible that much larger parts of the *A. catenella* genome consist of structural repeats than in *A. minutum*. This may also be the case with *A. pacificum* and *A. australiense*, which have larger genome size.

The genome sizes of *A. minutum* strains appear to be smaller and less varied than the genome sizes of species of the former *Alexandrium tamarense* species complex, which is consistent with the previous measurements in *A. minutum* (77, 155). Both *A. catenella* and *A. pacificum* are known for a large genome size of 64.7±7.7 pg (155), and our results found that the size can be even bigger, as several strains of *A. catenella* have a larger genome than 80 pg, and a strain of *A. pacificum* (CS798) has a genome size of 130.9 pg. Genome sizes of the former *A tamarense* species complex were highly variable, particularly for *A. pacificum*. The high genome size variability suggest the possible polyploidy, a theory that has been proposed before (77). Specialised ribosomal chromosomes have been observed in the former *Alexandrium tamarense* species complex (155), which is unique for organisms with a large genome. However, it is important to note that such a dedicated chromosome was not found in *A. minutum* (155). Whether such a distinctive feature may be associated with a higher adaptability to environmental stresses in *A. pacificum* and *A. catenella* remains unknown.

To conclude this chapter, genome size of species of the former *A. tamarense* species complex were found to be large and highly variable (up to 130 pg), and significantly correlated with the number of rDNA copies per cell. Evidence of an active dosage effect was found across the *Alexandrium* species, as the highest PST producing *A. catenella* possesses more *sxtA4* copies than the other examined species, and *A. australiense* strains with more *sxtA4* copies evidently produced more PSTs. However, *A. minutum* was found to consistently produce a higher PST amount per cells, despite lower copies per cell of *sxtA4*. It is also important to consider the influence of the copy number variations of other genes in PST-biosynthesis pathway, as well as regulations at a transcriptional or translational level. Finally, the copy numbers of *sxtA4* were much less variable than those of rDNA in the studied species. rDNA copies per cell were found to vary by up to 5 orders of magnitude in *Alexandrium* species, suggesting that environmental quantification using rDNA as a barcoding gene may be subject to significant inaccuracies.

Chapter 4. Optimising a pipeline for *in situ* rapid detection of Paralytic Shellfish toxin-associated dinoflagellates

4.1. Abstract

Paralytic Shellfish Toxins (PSTs), a group of toxins synthesised by dinoflagellates in marine habitats, have caused significant problems for shellfish farmers worldwide. Furthermore, the occurrence, frequency, and intensity of large-scale blooms of PST-producing dinoflagellates appear to be increasing in several harvest areas around the world. An early-warning platform that can inform farmers about impending blooms can help them apply mitigation strategies, thus allowing them to minimise economic losses. Ideally, such a platform should detect the presence of PST-associated microalgae in the water column before they accumulate in the shellfish. Additionally, the platform should be standardised and simple enough for use by the farmers *in situ*. In line with the increasing number of portable in-field qPCR platforms, we have devised and tested a pipeline based on the detection of the *sxtA* gene, which initiates the biosynthesis of PSTs. This pipeline has been designed for on-farm application, using highly efficient (above 90% cell recovery) standardised sampling and sample processing protocols, and lyophilised qPCR reagents. A qPCR assay based on *sxtA* (Phytoxigene™ DinoDtec) was found to be specific, sensitive and efficient. Finally, a test of several preservative buffers indicated that Longmire's buffer did not inhibit qPCR reactions.

4.2. Introduction

PST- producing algal blooms have affected shellfish harvest areas worldwide (48, 112, 113, 176), with financial impacts through farm closures and product recalls. For example, blooms of the species *A. catenella* (previously known as *A. fundyense*, *A. tamarense* Group1), in Tasmania, Australia have been persistent since 2012 (19, 47). Such instances have become increasingly unpredictable and toxic since then, reaching an unprecedented level of 150 mg kg^{-1} of total PSTs in mussels in 2017 (47, 177, 178).

In Tasmania, Australia, quantification of total PSTs is based on the Lawrence method (179). According to the New South Wales (NSW) and Tasmanian Shellfish Quality Assurance Programs, mandatory closure occurs when total PST concentration reaches 0.8 mg kg^{-1} of shellfish flesh (180, 181). Although this system has been largely successful in guaranteeing consumer safety, it does not provide sufficient information fast enough for farmers to prepare themselves adequately against the forthcoming impact(s) of the bloom. Sample transport, processing, and analysis of PST concentration in shellfish meat can take up to 12 days (47), leaving little or no time for the farmers to react. Furthermore, by the time results are available, shellfish farmers only receive the information regarding the current status of their product, without the means to predict the toxin content in the future.

The current phytoplankton monitoring system in Tasmania relies on the characterisation of morphological characteristics using light microscopy. However, species such as *A. pacificum* and *A. australiense* encompass strains that can vary in their PST production (41, 46, 143), despite being almost morphologically identical to the toxic *A. catenella*. Moreover, less toxic or non-toxic *A. pacificum* and *A. australiense* species may co-occur with *A. catenella* (47), leading to potential false positives or negatives. False positives may result in unnecessary closure, while false negatives put the farmers at greater risk of product recall. Therefore, a reliable early-warning system that can rapidly and accurately identify the threat of impending toxic blooms would allow for shellfish farmers to better apply mitigation strategies.

The idea of exploiting the DNA extracted from the environment for monitoring the presence of microorganisms has existed for almost two decades (182). This approach can bring advantages such as improvement in sensitivity and specificity with smaller logistical costs and faster identification of PST-associated dinoflagellates (within hours if necessary instead of weekly (180)). Consequently, action can be taken prior to the accumulation of toxins within shellfish to avoid potential product recall. Apart from the specificity, a further advantage of utilising environmental DNA (eDNA) is the possibility to perform additional analyses such as metabarcoding, or biochemical pathway recruitment through metagenomics.

Quantitative polymerase chain reaction (qPCR) assays, such as rDNA gene-based assays (102, 104, 144, 164) or toxin gene-based assay (42), have been used to detect toxic species when present at a very low concentration (108, 145, 146, 148). Cryptic species such as *A. catenella* can be detected, and more importantly, discriminated from other important species (40, 164). However, as discussed in Chapter 2 and other studies (148, 154), ribosomal DNA-

based qPCR assays can result in discrepancies when compare to microscope counts. This is likely because, as shown in Chapter 3, the rRNA gene copy numbers can vary by several orders of magnitude within the same species. An assay based on a toxin gene such as *sxtA* (42) can provide a more accurate option, as copy number variability within a species is much lower than in ribosomal sequence targets. Moreover, from a monitoring point of view, the indication of toxic species presence is more important than the identity of the species itself. For this reason, a commercial *sxtA*-based qPCR assay, Phytoxigene™ DinoDtec, has been developed.

The use of eDNA for phytoplankton bloom monitoring has been discussed previously (103, 106, 164, 183). Currently, the molecular work is carried out in dedicated molecular facilities, where molecular biology instruments and tools are readily available. Recently, the portable qPCR platforms such as MyGoMini (IT-IS Life Science), Mini8 (MoBiTec), Mic qPCR Cycler (Biomolecular Systems), and Liberty16 (Ubiquitome) have been emerging as viable options for *in situ* detection. With these platforms available in the market, quantification of target genes *in situ* is now possible. However, a simplified and standardised pipeline for the operation of qPCR screening on shellfish farm has not been developed to date.

Phytoxigene™ DinoDTec is a qPCR assay designed for the detection and quantitation for the presence of *sxtA* toxin producing genes, responsible for the production of saxitoxin (PST) in dinoflagellates. The assay comes in a lyophilised form consisting of enzymes, TaqMan-based probe, primers, dNTP and Internal Amplification Control (IAC). Once rehydrated with 80 µL of PCR grade water, the mix is enough to carry out four tests.

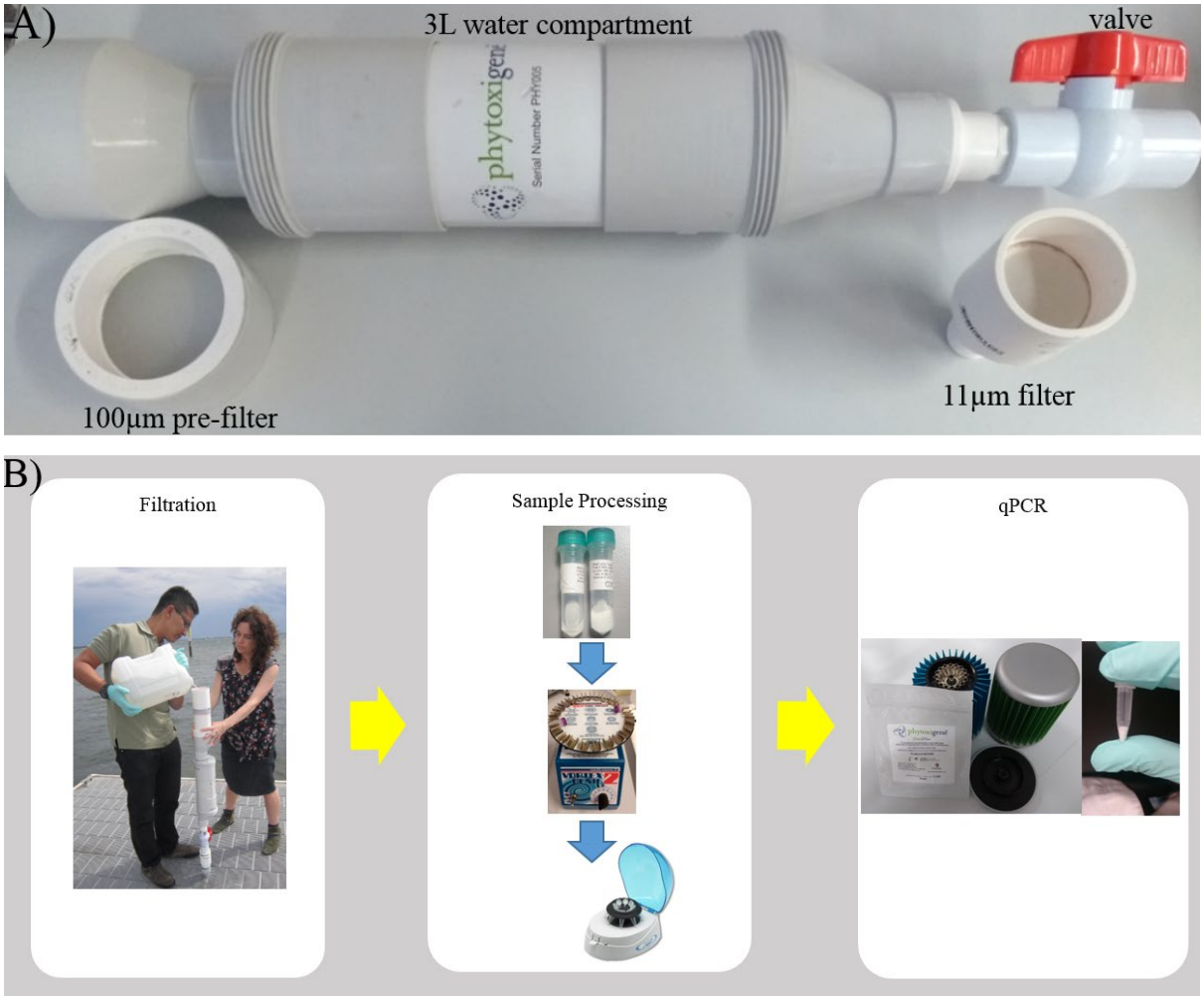


Figure 4. 1.A). Disassembled gravity operated filter device, consisting of the 100µm pre-filter, the 3L water compartment, and the 11µm filter to trap the phytoplankton. B). Pipeline for DinoDtec on-site application.

In this project, we developed a simplified and standardised protocol that allows for tests to be performed by shellfish farmers, yielding results within ~2 hours. The pipeline can be broken down into four key steps: i) cell capture through filtration, ii) cell preservation, ii) DNA extraction and purification, and iv) qPCR analysis and data interpretation. The protocol consists of a gravity-based standardised filter, cell lysis using commercially available BioGX lysis tubes, and a commercially available *sxtA*-based qPCR assay (DinoDtec, Diagnostic Technology). Lab-based tests for the efficiency of the filtration device, preservative buffers, and lysis tubes were carried out together with the sensitivity and accuracy comparison between the Phytoxigene™ DinoDtec assay vs light microscopy and *sxtA4* SYBR Green based qPCR assay using samples collected from the Gulf of Maine, USA,

4.3. Materials and Methods

4.3.1. Recovery efficiency of gravity operated sampler

The sampler was built to filter 3 L of seawater in two stages. The first stage is the 100 µm (pore size) nylon fabric, while the second stage consists of a 11 µm nylon fabric designed to capture and retain dinoflagellate cells. Three L of 5 µm-filtered seawater spiked with 100, 1000, and 10000 cells of *Alexandrium catenella* was loaded into the sampler. The valve was then opened to allow the water to flow through and the cells to be retained on the 11 µm filter. The filtered seawater was collected with a squeeze bottle. The 11µm filter was then unscrewed from the water compartment, and back-flushed using 50 mL of filtered seawater. A Sedgewick-Rafter counting chamber was then used to count 1 mL of the concentrated sample to obtain an estimation of the number of dinoflagellate cells per mL of sample. This was then multiplied by a factor of 50 to obtain the total number of cells present in 3 L of seawater. Percentage differences were then calculated from the counts before and after the filtration.

4.3.2. DinoDtec assay specificity and efficiency test

The specificity of the assay was tested by performing qPCR reactions against the non-toxic species of *Alexandrium*. DNA was extracted from 50,000-75,000 cells of *Alexandrium* using FastDNA Spin kit for soil (MP Biomedicals) and eluted in 80 µL of elution buffer from the kit. Between 600-1000 cells were used for the qPCR reactions.

Efficiency tests were performed by developing standard curves from strains of *A. pacificum*, *A. catenella*, and *A. minutum*. Serial dilutions were carried out (1:2) seven times on three extracts for each strain with DNA amount equivalent to 3000 cells. Reactions were carried out in triplicate with the same temperature and instrument set up as the sensitivity test.

The DinoDtec (Diagnostic Technologies, Sydney, Australia) mix was rehydrated with 80 µL of nuclease-free water, and a 20 µL aliquot was mixed with 5 µL of sample DNA extracts to make up a total of 25 µL qPCR reaction. The fluorescence signal from the probe was quantified in the FAM channel, and the internal control was measured through Cy5 channel of the qPCR machine. The reaction was run with temperature set up as follows: 95°C for 2 min, followed by 45 cycles of 95°C for 10 s and 64°C for 45 s on a Step One Plus (Life Technologies) qPCR platform. PCR-grade water was used as a No Template Control (NTC).

4.3.3. Preservative reagents tests

Inhibition of the qPCR reaction on commonly used preservative buffers; Longmire's buffer (184), RNA Later (Sigma Aldrich) and Iodine Lugol's (Sigma Aldrich) was compared between samples undergoing DNA extraction and the crude lysate from the BioGX lysis tube.

About 31,000 cells of *A. catenella* strain ATTR/F were fixed to an 8µm pore size nitrocellulose filter by syringe filtration (Merckmillipore, Massachusetts, US) in six replicates for each buffer. The samples were dried by pumping air through the syringe. For each preservative, triplicate filters were put inside lysis tubes (BioGX, Birmingham, USA). 1 mL of the preservative buffers was then added to the lysis tube, and the tubes were vortexed for 5 min in full speed on a Vortex-Genie® 2 (Scientific Industries, New York, USA) adapter, and centrifuged at 1000g for 1 min. The other set of triplicate filters were inserted into the lysis tube from FastDNA for Soil (MP Biomedicals), and 1 mL of the preservative buffer was added to the tube. The rest of the extraction steps were carried out according to the manufacturers' protocols. The qPCR reactions were performed with the same conditions as described in section 4.3.2, except that *sxtA4* (42) assay was used and only 1 µL sample DNA was used.

4.3.4. DinoDtec assay efficiency test using cell lysate as template

A standard curve was developed with the *sxtA4*-based DinoDtec assay to look at the performance of the assay using the template processed from the lysis tubes. The tubes contained beads and 500 µL of buffer solution. An 8 µm nitrocellulose filter (Merck Millipore) was attached to a reusable filter holder. The culture of *A. pacificum* strain CS300 with total cell number of 8000 was serially diluted (1:2 dilution) five times in triplicate. The cells were embedded to the filter by syringe filtration, and the filter was dried by pumping air through the filter. The filters were then inserted into BioGX Lysis tube, and vortexed at maximum speed using a Vortex-Genie® 2 (Scientific Industries, New York, USA) for 10 min. The tubes were then centrifuged for 1 min at 1000g to pellet the debris. 5 µL of the supernatant from each tube was transferred into the qPCR reaction plate, represent 80, 40, 20, 10, 5, and 2.5 cells μL^{-1}. DinoDtec reaction was carried out with the same condition as mentioned in section 4.3.2

4.3.5. Comparative analysis of light microscopy cell count with DinoDtec pipeline for the quantification of an *A. catenella* bloom in Salt Pond, Massachusetts, USA.

Samples of an *A. catenella* bloom were taken from Salt Pond (Eastham, MA USA), near Nauset Marsh between 4th March – 22nd May 2013. Samples were taken from depths of 1 m and 5 m from around the centre of the pond using a 5 L Niskin bottle in triplicates. From each bottle, 4 L seawater was concentrated using 20 µm Nitex mesh, and then resuspended in 20 mL of filtered seawater. The samples were divided into two 10 mL concentrates, with one part was used for qPCR analysis and the other part for cell count analysis with light microscopy (152).

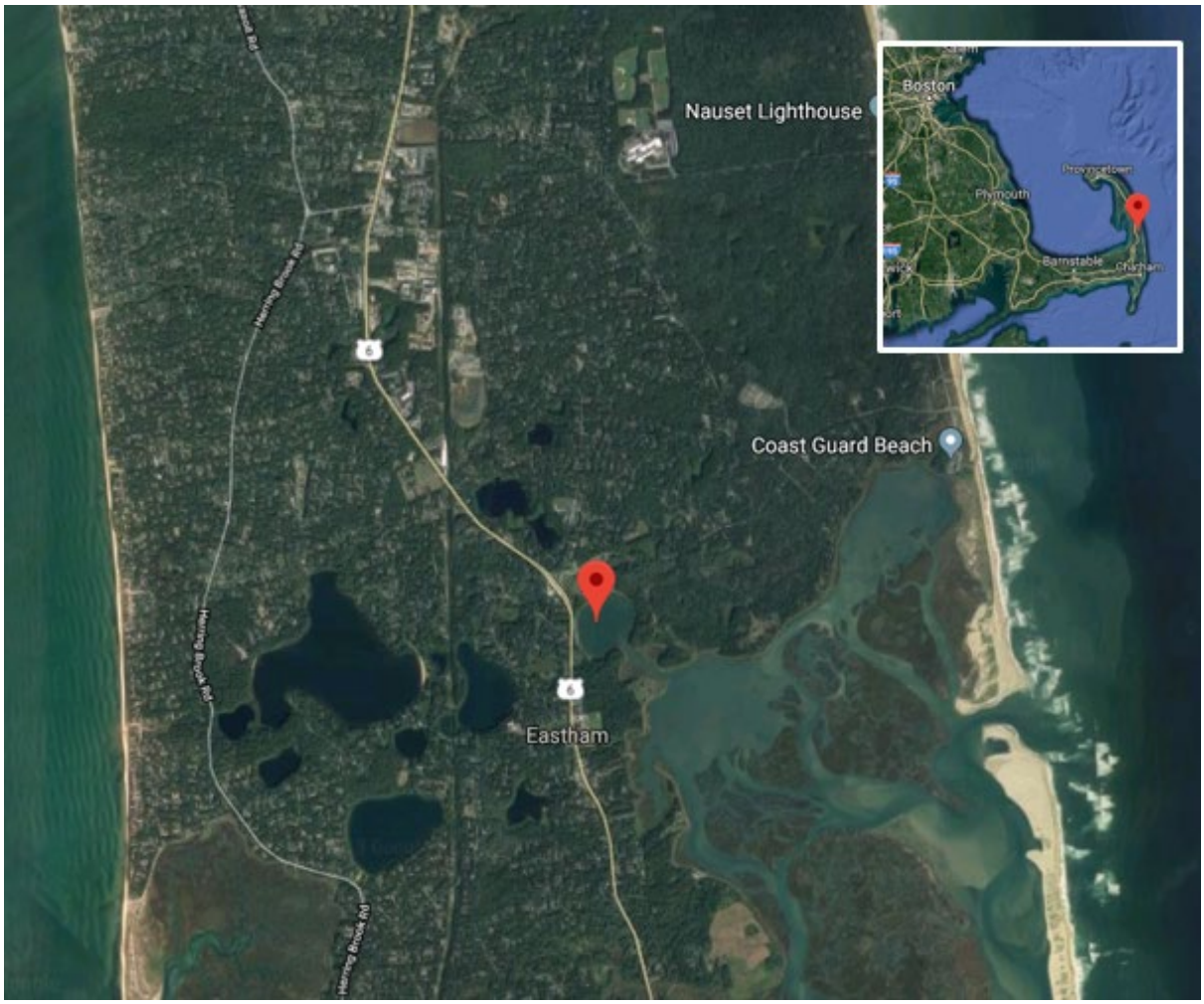


Figure 4. 2. Sampling location in Salt Pond, Massacchusetts, USA.

The cell count concentrates were fixed with 5% unbuffered formalin during transport to the Anderson laboratory at Woods Hole Oceanographic Institution. The cells were then centrifuged, resuspended in cold methanol, and stored at -20 ˚C until staining with a species-

specific fluorescent oligonucleotide probe (NA1; 5'-AGT GCA ACA CTC CCA CCA-3') (152). Half of the volume of the methanol suspension, which is equivalent to 1 L of raw seawater or 400 cells (whichever has the less volume of the methanol suspension) were fixed to a 5.0 μm Durapore filter for staining. Tenfold serial dilutions were carried out to apply 400 cells to filters in a volume of 1-7 mL for the very concentrated samples. After staining, filters were mounted on slides, and all *A. catenella* cells were counted. A parametric bootstrap model of each count was made using MATLAB to characterise the uncertainty associated with microscopy-based estimation.

For the qPCR analysis, phytoplankton samples were extracted with the FastDNA Spin kit for soil (MP Biomedicals), according to the manufacturer's protocol. In the cases where large amounts of biomass were visible, the filters were dissected into two halves using a scalpel, and DNA was extracted separately on each half. To improve the elution and recovery of DNA from the binding matrix, DNA was eluted in 200 μL of PCR-grade water. After elution, DNA was vacuum dried at 55 C (Concentrator plus, Eppendorf) and resuspended in a final volume of 40 μL. If two extractions had been made for one sample, they were pooled and resuspended in 40 μL. The final qPCR assay used 1 μL per replicate. DNA quality and quantity were determined using Nanodrop (Thermo Scientific).

The DinoDtec-based qPCR reactions were carried out as mentioned previously, with 1 μL of template DNA and additional 4 μL PCR-grade water was added to the reaction to ensure that the same sample concentration was used for both DinoDtec and SYBR-based reactions. The SYBR- based test was carried out with 20 μL of total reaction consisting of 10 μL of Sybr Select Master Mix (Life Technologies), 1 μL of template DNA, and 1 μL of 0.5 μM of *sxtA4* assays. Assays were performed in triplicate under the following temperature conditions: 95°C for 10 min, 40 x 95°C for 15 s and 60°C for the 30 s. Melt curve analysis was performed at the end of each run by increasing the temperature to 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. qPCR analysis was carried out with a Step One Plus (Life Technologies) platform.

4.4. Results

The recovery percentage of our gravity operated sampler was measured for approximately 100, 1000, and 5000 cells (Table 4. 1). The efficiency for all counts was above 90%. The efficiency was lowest when the filter was used to recover 120 cells (91.67%). Highest recovery efficiency was achieved while recovering approximately 1000 cells (95.38%).

Three preservative agents were assessed for potential inhibition effect on the qPCR reaction (Table 4. 2). The C_q of the sample preserved with Longmire's buffer was very similar to the non-preserved sample (28). The C_qs from the samples preserved with RNALater and Lugol's were 4-5 units higher than the non-preserved sample. The samples processed with BioGX lysis tube did not amplify when preservative buffers were added to the tubes.

Table 4. 1. Cell Recovery Efficiency of Phytoxigene™ Sampler

Avg Initial Cell Number	Recovered Cell Number	% Efficiency
120	110 ± 20	91.67%
996	950 ± 50	95.38%
4780	4510 ± 225	94.35%

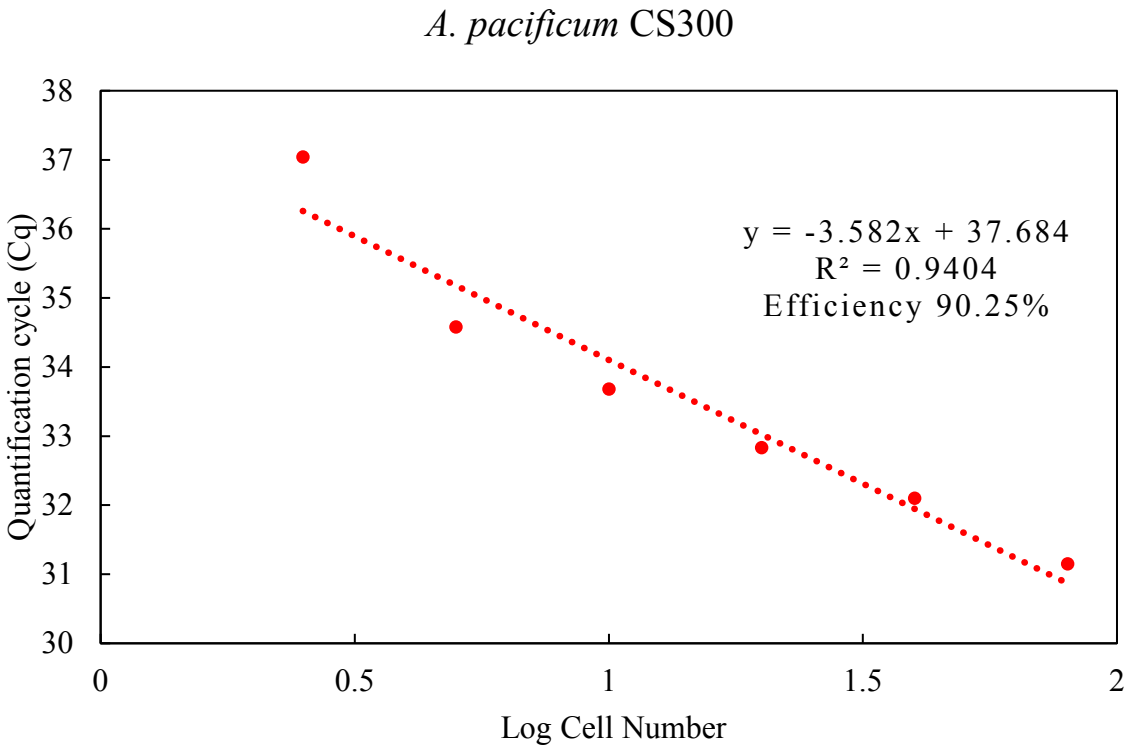


Figure 4. 3. Standard curve of DinoDtec assay using lysed cells of *A. pacificum* strain CS300 as template DNA, presenting the quantification cycle (y-axis) versus the known cell number in log scale (x-axis).

Table 4. 2. Inhibition Effect of Preservative Agents to qPCR Reaction

Preservative buffer	Average Cq FastDNA	Average Cq BioGX Lysis Tube
Longmire's Buffer	28.59 ± 0.09	-
RNA Later-Sigma Aldrich	35.22 ± 1.68	-
Lugol's Iodine-Sigma Aldrich	32.27 ± 0.4	-
No preservative	28.99 ± 0.25	23.35 ± 0.03

The DinoDtec assay is specific for toxin-producing species of *Alexandrium* (Table 4. 3). qPCR tests against confirmed non-toxic species showed no amplification. The efficiency of the standard curves across species and strains was within an acceptable range of 90-110%, with a regression coefficient of 0.99 or higher (Table 4. 4) and detection limit of 10 cells L^{-1} for all species tested.

The efficiency of the standard curve developed from *A. pacificum* cells strain CS300 was 90.25%, which was within the acceptable range. The quantification cycles obtained from the Internal Amplification Control (IAC) were ~32, with less than 1 unit difference to the negative control. The pipeline could detect about 83 cells L^{-1}.

Overall, quantification of *Alexandrium* cells with light microscopy and qPCR were closely correlated ($R^2 = 0.7318$, Figure 4. 4). The correlation between light microscopy and the quantification cycle was stronger at high cell densities compared to low cell densities. The correlation between quantification cycle of DinoDtec and SYBR-based qPCR were strong across the cell concentrations ($R^2 = 0.9659$, Figure 4. 5).

Table 4. 3. Specificity Test of Phytoxigene™ DinoDtec

Species Name	Strain	DinoDtec Response
A. affine	CCMP112	−
A. concavum	CAWD52	−
A. leii	CCMP2933	−
A. margalefi	CS322	−
A. fraterculus	CAWD97	−
A. pseudogonyolax	CAWD54	−
A. pacificum	CS300	+
A. catenella	TRIA-E	+
A. minutum	CS324	+

+, Target sequence amplified;−, target sequence not amplified

Table 4. 4. Standard Curves Efficiency of Phytoxigene™ DinoDtec

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

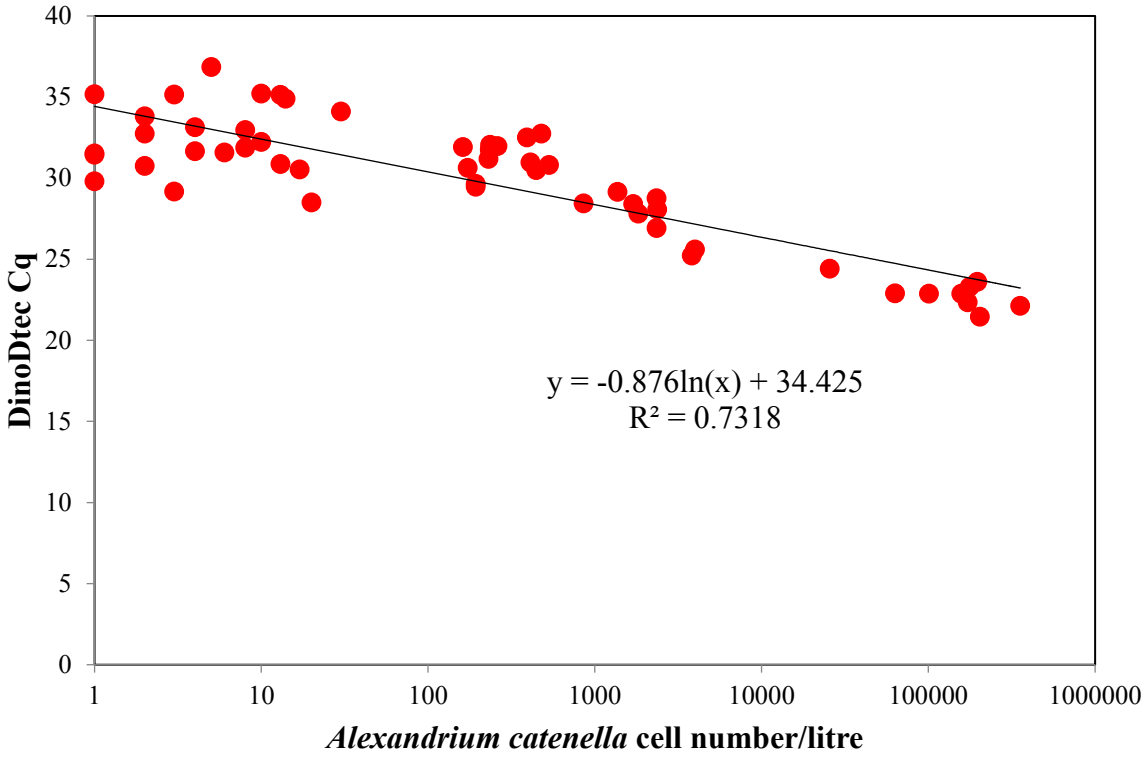


Figure 4. 4. Relationship between cell quantification using DinoDtec assay and light microscopy for bloom samples from Salt Pond, Gulf of Maine.

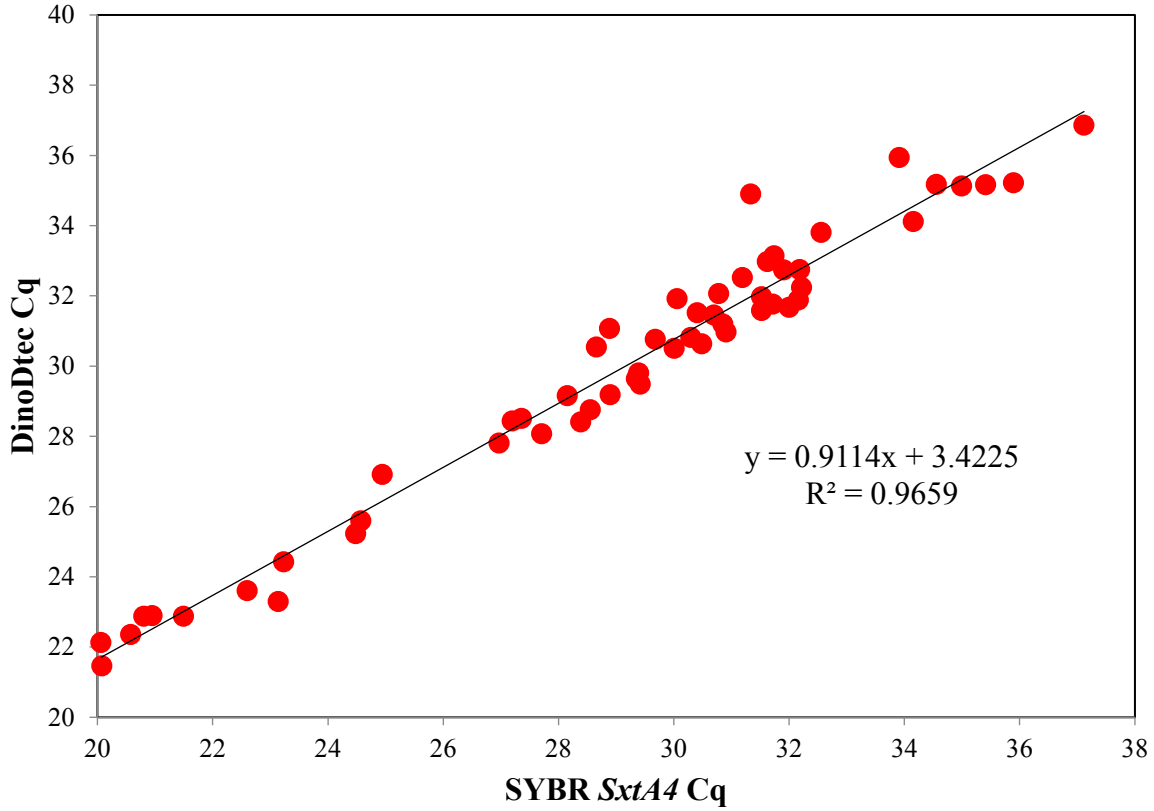


Figure 4. 5. Quantification cycle (Cq) correlation between Phytoxigene™ DinoDtec and SYBR-based assay of the bloom samples from Salt Pond, Gulf of Maine.

4.5. Discussion

In this thesis, a commercialised qPCR assay has been tested to detect the toxin gene responsible for PSP-associated bloom by targeting the *sxtA4* domain of saxitoxin biosynthetic pathway (109). The assay exists in a lyophilised form, enabling long-term storage at room temperature, making transport to the field easier. It contains an enzyme, probe, primers, dNTP and Internal Amplification Control (IAC) target. Each vial can be rehydrated with 80 µL DNA and RNA-free water and can accommodate four tests.

The first stage of our pipeline is the sample collection step. For environmental sample collection, filtration has been the preferred method to concentrate the phytoplankton cells compare to centrifugation. The sampler is gravity operated, allowing it to be used in the field without the need for an electricity supply (as is the case with a peristaltic pump). The sampler has been designed to accommodate 3L seawater, which is a greater volume than that used in current phytoplankton monitoring protocols (180, 181). Using a larger volume allowed us to

improve the sensitivity of the pipeline. Once the 11 μm mesh was back flushed with 50 mL of seawater, the samples can be concentrated onto an 8 μm nitrocellulose filter via syringe filtration and lysed for qPCR, or counted manually using light microscopy.

We tested the filter at low to mid-range cell densities (100-10,000 cells), as this is the likely situation to be encountered in the field when a bloom is beginning to develop. In this scenario, the recovery efficiencies of the sampler were higher than 90% for all measurements. The loss of cells may be caused by attachment of cells to the pre-filter membrane. Additionally, we performed cell counts using a Sedgewick-Rafter counting chamber, and thus it is important to consider inherent instrument error associated with this method (e.g. see 154, 185).

Sample preservation is necessary in the event that a qPCR instrument is not available for immediate analysis upon sample collection. Over a large sampling area that may require a lengthy sampling period, qPCR analysis simply cannot be performed that same day. In this event, there are several methods and reagents available to fix samples for downstream analyses, however cryopreservation is widely-considered the optimal approach for DNA preservation (189). However, transporting samples under liquid nitrogen can be expensive, and carries additional safety concerns such as e.g. the risk of skin burns upon exposure. Furthermore, freezing and thawing can affect DNA integrity (190), thus inert preservative buffers should be the primary choice for sample filter preservation.

RNALater and Lugol's iodine solution are two of the most commonly-used preservative buffers (191, 192). Previous studies have made conflicting statements about the inhibitory effects of Lugol's on PCR reaction (102, 193). Furthermore, cell degradation over time is also a known issue with the application of Lugol's for cell preservation purposes (191). Our test indicated that use of neutral Lugol's caused inhibition to the qPCR reaction, demonstrated by the higher Cq compared to the control. RNALater is approved for storing tissue samples for up to four weeks at 2-8° C, 7 days at 15-25° C or 1 day at 37° C (194). A previous study also suggested minimum PCR inhibition on cell samples stored with RNALater for up to 5 months at 4° C (192). This contradicts the findings of our study, suggesting that the inhibition is greater using RNALater compared to the two other reagents used.

Longmire's buffer is a lysis buffer formulated initially to preserve samples from mammalian tissue (184, 189, 195). However, recent studies have suggested that it can be used to preserve

filtered freshwater eDNA samples for 14 days (196) or even 150 days (197) without refrigeration. The use of Longmire's buffer for the preservation of microbial eDNA also appears to inhibit downstream DNA analysis the least (198). Such results are consistent with our findings, collectively indicating that Longmire's buffer may be the optimal buffer for preservation of phytoplankton samples.

However, all of the tested reagents, including Longmire's, appeared to inhibit the qPCR reactions for the samples processed by the BioGX lysis tube. Components of Longmire's buffer, such as SDS, denatures proteins, including DNA polymerase. Thus, it is advisable to use the lysis tube method only for in-field application of qPCR, immediately after cell lysis. Improvements can, however, be made by applying a cleanup process before qPCR reaction preparation.

The next stage of the pipeline is the *sxtA*-based assay - the sensitivity and specificity of which have been determined in this thesis. The DinoDtec assay is specific to the toxic species of *Alexandrium* (

Table 4. 3), so the risk of false positive using this approach is very low. In a phytoplankton monitoring scenario, the impact of false positives could be significant, as they could trigger unnecessary closure and delay of shellfish harvesting. For studies of bloom dynamics, this could result in the incorrect identification of physical parameters underlying a given toxic bloom's development.

The DinoDtec assay is also highly-sensitive when used for samples extracted with FastDNA Spin kit for soil (MP Biomedicals). It can detect *A. catenella* cells at a concentration of 1 cell L^{-1} (Figure 4. 4), with a high coefficient of determination (Pearson $R^2 = 0.9659$), compared to SYBR-based analysis. From chapter 3, the *sxtA4* copy number is higher in *A. catenella* strains in comparison to other toxic strains of *A. pacificum*, *A. australiense*, and *A. minutum*. Previous studies have also suggested that the *sxtA4* copy number is far lower in *A. ostenfeldii* in comparison with *A. catenella* (107). Thus, it is expected that the sensitivity of the DinoDtec assay will be lower with these particular species. However, our findings from chapter 3 indicate that *A. catenella* has the highest synthesised total PSTs $cell^{-1}$ amongst the toxic species, meaning that despite the limit of detection being higher for other species, the measured amount of PSTs in shellfish due to *A. minutum* is also going to be lower.

A widely-used method to test the efficiency of a qPCR assay is through measurement of slope of a standard curve (199). Through this approach, we showed that the DinoDtec assay is highly-efficient (Table 4. 4) when tested across different species of toxic *Alexandrium*, similar to the previous findings (42). The efficiency varied across strains of *A. pacificum*, but all were still within the acceptable limits (90-110%). It is clear from Chapter 3 that *A. pacificum* exhibits higher intraspecific variability on *sxtA4* gene copy numbers compared to other *Alexandrium* species tested. Pipetting error for samples with lower copy number might be higher in comparison to strains with higher copy numbers. The high amplification efficiencies also showed that 60°C is a suitable annealing temperature for the DinoDtec assay.

One of the key challenges applying qPCR analysis to environmental samples is the removal of substances that could potentially inhibit amplification reactions. The range of non-organic or organic substances capable of inhibiting the enzymatic reactions in PCR have been well-described (186). While shellfish farms are usually located in areas of relatively clean water, substances such as polysaccharides (186) from the phytoplankton cell structures and traces of humic substances are known to inhibit PCR reactions (146). Polysaccharides can mimic the

structures of nucleic acids, while humic substances can inhibit the enzymatic activity of DNA polymerase and bind to the template (187). There is also evidence that humic acid can act to quench fluorescence dyes such as SYBR, yet probe-based assay such as DinoDtec appear to be unaffected by this issue (188). Samples from oyster farms, which are typically located in shallow estuaries, may prove more prone to contamination by humic substances from the sediments compared to mussel farms.

In a well-equipped molecular laboratory, various DNA extraction protocols can be applied to remove inhibitory compounds from samples (186, 187). However, this is not the case when DinoDtec is utilised on the farm. The use of the lysis tube, such as BioGX Lysis tube combined with centrifugation can therefore offer an alternative method. In this protocol, we lysed the cells and spun the tubes using a microcentrifuge to precipitate cell debris. We then used the cell lysate for the qPCR reaction. The IAC allows the monitoring of inhibitors' effect on qPCR amplification. The overwhelming presence of the inhibitors will result in increasing Cq or inhibit the reaction of the IAC entirely. In a case when the IAC is inhibited, serial dilution must be carried out to reduce the concentration of inhibitors.

The standard curve we developed from a sample processed by the lysis tube showed that lysis tube can be used as an alternative to DNA extraction protocols. Without the clean-up process, the Cq for the IACs were stable, showing that there was very minor inhibition. Nonetheless, reduced efficiency could be observed when comparing the efficiency of qPCR reactions with template DNA from strains using DNA extraction vs the cell lysate from BioGX lysis tube. Even though reduced efficiency was expected due to the absence of the DNA clean-up process, the percentage efficiency was still within acceptable range. The sensitivity also appeared to be lower, with a higher limit of detection observed in the lysis tube standard curve. Despite the inherent limitations, the pipeline can still detect the cells at a concentration of 83 cells L^{-1}, which importantly is representative of pre-bloom concentrations.

With the mesh size of 11µm, the Phytoxigene™ sampler could concentrate not just PSP associated microalgae, but also species responsible for diarrhetic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP). With the availability of assays specific to *Pseudo-nitzchia* (200, 201) (202) and *Dinophysis* spp, (203) it may also be possible to monitor the presence of these species in the future. Further study on the clean-up of cell lysate from lysis tubes and long-term archiving of eDNA samples obtained from lysis tubes is necessary to confirm this result, and further studies on diversity can be undertaken at a later date.

In conclusion, we have established a working pipeline for the detection and quantification of PST-producing dinoflagellates that is applicable for use in the field. The recovery efficiency of the sampler is high, and the DinoDtec assay is sensitive, specific and efficient. Lysis tube can replace DNA extraction kits for a simple sample processing in the field. Moreover, Longmire's buffer can be used to preserve the sample filter prior to DNA extraction step in the lab without inhibiting qPCR reaction.

Chapter 5: The development of an on-farm predictive risk management tool for PST-producing dinoflagellates in Tasmanian waters.

5.1. Abstract

The species *Alexandrium catenella* (Group 1 genotype), producing Paralytic Shellfish Toxins, has been present annually in high abundances in shellfish aquaculture intensive areas of the east coast of Tasmania since 2012. The species *Gymnodinium catenatum*, *Alexandrium pacificum*, and *Alexandrium australiense*, which can also produce Paralytic Shellfish Toxins also occur in this area. High abundances of *Alexandrium catenella* are a significant threat to the shellfish industry, and may also have extended impacts upon other industries such as tourism. An effective early-warning system to indicate when *A. catenella* abundance is increasing would therefore provide valuable additional time in which to make management decisions. An in-farm molecular genetic-based assay was developed and used to monitor the presence of PST-producing dinoflagellates. This has allowed a mussel (*Mytilus galloprovincialis*) producer in Tasmania to detect the presence of PST-producing dinoflagellate species while still in relatively low abundance, before paralytic shellfish toxin detection in shellfish. Multiple Pearson correlations have been used to infer the temporal relationship between the level of PST-associated gene in the seawater and the Paralytic Shellfish Toxin accumulation and depuration rate in *Mytilus galloprovincialis*. A spatial survey during an *A. catenella* bloom along the east coast Tasmania showed the irregular surface distribution and cell density stratification across depth, with certain implications on future monitoring strategies.

5.2. Introduction

The accumulation of Paralytic Shellfish Toxins (PSTs) in farmed or wild shellfish has caused significant financial and public health problems worldwide, including the shellfish harvesting-intensive area in the east coast of Tasmania, Australia (48, 118, 119). Before

2012, dinoflagellate blooms in Tasmania were characterised by the moderately toxic species *Gymnodinium catenatum* and *Alexandrium pacificum* (27, 28, 118, 204). The unexpected occurrence of the PST-producing species, *Alexandrium catenella* (previously called *A. fundyense* or *Alexandrium tamarense* group 1) in 2012, directly impacted the mussel and oyster industry, and resulted in A$23 million loss to the Tasmanian local economy (19). Key elements contributing to this loss of revenue were the failure of phytoplankton monitoring efforts to provide results in real-time, and failure to detect whether a PST-producing species of *Alexandrium* was present (19). Since 2012, *A. catenella* has been appearing with increasing intensity (47). Moreover, the more recent identification of PST in non-conventional vectors such as crab and lobster viscera indicate that this issue may be spreading to other industries in Tasmania (47, 205). While 2013 and 2014 were marked with low intensity blooms (47), 2015-2018 were marked with increasing bloom intensity and toxicity. In 2015, 4 people were hospitalised due to Paralytic Shellfish Poisoning (PSP) from wild catch oyster (47, 48, 206). In 2017, the highest concentration of PST ever recorded in Tasmania (150 mg kg^{-1}) was found in a mussel sample from Bicheno, which was > 150 times the regulatory level of 0.8 mg kg^{-1}(177).

The severe impact of this PST-producing bloom highlights the importance of a monitoring program that allows for the accurate and rapid identification of all PST-producing species, thus providing enough response time for farmers to implement management strategies. In particular, in a region in which multiple PST producing species can occur, including *G. catenatum* and several *Alexandrium* species, a method that can quickly detect all species is important. Previous studies have established the efficacy of detection tools using a molecular approach to identify PST-producing species, which is based on the detection of *sxtA4* gene (42, 108, 110). An on-farm detection platform can allow for the results to be obtained within hours of water sample collection, rather than days, which is the time required when shipping to an off-site laboratory for analysis.

Mathematical models have been developed that incorporate the fluctuation of toxic cell numbers over time, along with nutrient and physical data. These models can be utilised to observe the relationship between nutrient availability (207, 208) and physical factors (209, 210) on the growth of PST-associated species and bloom development. Measurement of PST accumulation and depuration has also been developed in a lab culture setting (97, 211). However, a model linking the abundance of *Alexandrium* spp. with PST accumulation and depuration *in situ* has not been developed. The integration of the data on *in situ sxtA4* copies

in the water column with information on PST accumulation and depuration in shellfish can be used to characterise the temporal relationship between these variables.

In chapter 4, I developed and assessed the efficacy of a commercialised pipeline, Phytoxigene™ DinoDtec based on the gene *sxA4,* which is the first gene in the biosynthesis pathway of PST, has been carried out. However, the use of this pipeline for the early detection and spatial characterisation has not been shown *in situ*. In this study, almost 2 years of weekly data collection from a commercial mussel (*Mytilus galloprovincialis*) farm were used to determine the temporal relationship between *sxtA4* and PST accumulation. We also used the DinoDtec pipeline validated in the previous chapter to look at the spatial and cross-depth profile of *A. catenella* cells in the middle of 2016 bloom along the east coast of Tasmania, Australia.

5.3. Materials and Methods

5.3.1. Spatial and cross-depth characterisation of 2016 *Alexandrium catenella* bloom

5.3.1.1. Sample collection

Seawater samples were collected during the peak of an *Alexandrium* bloom between 26-28 August 2016 onboard the RV Southern Cross. Samples were collected along inshore-offshore transects on the east coast of Tasmania (Table 5. 1). A ten-litre Niskin bottle was used to collect seawater from multiple depths at 18 stations. Water samples were collected from the surface only in Blackman Bay sites (BB1 and BB2) and six depths in Marion Beach (MB1) site (0 m, 2 m, 5 m, 7 m, 10 m, 15 m). In three Coles Bay estuarine stations (CB3-CB5), seawater was collected from the surface and three meters depth. At station CB1, samples were collected from 0 m, 2 m, 5 m, and 7 m, whereas only surface water was collected from CB2 station. Samples were also collected from five stations around Spring Bay (SB1-SB5). In three of these stations (SB1-SB3), seawater was collected from seven depths (0 m, 2 m, 5 m, 7 m, 10 m, 15 m, and 30 m). In the shallower SB4 and SB5 stations, samples could only be collected from six depths (0 m, 2 m, 5 m, 7 m, 10 m, 15 m) and five depths ((0 m, 2 m, 5 m, 7 m, 10 m) respectively. In the estuary of Little Swanport, surface water was collected in three sites (OB3-5), whereas it was deep enough to collect samples from the surface, 2 m and 5 m depths at station OB2. The station OB1 was the only oceanic station in which seawater was collected from the surface, 2 m, 5 m, 7 m, 10 m, 15 m, and 30 m. Temperature, salinity,

and pH measurement across depth in all stations were collected with a Seabird SBE 19 PlusV2 CTD (Sea-Bird Scientific, Washington, USA).

Table 5. 1. Co-ordinates of Southern Cross RV Stations

Site	latitude	longitude
BB1	-42.877	147.863
BB2	-42.843	147.884
MB1	-42.836	147.91
SB1	-42.598	148.235
SB2	-42.545	148.13
SB3	-42.558	148.034
SB4	-42.566	147.925
SB5	-42.549	147.925
OB1	-42.318	148.113
OB2	-42.307	148.006
OB3	-42.318	147.984
OB4	-42.315	147.984
OB5	-42.336	147.976
CB1	-42.114	148.24
CB2	-42.08	148.163
CB3	-42.079	148.183
CB4	-42.081	148.206
CB5	-42.093	148.238

Three L of collected seawater from all the stations were filtered using a gravity-based system Phytoxigene™ Portable Sampler (Figure 5. 1). The seawater was poured from the top until the chamber was full. The valve was then released to allow the seawater to flow through the 10 µm mesh. The flow-through seawater was collected with a squeeze bottle, and the cells embedded in 10 µm filter were back flushed with 50 mL of filtered seawater. The cell concentrate was collected into 50 mL centrifuge tubes. After that, the tubes were stored in a closed portable cooler in a shaded, cool location throughout the day trip. Once we reached land, the concentrates were syringe-filtered onto 25 mm 8 µm nitrocellulose filters (MerckMillipore), and the filters were dried by pumping air through the filter two times. The filters were transferred to 2 mL cryogenic tubes, and 1mL of Longmire's buffer (184) was added to each tube. Samples were stored at room temperature until extraction.

5.3.1.2. Alexandrium cell count

Lugol's iodine-preserved samples were settled until all cells sank onto the bottom of the 1L bottle. The overlying liquid was then discarded until only 10 mL remained. The cells and the liquid were mixed by stirring, and 1 mL was loaded into a Sedgewick-Rafter chamber. The concentrated sample was counted under an inverted light microscope (Leica, Wetzlar, Germany).

Figure 5. 1. The Phytoxigene™ DinoDtec pipeline filtration system (left), and on-site qPCR set up (right)

5.3.1.3. DNA Extraction

The samples from RV Southern Cross RV were extracted using the Powersoil® DNA Isolation kit (Qiagen). For the bead beating step, nitrocellulose membrane filters with embedded phytoplankton cells were gently folded or rolled and placed into bead lysis tubes from the kit, with the filtrate side facing inwards into the tube. The tubes were placed into Vortex adapter for 24 (1.5 – 2.0 mL) tubes (Qiagen, Venlo, Netherlands) attached to Vortex-Genie® 2 (Scientific Industries, New York, USA). The vortex was run at maximum speed for 10 min. The remaining protocol was conducted according to the manufacturer's suggestions.

5.3.1.4. Toxin gene quantification with qPCR in seawater column

DinoDtec kit (Diagnostic Technology, Australia), developed based on primers targeting the *sxtA4* gene (109), was used to quantify PST-related genes in seawater samples. The mix was rehydrated with 80 µL of nuclease-free water, and a 20 µL aliquot was mixed with 2 µL DNA sample and 3 µL PCR grade water to a total of 25 µL per qPCR reaction. Fluorescence signals from the probe were quantified in the FAM channel and the internal control was measured by Cy5 channel. The reaction was run with temperature settings starting at 95°C for 2 min, followed by 45 cycles of 95°C for 10 s and 64° C for 45 s on MyGoMini portable thermocycler platform (IT-IS LifeScience, Cork, Republic of Ireland). The Phytoxigene™ DinoNAS standards were used to develop the standard curve for *sxtA4* copy number quantification. Phytoxigene™ DinoNAS is a DNA standard for the DinoDtec kit developed by the National Measurement Institute (NMI).

5.3.2. Predictive model

5.3.2.1. Sample collection and qPCR analysis

Approximately 10 L of seawater was collected at 5 m depth from Spring Bay Seafoods lease (42.59, 147.97) every Sunday starting on the 22nd May 2016 until the 30th October 2017. The lease is located at the same site as SB3 station (Table 5.1). A peristaltic pump was used to collect and transfer the seawater into a clean plastic jerry can. Seawater was then transported to the hatchery, where subsequent filtration was conducted.

The Phytoxigene™ Portable Sampler was used to concentrate phytoplankton samples as performed during the Southern Cross trip. However, the syringe filtration was carried out immediately after the cells were back-flushed with 50 mL filtered seawater. The nitrocellulose filter was inserted into a BioGX Lysis tube, and the lysis tube was attached Vortex adapter for 24 (1.5 – 2.0 mL) tubes (Qiagen, Venlo, Netherlands) attached to Vortex-Genie® 2 (Scientific Industries, New York, USA) Vortex. The vortex was run at maximum speed for 10 min. Lysis tubes were then centrifuged at 1000g for 1 min to precipitate the cell debris. DinoDtec mix was prepared the same way as previously described. Twenty µL of DinoDtec mix and 5 µL of cell lysate were transferred into a qPCR tube and run with the same temperature as previously along with the negative control (5 µL of PCR grade water to replace the sample) tube and Phytoxigene™ DinoNAS standard representing 5, 000 copies of *sxtA4*.

5.3.2.2. Measurement of PST concentration in flesh samples

PST content in mussel and oyster flesh was measured weekly by the Tasmanian Shellfish Quality Assurance Program (TSQAP). High-Performance Liquid Chromatography (HPLC) was used according to Lawrence method (179, 212).

5.3.2.3. Numerical procedure and parameter setting

The *sxtA4* copy numbers quantified from seawater column were compared to the total PST concentration in the mussel. The results between the 5th December 2016 until the 13th June 2017 are not included as the PST or *sxtA4* were not detected. Pearson correlations were established between the *sxtA4* copy number in seawater column and the PST concentration in the mussel tissue at 1, 2, and 3weeks lag times as well as correlation on the same day from all data from both 2016 and 2017.

5.4. Results

During the 2016 *Alexandrium* bloom (Figure 5.2), the number of *sxtA4* copies began increasing at Spring Bay on 3rd July 2016. Although the copy number then decreased to almost zero on 17 July 2016, it increased again in the following week. The PST levels in mussel started to increase on the week starting on 31st July 2016, which is about four weeks after the first *sxtA4* copies were observed. The PST concentration then increased until the week starting on 25th September 2016, after which it continuously decreased (Figure5.2).

In 2017, the rapid increase *sxtA4* copies L^{-1} number started on the week of the 21st August 2017, and the increase continued until 2nd October 2017 (Figure 5. 3). PST concentrations began to elevate during the week commencing on the 5th September 2017. The PST concentration reached its peak on the 10th October 2017 (140 mg kg^{-1}), after it which it gradually declined. This was correlated to decreasing numbers of *sxtA4* copies during this time. As cell numbers were extremely high on the week starting on 10th October 2017 and 17th October 2017, Spring Bay Seafoods was not operational and sampling was suspended. Hence, no qPCR data was collected.

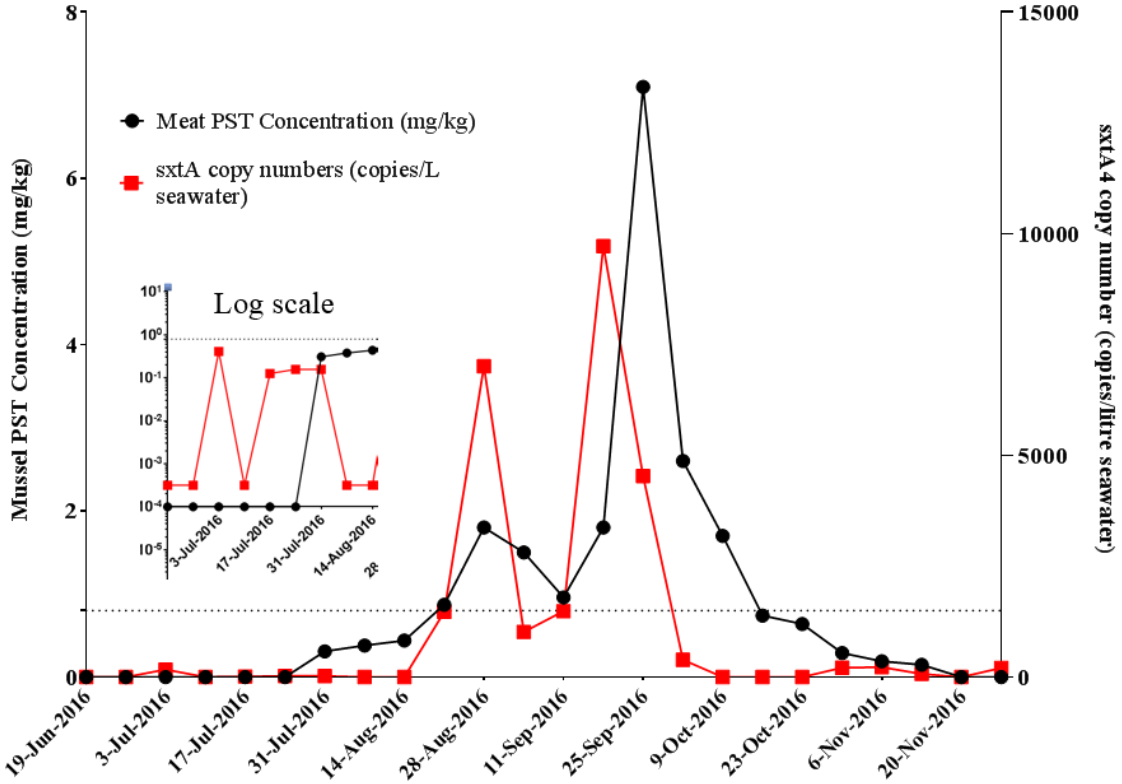


Figure 5. 2. The copy number of *sxtA4* L^{-1} of seawater and the total concentration of PST in mussel tissue during the 2016 *Alexandrium* bloom from a mussel farm SpringBay Seafoods lease. The inset shows the log scale of the graph showing detection of the cell before toxin accumulation occurred in the mussels.

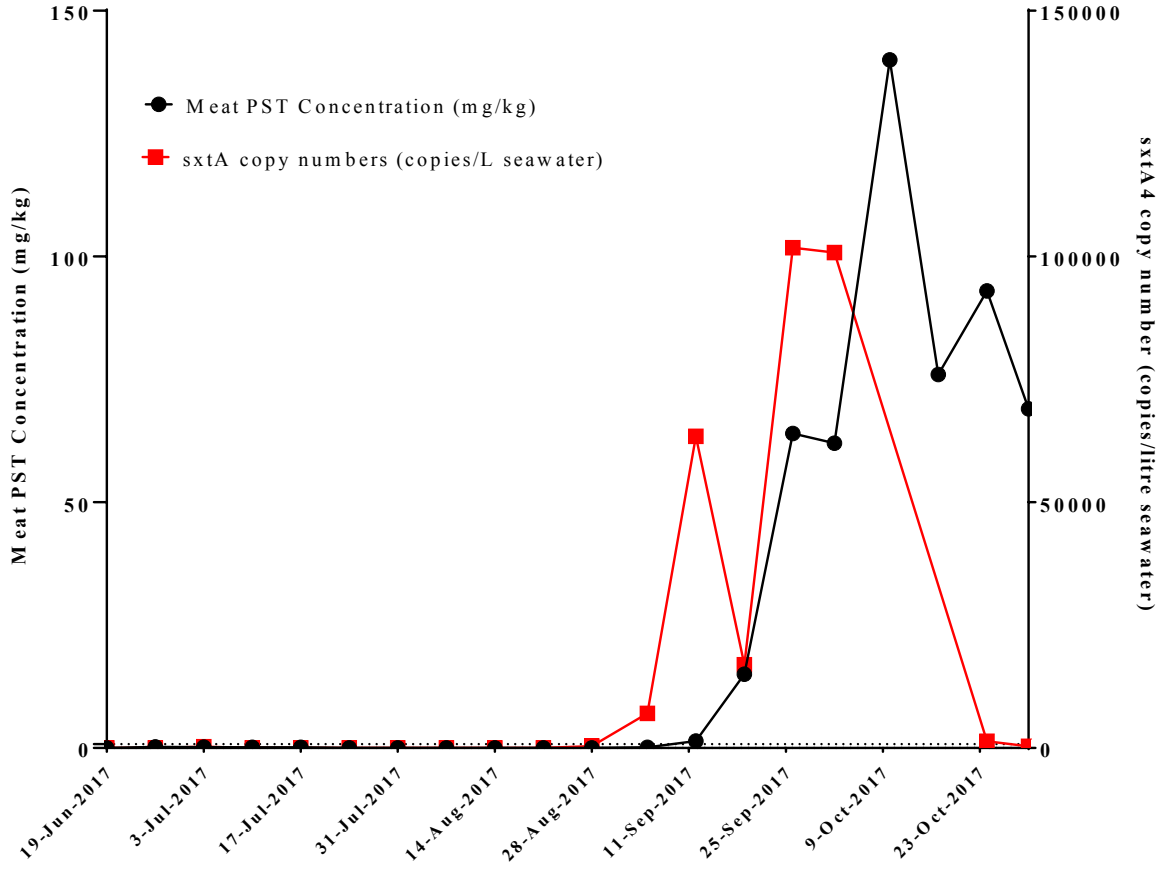


Figure 5. 3. The dynamics of *sxtA4* copy number in seawater column and the concentration of PST in mussel meat starting from 24 April 2017 to 30 October 2017.

Table 5. 2. Pearson Correlation between *sxtA4* Copy Numbers and PST in Mussel

Lag time (weeks)	Pearson r
-3	0.701
-2	0.945
-1	0.778
0	0.538

The results of the Pearson correlations between *sxtA4* copies L^{-1} in the water column and the PST concentration in mussel tissue at 4 different time points showed that the strongest correlation occurred with a 2 week lag time (Table 5.2). The weakest correlation occurred with no lag time.

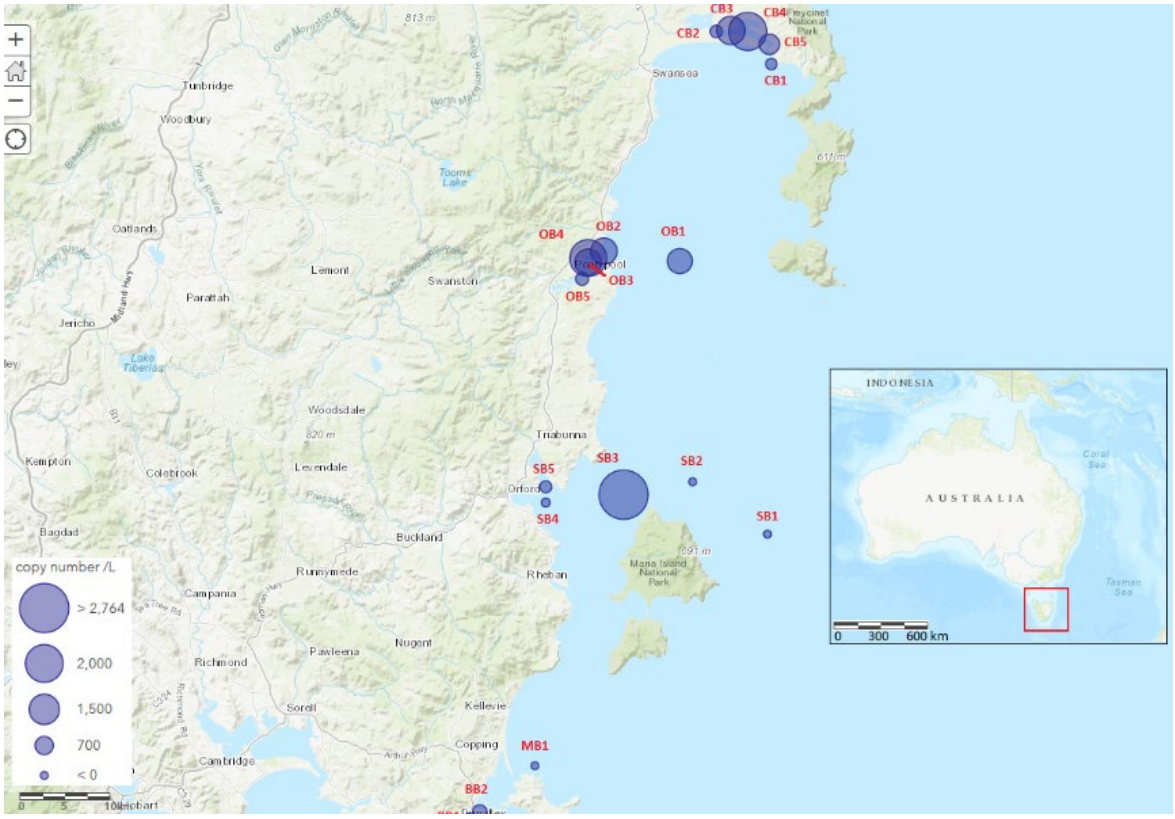
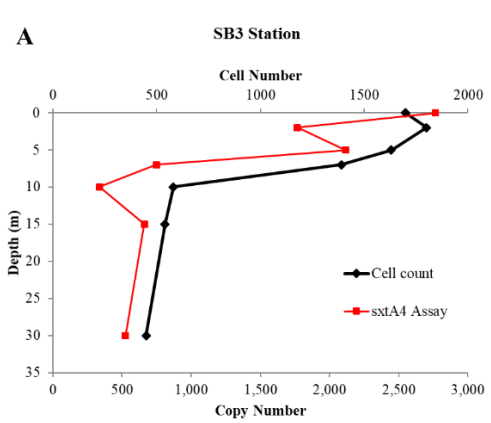


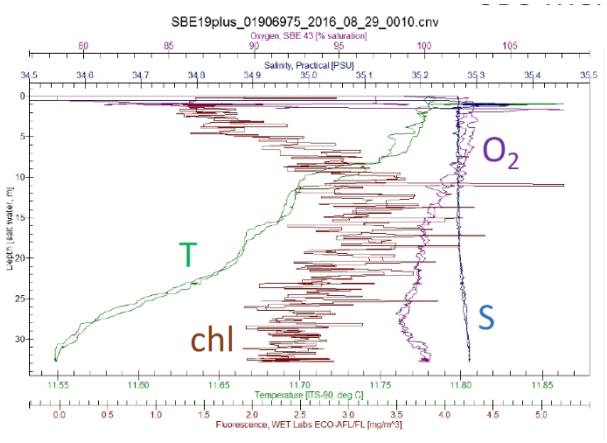
Figure 5. 4. The sampling stations of the RV Southern Cross survey along the east coast of Tasmania, Australia, with the concentration of surface-*sxtA4* copy numbers detected at each site during the peak (26-28 August) of *Alexandrium* bloom in 2016.

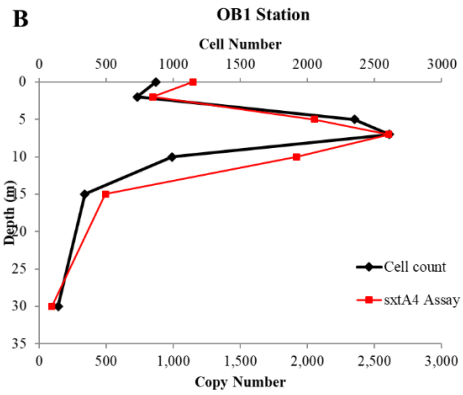
The copies of *sxtA4* detected on the surface in Coles Bay1-5 (CB1-CB5) stations were high, except for site CB1 (306 copies L^{-1}), located in the outermost side of the bay, and CB2 (238 copies L^{-1}), in the innermost side of the bay (Figure 5. 4). More than 1000 copies L^{-1} of *sxtA4* sequences were detected from the other surface samples from this transect (1368 copies L^{-1} for CB3, 2017 copies L^{-1} for CB4, 1090 copies L^{-1} for CB5). In the Oyster Bay (OB) transect (OB1-OB5 stations), the lowest *sxtA4* copy was detected in the inshore OB5 station (337 copies L^{-1}). However, a relatively high number of copies of *sxtA4* were detected in the innermost site OB3 (1232 copies L^{-1}). The highest number of *sxtA4* copies identified in this transect were from OB4 station (1939 copies L^{-1}). The number of *sxtA4* copies in the outermost station of this transect (OB1) was 1147 copies L^{-1}, whereas OB2 was 1242 copies L^{-1}. In Spring Bay transect (SB1-SB5), there were relatively lower copies of *sxtA4* quantified in SB4 (85 copies L^{-1}) and SB5 (277 copies L^{-1}) stations, and no *sxtA4* was detected in

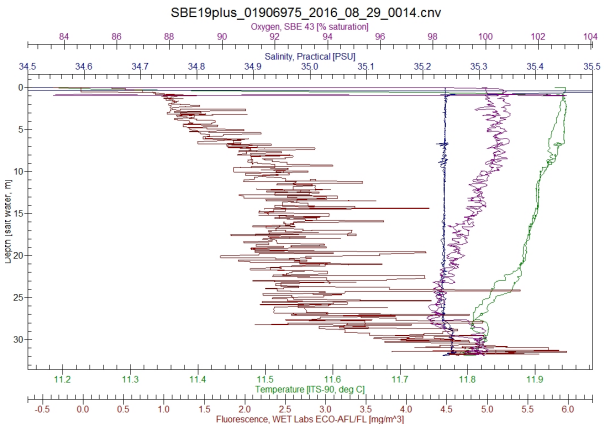
offshore stations of SB1 and SB2. The highest number of *sxtA4* identified in this transect was found at the SB3 station, which was 2764 copies L^{-1}. In the Blackman Bay area, *sxtA4* could only be detected in BB2 station at a low amount (452 copies L^{-1}). In other stations, BB1 and MB1*, sxtA4* sequence was not found (Figure 5. 4).

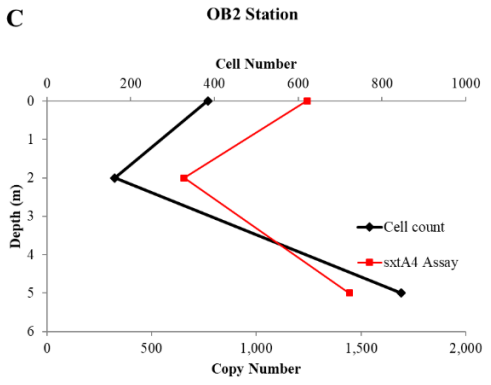
At the deeper (>30m) stations of SB 3 (Figure 5. 5A) and OB 1 (Figure 5. 5B) a stratification of *A. catenella* abundances was noticeable for the cell density compared to depth. However, there were only negligible differences in physical parameters across depth at both sites. Most cells were detected at a depth of 5-10 m. These patterns were consistent with both qPCR and light microscopy results. The cell numbers on both stations appear to decrease at a depth below 10 m. In station at shallower depths (<10 m) (Figure 5. 5C-D), the highest number of cells were found at a depth of around 5 m. In estuarine sample stations (<5m) (Figure 5. 5E-G), the *Alexandrium* cell numbers did not vary greatly between the surface and down to 3m. Furthermore, there appeared to be no consistent cell distribution pattern across estuarine sample stations. One site, CB1, showed a difference in abundance estimates between methods (Figure 5. 5D).

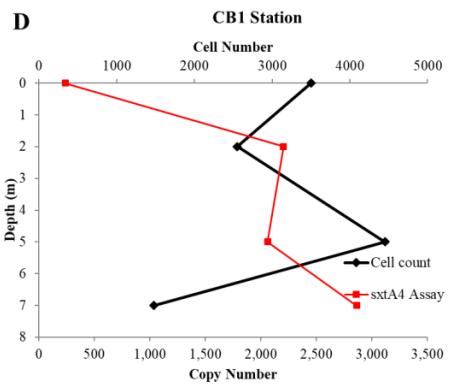

A
SB3 Station
Cell Number
Depth (m)
Cell count
sxtA4 Assay
Copy Number


SBE19plus_01906975_2016_08_29_0010.cnv
Oxygen, SBE 43 [% saturation]
Salinity, Practical [PSU]
Depth [salt water, m]
O2
T
chl
S
Temperature [ITS-90, deg C]
Fluorescence, WET Labs ECO-AFL/FL [mg/m^3]


B
OB1 Station
Cell Number
Depth (m)
Cell count
sxtA4 Assay
Copy Number


SBE19plus_01906975_2016_08_29_0014.cnv
Oxygen, SBE 43 [% saturation]
Salinity, Practical [PSU]
Depth [salt water, m]
Temperature [ITS-90, deg C]
Fluorescence, WET Labs ECO-AFL/FL [mg/m^3]


C
OB2 Station
Cell Number
Depth (m)
Cell count
sxtA4 Assay
Copy Number


D
CB1 Station
Cell Number
Depth (m)
Cell count
sxtA4 Assay
Copy Number

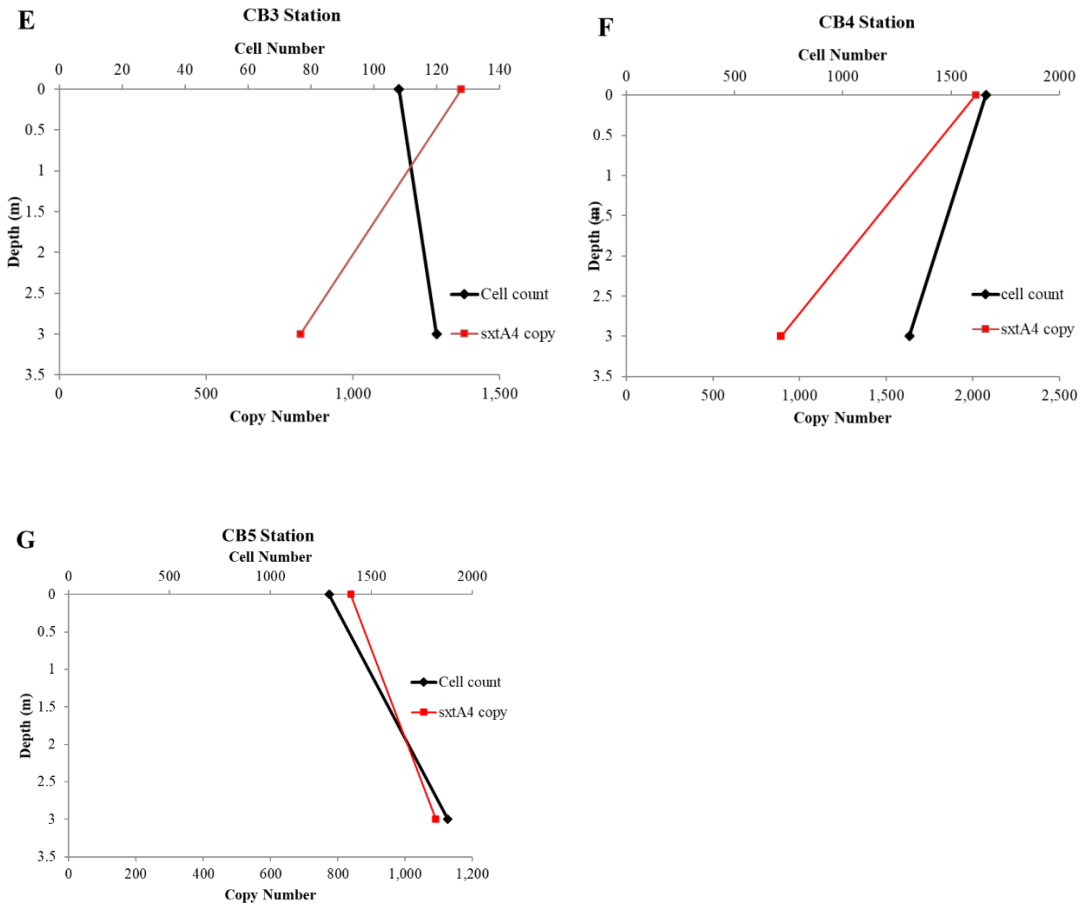


Figure 5. 5. Cross-depth quantification of *Alexandrium* cells counted using light microscopy and DinoDtec *sxtA4*-based qPCR. SB3 and OB1 stations are the oceanic stations, and OB2, CB1, and CB3-5 were the estuarine sites. CTD data are shown for the oceanic stations of SB3 and OB1.

5.5. Discussion

Assays based on the *sxtA4* sequence have been successfully utilised as an indicator of shellfish toxicity in oyster (149). In this study, we have used qPCR assay targeting the same gene (42, 70), as an early warning indicator of the presence of PST-producing species on a mussel farm in Spring Bay, Tasmania. In a study of ~18 months of weekly sample analysis at the mussel farm, covering two annual *A. catenella* blooms, a lag period of ~2 weeks showed the highest correlation between *sxtA4* L^{-1} and PST mg kg^{-1} in mussel tissue (Figure 5. 2, Figure 5. 3, Table 5.2). However, at a certain condition, PST accumulated in *M. edulis* could exceed the regulatory level within 1 h (215). A Pearson correlation with a lower r value was

also found when modelling the *sxtA4* copies L^{-1} and PST mg kg^{-1} in mussel tissue (Table 5.2), suggesting that up to 3 weeks' early warning may be possible.

Feeding studies related to PST in shellfish have found that uptake, PST biotransformation and depuration rates can differ greatly between shellfish species (213-216). In green-lipped mussel, the accumulation of PST exceeding the regulatory level was reached within 2 days of exposure with significant amount of toxins were removed within three weeks (214). Our result with *M. edulis* is consistent with the previous laboratory study, showing about two-week lag time between the addition of dinoflagellate cells to the water, and the accumulation of toxin in the mussel's organ (217). Australian abalone uptake PST at about ten times slower rate than mussel, but with comparable depuration rate (218). In scallops, the accumulation and depuration is much slower than mussel, and can take several months (215). In Pacific oyster (*Crassostrea gigas*), the PST level higher than regulatory limit can be reached in three weeks when the concentration of *A. minutum* is between 9 and 140 cell L^{-1} (219).

The rate of PST accumulation and depuration may also differ depending on factors such as the ploidy level of the shellfish (220) and the ambient water temperature (220). Depuration in *Mytilus galloprovincialis* is additionally influenced by size, age, soft tissue weight, and reproductive stage (221). For example, increased acidification and increased temperature could potentially cause lower maximum PST accumulation, but slower depuration in *Mytilus galloprovinciali* (222). Farmed mussels are grown from the same batch, which means that they have a similar size, age, and weight, potentially minimising differences among individuals.

Given the differences among species and related to environmental factors, it is likely that the optimal lag time established between *sxtA4* copies L^{-1} and PST mg kg^{-1} established in this study will be specific to the growth of mussels impacted by *A.catenella* during the Austral winter, which on the East Coast of Tasmania, is generally characterised by water temperatures of 10-15 ºC.

During 2016 and 2017, the detection of *sxtA4* copies L^{-1} was used to make decisions regarding the management of Spring Bay Seafoods, leading to an early voluntary harvesting closure before the farm received official notice from the Tasmanian Shellfish Quality Assurance Program that PSTs had been detected in shellfish and that therefore harvesting must cease. The company was then able to switch shellfish production to another production site that was not impacted by PSTs. As a result, production continued uninterrupted

throughout the period. Weekly tests during the *A. catenella* bloom period in 2016 and 2017 (Figure 5. 2 and Figure 5. 3) proved beneficial for the farmer as the fluctuation of the PST concentration in the meat coincided, albeit to a delayed extent, with the fluctuation of the detected *sxtA4* in the seawater column. The decline of the *sxtA4* copy number was an early indication of the decline of the bloom and the decrease of PST-content in the shellfish meat. The decline of *sxtA4* copies gave the farmer valuable insight, allowing them to prepare to continue the production in the affected area, about two weeks before the PST-level decreased to below the regulatory limit (0.8 mg kg^{-1}).

During 2016, the decrease of *sxtA4* copies L^{-1} occurred from the week starting on 25th September 2016 (Figure 5. 2), while PST mg kg-1 in mussel tissue was still above the regulatory limit. During this period, a spring diatom bloom occurred (data not shown). While weekly samples were adequate to inform management decisions and modelling, more frequent sampling may provide information on finer scale dynamic shifts in *A. catenella* and improve the resolution of the results.

To determine the best sampling site and the physical parameters underlying stratification, we examined the vertical distributions of cells in the middle of a bloom. The vertical profile of A. *catenella* cell abundance across depth indicated a cell distribution stratification pattern in oceanic stations during August 2016 (Figure 5. 4). The highest *A. catenella* cell abundance and *sxtA4* copies were observed at a depth of 5-10 metres below the sea level, decreasing in deeper samples. Such a vertical trend was also observed in bloom in the Gulf of Maine in 2001 (223), where the highest cell number was found at a thin layer depth of 10-11 metres. However, there were negligible fluctuations of measured physical parameters (salinity, pH, temperature, and Chl-α) from the surface to bottom, suggesting physical factors did not cause this stratification. It has been proposed that phytoplankton cells can synthesise Extracellular Polymeric Saccharide (EPS) to promote cell buoyancy (224), resulting in accumulation of viable cells near the seawater surface, promoting the uptake of sunlight and nutrients.

The vertical distribution trends imply that depths from 5-10 m is the ideal sampling point for cell quantification in oceanic stations. It is still unknown whether growing the mussel deeper will affect PST uptake by the mussels. Hence, further experimentation is needed to test the potential effectiveness of such mitigation steps.

The lack of stratification is evident across estuarine stations (Figure 5. 5 E-G). From a qPCR sample collection point of view, the surface water sample should represent the abundance of

the cell in a particular station. Deeper sampling depths nearer the surface of the estuarine may give poor results, as qPCR inhibitors such as humic acid may be collected from the bottom estuarine sediment. Such factors may also explain the irregularity between cell counts and *sxtA4* qPCR.

On the sampling day in August 2017, the spatial distribution of *sxtA4* copies L^{-1} was patchy (Figure 5. 4), and no pattern was observed between oceanic and estuarine stations. It is likely that factors such as upwelling and downwelling, rainfall, and current flow affected the spatial cell distribution (225, 226). As qPCR is relatively fast and cheap to implement, additional sampling stations and replicates can be used by phytoplankton monitoring agency, to increase accuracy and precision of mapping efforts.

In conclusion, in this study, a qPCR assay based on the gene *sxtA* was used to conduct weekly monitoring on a mussel farm in Tasmania, Australia. Based upon the lag between increases in cell numbers and subsequent accumulation of PST in mussels, this method provides 2 weeks early warning in this region during the Austral winter. Based on the stratification pattern of the cells across depth, 5-10 metres is optimal for sampling. Due to the patchy spatial cell abundance distribution, more sampling stations are required to provide better resolution.

Chapter 6. Outlook for qPCR-based quantification of PST-associated dinoflagellates

6.1. An in-farm qPCR pipeline for the monitoring and management of PSTs

Species identification based on the discrimination of morphological characteristics, using light microscopy, is the current standard phytoplankton monitoring method for the detection of potentially harmful microalgae species in shellfish harvesting areas in Australia, and internationally. However, discriminating cryptic species of the former *Alexandrium tamarense* species complex (40) is an impossible task with light microscopy, as their morphologies are very difficult to distinguish, and misidentification can result in unnecessary harvesting closure or a threat to public health. In 2012, due to a failure of marine harmful algal and biotoxin monitoring, Japanese Import Authorities detected a high level of PST in exported mussels from Tasmania and instigated a ban on importing any Australian shellfish for one year (19, 47). A review conducted subsequently indicated the failure to detect *A. catenella* in phytoplankton samples in a timely manner as one of the main causative factors (19).

Despite their overlapping or identical morphological features, the phenotypic characteristics of *A. catenella, A. australiense,* and *A. pacificum*, such as PST production, can be very different. An example shown in this thesis is the PST content per cell, which can be very high in *A. catenella*, and low or even zero in *A. australiense* (Chapter 3). These three species, along with another PST-producing species, *Gymnodinium catenatum*, co-occur in Tasmania, as they do in many other places worldwide (136, 138, 164). This means that more accurate methodology such as a molecular genetic detection is required for aquaculture monitoring. Detection of marine organisms using environmental DNA (eDNA) is becoming increasingly common and has begun to be used for commercial monitoring by the aquaculture industry (227).

In this thesis, I have assessed the specificity, sensitivity, efficiency, and accuracy of qPCR assays targeting the rRNA genes and the gene *sxtA4,* which encodes the synthesis of saxitoxins (STX). The *sxtA4* qPCR assay and all except one rRNA gene-based assays were found to be sensitive, specific, and efficient. However, the qPCR assays based on rDNA showed an overestimation of the absolute value of the *Alexandrium* species present, a problem that has been previously recognised (154). The quantification of the absolute number of genomic copies of rDNA and *sxtA4* showed variation among strains for both target genes. The variation for rDNA copies within the same species of *Alexandrium* was found to be up to 3-5 orders of magnitude, suggesting that estimates of the cell abundance could be over or underestimated by several orders of magnitude, if the number of genomic copies of rDNA in the natural population differed from that used in the strain used for the standard curve.

The number of genomic copies of *sxtA4* also varied, by up to an order of magnitude within the same species, which was significantly less than that observed among strains for rDNA copies. Therefore, quantification using the *sxtA4*-based qPCR assay was found to be much more likely to reflect the actual environmental abundance of cells. It is possible that the use of locally isolated strains of *Alexandrium* to develop a standard curve for the rDNA-based and *sxtA* based assays could improve the accuracy of the quantification. However, the validity of this has not been tested, as it is also possible that copy numbers of rDNA genes may vary within a population, and between years at the same site. The results of this study suggest that a standard validation step for the use of a qPCR assay should be to compare counts using multiple methods for a subset of the sample, in order to calculate the copy number of the target genes before applying a standard curve.

Using a qPCR assay targeting the gene *sxtA*, I describe a pipeline developed for use on farm by a commercial mussel farm. Using this pipeline, *sxtA* copies indicative of the presence of PST-producing species were found to be present two weeks before detectable PSTs were found in mussel tissue. Given that the PST analyses itself required several days, acquiring the information on *sxtA* presence and abundance two weeks previously allowed the aquaculture operator to make a significant management decision, in this case to voluntarily close harvesting and resume harvesting from another mussel area. The weekly *sxtA* qPCR analyses showed the continued abundance and decline in abundance of *A.catenella*, providing information used to determine when production could resume in the affected area.

6.2. Molecular mechanisms of saxitoxin biosynthesis

Gene regulation in dinoflagellates remains elusive. Studies to date have shown that the majority of genes appear to be regulated in unconventional ways, such as post-transcriptionally (57)**,** rather than transcriptionally. As a result, non-traditional approaches are required to understand gene regulation in dinoflagellates. In this study, I investigated the dosage effect in relation to copies of *sxtA4* in species of *Alexandrium* producing PSTs, and to analyse the implication of the variation in the genome size across several *Alexandrium* species. A significant positive correlation was found between the number of *sxtA4* copies $cell^{-1}$ and PSTs produced across the species of *Alexandrium*. Nonetheless, other factors not investigated in this study such as transcriptional and translational level and the genomic copy number of other genes in the PST biosynthesis pathway may influence the amount of synthesised PSTs per cell. The enumeration of copies of other genes in the PST biosynthesis pathway may allow for a broader perspective regarding the dosage effect across the species of *Alexandrium*. Combining the copy number data with proteomic and transcriptomic data may give a broader insight into the regulation of PSTs synthesis.

Very high variability in genome size was observed in the species of *Alexandrium* spp. examined in this study. We observed higher variability in the genome size of the species formerly described as *Alexandrium tamarense* species complex compared to *A. minutum*. The variability is exceptionally high in *A. pacificum*, which may indicate the case of polyploidy. Our study also confirmed previous findings regarding the positive correlation between the genome size and the rDNA copy number.

6.3. Future directions

With the emergence of small, portable qPCR platforms within the past 10 years, it is now becoming feasible to conduct qPCR in the field. qPCR is a flexible platform, which can be used to identify multiple target genes. The flexibility of qPCR can be beneficial for the aquaculture industry, as other toxins produced by marine microalgae such as okadaic acid or domoic acid, as well as certain species of bacteria (*Escherichia coli*, *Vibrio sp*) and viruses (norovirus, Pacific Oyster Mortality Syndrome (POMS)) can also affect the shellfish production line.

The integration of *sxtA* based qPCR assay pipeline into a phytoplankton monitoring program can be a possibility in the future. The availability of a liquid handling robot and the qPCR platforms that can accommodate a 384 well-plate can significantly enhance the sample processing capacity. In chapter 5, the spatial patchiness of the *Alexandrium* cells was shown. With the availability of methods with a high sample processing capacity, more spatial replicates can be collected and analysed to get a better spatial resolution.

Appendices

Appendix A. Strains used for genome size measurement and copy number quantification

Species	Strain	Origin	Isolator
A. pacificum	ACSHO2	Sydney Harbour, NSW, Australia	S. Murray
	CAWD44	Tauranga, New Zealand	L. Mackenzie/J. Adamson
	ACTRA02	Triabunna, Tasmania, Australia	C. Bolch
	CS798	Port River, Adelaide, South Australia	N. Parker
	CS315	Port Phillip Bay, Victoria, Australia	S. Blackburn
	ACCC01	Cowan Creek, New South Wales	Unknown
	CS300/01	Samchonpo, Korea	C. Bolch
	CS314/8	Port Phillip Bay, Victoria, Australia	O. Monestrup
	CS313/1	Port Phillip Bay, Victoria, Australia	S. Blackburn
	CS316/3	Ballast water, Kashima, Japan	Unknown
	CS319	Ballast water "Golden Crux", Singapore	Unknown
A. catenella	STH-M	St Helen, Tasmania, Australia	C. Bolch
	AF1531	Unknown	C. Bolch
	TRIA-F	Triabunna, Tasmania, Australia	C. Bolch
	STH1608	St Helen, Tasmania, Australia	C. Bolch
	ATTR/F	Triabunna, Tasmania, Australia	C. Bolch
	STH-D	St Helen, Tasmania, Australia	C. Bolch
	STH1604	St Helen, Tasmania, Australia	C. Bolch
	STH-F	St Helen, Tasmania, Australia	C. Bolch
	STH-A	St Helen, Tasmania, Australia	C. Bolch
	AF1532	Unknown	C. Bolch
	STH1625	St Helen, Tasmania, Australia	C. Bolch
	ATMP7E8	Salt Pond, Massachussets, US	Unknown
	TRIA-E	Triabunna, Tasmania, Australia	C. Bolch
A.australiense	ATCJ33	Cape Jaffa, South Australia, Australia	M. de Salas
	ATNWB01	North West Bay, Tasmania, Australia	C. Bolch
	AABP-B	Unknown	Unknown
	AADVN-1	Unknown	Unknown
	AT-YC-H	York Cove, Tasmania, Australia	C. Bolch
A.minutum	RCC4873(1252)	Iroise Sea, France	L. Guillou
	CCMI1002	Bantry Bay, Ireland	Unknown
	RCC4877	Iroise Sea, France	L. Guillou
	CS324	Port River, Adelaide, South Australia	S. Blackburn/J. Cannon
	De1231	Unknown	L. Guillou
	De1699	Unknown	L. Guillou
	De1215	Unknown	L. Guillou

RCC4874 (1254)	Iroise Sea, France	L. Guillou
De1196	Unknown	L. Guillou
De1236	Unknown	L. Guillou
RCC4879(1262)	Iroise Sea, France	L. Guillou
AM89BM	English Channel, France	L. Guillou
RCC3145	English Channel, France	L. Guillou
RCC4872(1251)	Iroise Sea, France	L. Guillou

Appendix B. gBlocks® sequences and primers for gene copy number quantification

Species	Target gene	Primers	References	gBlocks® Sequence
A. pacificum	*sxtA*	5'-ctg agc aag gcg ttc aat tc-3' 5'-tac aga tmg gcc ctg tga rc -3'	Murray et al. 2011	cttcgggttg gactacgcgg agaacaacat catctacgcc gggcagctga gcaaggcgtt caattcgccc ggcggattcg tcagctgtgc gcgcgagacc gacgagaatt tcggcgttct gaacttggcc aagaactcga acacactcgt gttcacaggg ccgatctgta ctgccggcct gtcgagtgcg aagacgacct
	LSU rRNA	5'-tcc tca gtg aga ttg tag tg-3' 5'-gac aag gac aca aac aaa tac-3'	This publication	aatgagtttg tatttgctaa acacaaagta aacagacttg atttcctcag tgagattgta gtgcttgctt aacaatgggt tttggctgca agtgcaataa ttcttgcttt gtgtgccagt ttttatgtgg acatttgatt acctttgcac atgaatggta attttcctgc ggggtgtgga ttgcatatgc atgtaatgat ttgcatgttt gttaaatgtg tctggtgtat ttgtttgtgt ccttgtcctt gaggttgctt tctcccttgg gcttacatgc
A. catenella	LSU rRNA	5'-tga ttt gct tgg tgg gag tg-3' 5'-caa gga agg aag cat ccc c-3'	This publication	gagtttgtat ttgctgaaca caaagtaaac agacttgatt tgcttggtgg gagtgttgca cttgcttgac aagagctttg ggctgtgggc gtaatgattc tttctttgca tgccagtttc tatgtgtaca tctgattacc tttgcacatg aatgataagt ctcctgtggg gggtggattg catgtgcatg taatgatttg tgttttgata aatgtgtctg gtgtatgtgt gtgtgttcct gtgcttgggg atgcttcctt ccttggactt acaagccctg acacacacat gctggcaaaa
A. australiense	LSU rRNA	5'-cgg tgg gtg caa tga ttc-3' 5'-gca gga aaa tta cca ttc aag t-3'	This publication	cttagtgaga ttgtagcact tgcttgacaa taggttttgg cggtgggtgc aatgattctt gctttgtatg ccagtttcta tgtggacatt tgattacctg tgcacttgaa tggtaatttt cctgcggggg gtggattgca tatgcatgta attatttgca
A. minutum	LSU rRNA	5'-aca tgg ata act gtg gta att cta tag cta a-3' 5'-gtt ggt tct gta act aat gac cac aac-3'	Toebe et al. 2013	gctcattaaa acagttataa tgcacttgat gatcgattgc ttacatggat aactgtggta attctatagc taatacatgc atccaaacct gacttctggg aagggttgtg gtcattagtt acagaaccaa cccaggctct gcttggaatt ttggtgattc atgatgactg aatgaattac

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